Final Report

Establishing biofumigation as a sustainable replacement to pesticides for control of soil-borne pests and pathogens of potato and horticultural crops

Ref 11120001

March 2014 – October 2019

Report Author: P. E. Urwin, University of Leeds

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Executive Summary

Soil-borne pests and pathogens are major constraints to crop production. Nematode control in agricultural crops is expensive, often ineffective and reliant on synthetic chemicals. Chemical control of soil-borne fungi is also problematic. New approaches with broad efficacy, which are suitable for use on a wide range of crops, are urgently required. Biofumigation involves incorporation into soil of glucosinolate-containing brassica plants for control of pests. Glucosinolates are hydrolysed by microbial and plant myrosinase enzymes to yield bioactive products, particularly isothiocyanates, when plant tissue is damaged. However, inconsistent efficacy and a lack of detailed data on optimal deployment of biofumigation under a range of agronomic situations prevent the widespread uptake of this technique.

Our project has addressed this knowledge gap by elucidating factors underpinning effective biofumigation. We have characterised how glucosinolate profiles of different biofumigant plants vary with developmental stage and with both agronomic and environmental factors. We have identified other secondary metabolites produced by some brassica plants that have toxicity towards nematodes. The effects of biofumigation on a range of pests and pathogens have been determined in glasshouse, polytunnel and field trials. In general, we found few significant effects of biofumigation on either nematodes or fungal pathogens outside of lab-based studies. Where a significant effect was found on potato cyst nematodes, it was dependent on soil type, with soil moisture a secondary factor.

We have developed a novel plant growth system to visualise a group of plant-feeding nematodes for which there is little information regarding the effects of biofumigation. We have evaluated the impact of biofumigation on non-target below-ground fauna.

Key findings

A number of key findings to date will either direct future research or influence biofumigation best practice.

- Seeding density has little effect on isothiocyanate-release potential (in terms of biomass and glucosinolate concentration) of biofumigant crops at incorporation.
- Optimum incorporation time for a biofumigant crop is when plants are flowering.
- The major biofumigant compound (allyl ITC) is lost rapidly from soil after brown mustard incorporation, but other, less well-studied chemicals (dimethyl sulphide and dimethyl disulphide) increase in soil over time and may contribute to pest/pathogen toxic effects.
- Volatile halide compounds are produced by roots of growing brassicas and are lethal to potato cyst nematode juveniles at very low doses.
- Standard glucosinolate extraction techniques were improved to provide a new method that gives cheaper, quicker and more accurate results.
- An imaging and detection system has been developed to monitor behaviour of nematodes in transparent soil microcosms. This can be used to assess effects of biofumigant chemicals.
- Two soil-borne fungal pathogens*, Rhizoctonia solani* and *Verticillium dahliae* are differentially sensitive to a range of isothiocyanates, but symptoms of *R. solani* on potato were not reduced by biofumigation with a brown mustard (ISCI99) in a glasshouse trial.
- In raised bed trials, there was a significant decline in viability of potato cyst nematodes both during growth of a brown mustard crop (ISCI99) and after its incorporation. This

outcome differed with soil type. There was a significant decline in viable J2 for cysts present in clay loam soil during growth of ISCI99 plants, compared to cysts in non-planted soil. The small reduction in viability for cysts in the sandy loam soil was not significant. A significant effect was again seen in the clay loam soil, but not in the sandy loam soil, following incorporation of the biofumigant. This effect was greater when incorporation took place at 30% soil moisture than at 10 % soil moisture.

- In polytunnel trials, using large 600 litre growth boxes filled with field soil known to contain high levels of FLN, biofumigation with either the brown mustard ISCI99 or the radish Bento had no significant effect on any of the growth parameters of subsequently cultivated raspberry plants, on the total number of nematodes in the soil, or on the number of *Pratylenchus* and *Longidorus* nematodes in the soil.
- Two field trials investigated the effect of biofumigation on potato cyst nematode, using the brown mustard 'ISCI99', the radish 'Bento' and the white mustard 'Ida Gold'. There were no significant differences in viability of the natural soil population of cysts between any of the biofumigant treatments, the non-biofumigant bristle oat control, or the fallow. For Trial B, there was a significant drop in number of viable J2s post-incorporation irrespective of treatment. We also found no evidence for a significant impact of biofumigation on viability of nematodes within bagged cysts, with large variability between replicates.
- Two trials investigated biofumigation with either a brown mustard (ISCI99) or a radish (Bento) prior to drilling a carrot crop. In one trial, there was no significant effect of biofumigation on subsequent yield of carrots or the proportion of FLN-damaged carrots. In the second trial, biofumigation with 'ISCI99' but not 'Bento' increased the proportion of pre-pack class carrots, but not the total yield. The % carrots displaying FLN damage was significantly reduced in the plots treated with 'ISCI99' when compared with the fallow plots but was not significantly different from the damage in plots treated with the non-biofumigant wheat.
- There is no evidence for a longer term detrimental effect of biofumigant growth or incorporation on abundance of non-target soil nematodes.

Widespread grower uptake of biofumigation is currently limited by several factors, including a lack of data on the factors affecting its consistent efficacy. The combined findings from this project will provide agronomists with the information required to offer evidence-based advice to growers on the suitability of biofumigation as a nematode management tool in different scenarios. If chosen as a management practice they will be able to devise optimal deployment strategies to ensure that biofumigation delivers maximum efficacy.

We have defined the brown mustard (ISCI99) and radish (Bento) biofumigant cultivars of choice based on their biofumigation potential and clarified the optimum growth stage for incorporation.

Importantly, we found that biofumigant seeding rates have no significant impact on biofumigation potential. This new knowledge suggests that grower costs can be minimised by reducing seeding rate without negatively affecting biofumigation potential.

We found that the main biofumigation compound is lost from soil within 24 hours after incorporation. This is important knowledge both for informing the most effective incorporation techniques and when designing realistic *in vitro* exposure assays with pests and pathogens.

Our finding that biofumigation was effective in clay loam but not sandy loam could affect the management advice given to growers, based on their soil type. However, we also find that

biofumigation effects on potato cyst nematode are highly variable, even within a single trial, making it difficult to confidently endorse it as an effective tool for management of PCN.

1. Introduction

Soil-borne pests and pathogens, including many nematode and fungal species, are major constraints to profitable crop production in the UK. The problem will be exacerbated in the immediate future by removal of approved chemical control strategies. New approaches are required that have broad efficacy and are suitable for use on a wide range of crops. Biofumigation involves the incorporation into soil of Brassicaceous plants, which produce a range of secondary metabolites including glucosinolates, able to control pests and pathogens. However, inconsistencies in efficacy and a lack of detailed empirical data on its deployment under a range of agronomic situations threaten the widespread uptake of this technique for sustainable pest control. The project, a collaboration across three academic institutions (University of Leeds, University of York and The James Hutton Institute), set out to address this knowledge gap by elucidating the fundamental biochemical and metabolic processes underpinning effective biofumigation strategies. It aimed to characterise the glucosinolate profiles of different biofumigant Brassicas, how these vary with plant developmental stage and environmental factors, and identify additional secondary metabolites that may be responsible for some of the observed effects of biofumigant plants. Outputs of the research would allow optimal deployment of biofumigation for maximum efficacy in a range of field conditions, providing a sustainable control option for both conventional and organic crop production.

1.1. Background

Nematode control in agricultural crops is expensive, often ineffective and reliant on synthetic chemicals that are being withdrawn from use (91/414/EEC). Chemical control of soil-borne fungi is also problematic. New, more broadly effective and sustainable approaches for pathogen control are needed.

One alternative control method that could be adopted by UK growers is biofumigation. This approach suppresses pests and pathogens by incorporating brassica green manures into soil. The effectiveness of biofumigation with brassicas as a pest control strategy is thought to rely mainly on a class of thioglucosides they contain called glucosinolates. These compounds are hydrolysed by microbial and plant myrosinases to yield bioactive products, particularly isothiocyanates, when plant tissue is damaged. The glucosinolate profile of crops varies between brassica species and cultivar, and may change significantly over the lifecycle of the plant. Some green manures also release other biocides including volatile halogen and sulphur-containing compounds that are nematicidal. The potential role of these compounds in biofumigation had not previously been examined and it is possible that in practice biofumigation relies upon a “cocktail” strategy in which greater efficiency is obtained through lower concentration, mixed biofumigant compound delivery.

Biofumigation has been employed for nematode control in some regions of the world but efficacy is very variable and there has been little concerted research aimed at understanding the range of factors that ensure optimum biofumigant production, release and efficacy. Furthermore, tests that explore the specific efficacy of any individual isothiocyanate at agricultural concentrations are rare to non-existent. Biofumigants may have a broader effective range than pathogenic nematodes such as the potato cyst nematode *Globodera pallida* as there are reports of efficacy against fungi and other invertebrate pests.

The original proposal aimed to address the outstanding uncertainly around the efficacy of biofumigation in the field, and to determine its utility for a wider range of nematode and fungal pathogens, through five main objectives.

Objectives

1. Establish optimal agronomic practices for the management of *G. pallida* using biofumigation.
2. Measure glucosinolate profiles of selected biofumigant plants and determine how they alter over the course of plant development and in response to environmental factors.
3. Characterise novel compounds, in addition to glucosinolates, in plant accessions shown to be effective against pests and pathogens.
4. Determine the effectiveness of biofumigation against trichodorids and longidorids on potato, carrot and raspberry where applicable.
5. Determine the potential of biofumigation to control soil-borne pathogens.
6. Analyse the impact of biofumigant crops on biotic aspects of the below ground food web.

2. Experimental Sections

2.1. Objectives 1 & 2

Objective 1 that involves field, raised bed and glasshouse trials to investigate a range of environmental and agronomic variables is inextricably linked with Objective 2, to measure the glucosinolate profiles of plants grown in those trials. Therefore, the results are combined into one section for this report.

2.2. 2014 Field trials

Seeding rate of biofumigant crops will vary the growth habit and may influence total biomass and glucosinolate concentration/total content. We compared the impact of different seeding rates for a range of biofumigant brassica accessions on biomass and glucosinolate profiles. We examined the glucosinolate profiles of three commercial *Brassica juncea* (brown mustard) cultivars: Vitasso, Scala and ISCI99, two *Raphanus sativus* (fodder radish) cultivars: Bento and Diabolo, and the *Sinapis alba* (white mustard) cultivar: Ida Gold.

The trial took place in a sandy-loam-soil-dominated field (coordinates: 53.000 371, −0.290 404). Nitrogen (90 kg) in the form of ammonium sulfate was added to the field, which was subdivided into randomized block plots (1.6 × 12 m). The mustard plants were grown at 4 seeding rates spanning the range recommended by seed suppliers: 6, 8, 10, and 12 kg/ha. The radish plants were sown at three commercially suggested seeding rates, 10, 15, and 20 kg/ha. All brassicas were harvested once 50% of the plants had flowered. Additional biomass data only was collected from plants grown at thousand grain weight seed rate aiming to provide 100 plants/sq metre.

Samples for glucosinolate analysis were collected at three growth stages: i) early rapid growth, ii) 50% maturity and iii) 50% flowering. For the mustards these stages corresponded to (i) 49 days post drilling), (ii) 64−68 days post drilling, and (iii) 79 days post drilling. For the radishes, these stages corresponded to i) 42 days post drilling), (ii) 52 days post drilling, and (iii) 58 days post drilling). Plants were sampled at 4 and 8 m intervals along each plot (1 × 0.5 m) to give a combined total sample area of 1 m^2 for each plot; 3−6 biological replicates were sampled for the biomass and glucosinolate analyses. The leaves and stems

were weighed, and subsamples were frozen and stored at −80 °C prior to processing. Frozen tissue was freeze-dried, then homogenised using a grinder and finally milled with ball bearings, sealed and stored.

Effect of seeding rate on biofumigant biomass

The first trial set up in July 2014 developed extremely quickly such that the first sampling time could not be carried out for the mustards. No samples were therefore taken for glucosinolate analysis; however this trial was taken to completion for analysis of biomass. In this first trial Loti was grown in place of Vitasso as the third *B. juncea* cultivar. Both biomass and glucosinolate analysis was carried out for the two radish cultivars in this trial. A second trial of mustards only was set up on 7th August 2014 with both biomass and glucosinolate analysis carried out for this trial. The trials were set up in 2 blocks (A and B) with three replicates per block for all cultivars and seed rates. Given their very different growth characteristics, the data for mustards and radishes were analysed separately.

Radish

Both radishes (Bento and Diablo) showed a significant increase in total biomass production with increasing seed rate. A satisfactory fit is provided by log transformation (Fig 1). Biomass production of Diablo was significantly higher than Bento when considering all seed rates (Fig. 2). The partitioning of biomass between leaves, stems and roots was analysed for those plants grown at thousand grain weight. There was no significant difference in biomass of the same plant parts between cultivars (Fig. 3a) and also no significant difference in stems produced per sq metre (Fig. 3b).

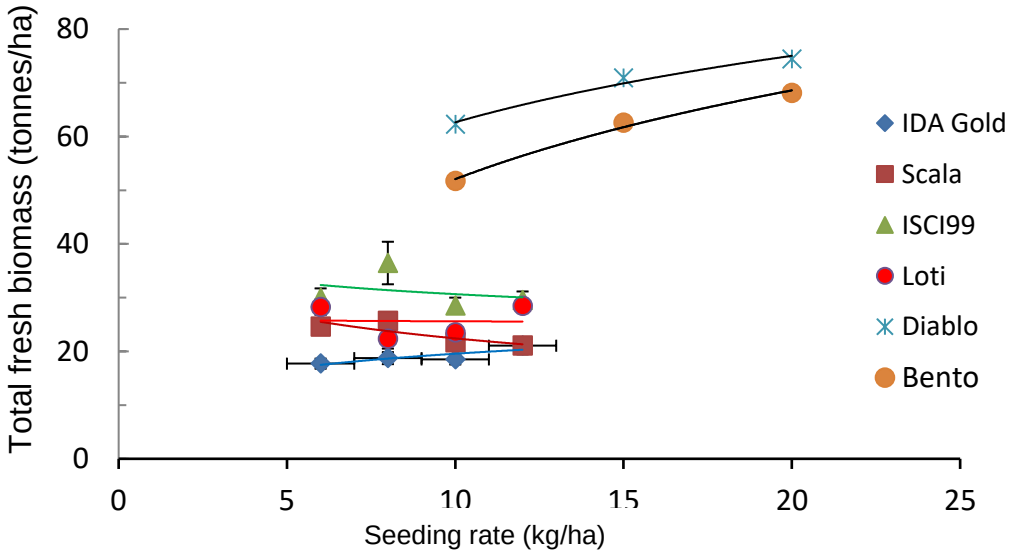


Figure 1: Combined fresh biomass of leaf, stem and root for Brassicas grown at a range of seeding rates. Biomass was taken at 50% flowering as for standard biofumigant incorporation. There is a trend of increased production of the two radishes with increasing seed rate. Values are means ± SEM and the trend for Bento and Diablo is significant after log transformation ($P < 0.05$ in both cases). There was no significant trend of seed rate on production for any of the mustard cultivars.

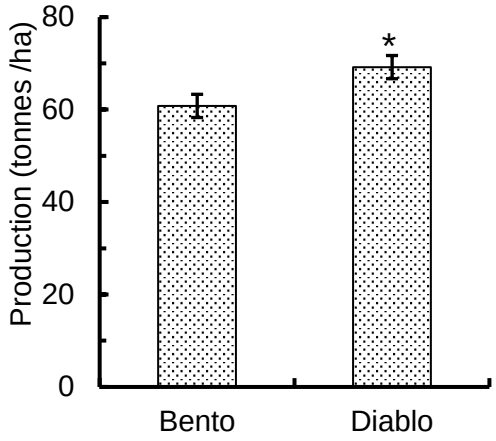


Figure 2: Total fresh biomass production of two radish cultivars (Bento and Diablo) at an adjusted seed rate of 14.4 kg/ha (univariate ANOVA). Values are means ± SEM and they differ significantly (P = 0.014).

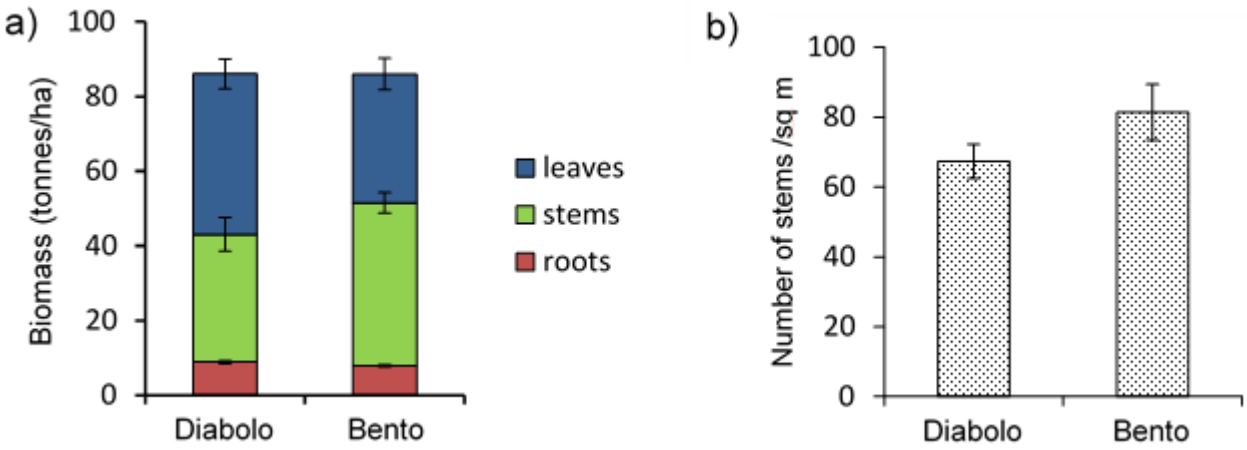


Figure 3: a) Yield of roots, stems and leaves for two radish cultivars when sown at thousand grain weight aimed at providing 100 plants/m^2. Values are means ± SE. No means for the same plant parts differ. **b)** Number of stems produced by two radish cultivars when sown at thousand grain weight aimed at providing 100 plants/m^2. Values are means ± SEM. The two values are not statistically significantly different

Mustard

For the mustards, univariate analysis with cultivar and block as factors and seed rate as the covariate established that the effect of block was not significant and there was no significant interaction between block and cv (ie. the effect of cv was similar for both blocks). The effect of seed rate was also not significant in the first trial and so the data is represented as a bar graph in Fig.4. Overall in this first trial, ISCI99 produced more total fresh biomass than the other three cultivars with Scala and Loti not different from each other but Ida Gold producing less biomass than the other three cultivars ($P < 0.05$; Pairwise comparisons, Univariate ANOVA). ISCI99 differs from the Ida Gold and Scala at $P<0.001$ and Loti at $P<0.007$).

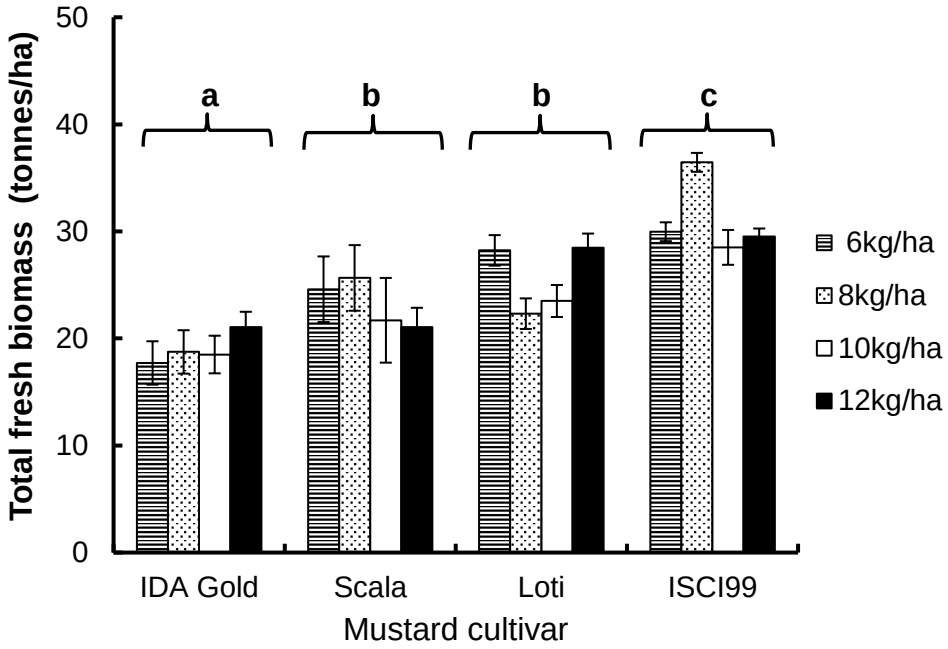


Figure 4: Production of combined fresh weight of leaves, stems and roots for four mustard cultivars at four different seeding rates in Trial 1, 2014. The overall difference between cultivar across the four seed rates differs by at least $P< 0.05$ when they do not share a letter a, b or c.

In the 2nd mustard trial, drilled one month later, Vitasso replace Loti, but the other cultivars remained the same. Data from trials 1 & 2 could not be merged as the same cultivars have significantly different biomass in the two trials. Nevertheless, the relative biomass for those common cultivars was relatively consistent ie. the ranking of ISCI99, Scala and Ida Gold was similar. On this occasion, the effect of increasing seed rate in the 2nd trial was linear for three cultivars but a quadratic fit was required for Vitasso. This suggests that 8-10 kg/ha is the optimum seed rate for Vitasso with 12 kg/ha possibly producing too much competition between the plants (Fig. 5).

Univariate ANOVA was carried out with cultivar as a factor and seed rate as the covariate. That analysis established that Ida Gold provided a lower harvest than the three other cultivars ($P = 0.014$, Bonferroni). The lower production of the white mustard Ida Gold relative to the three brown mustards is consistent with Trial 1 (Fig. 6). The equivalent total biomass data for Trial 1 is included in Figure 6 for comparison. For each cultivar that was included in both trials, the biomass was significantly higher in Trial 2.

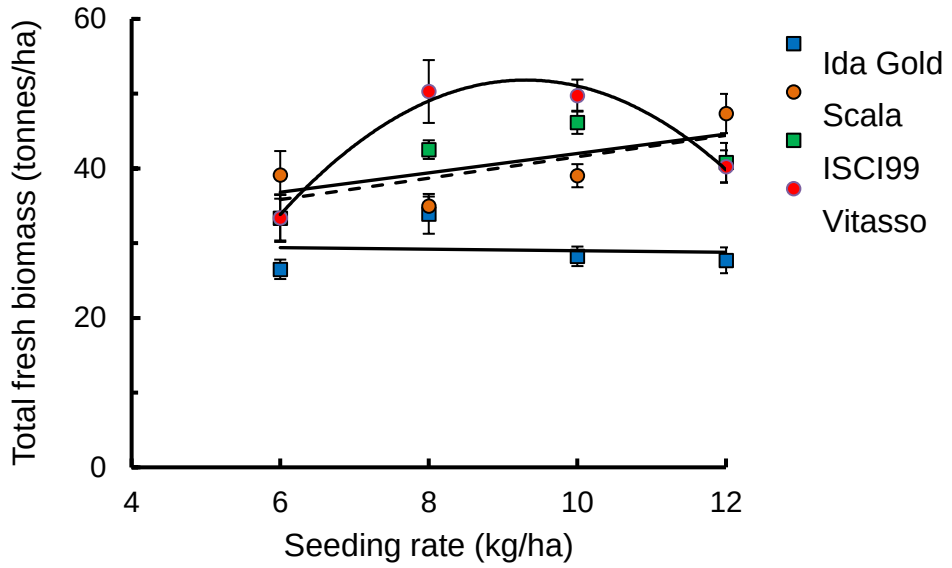


Figure 5: The effect of seed rate on production of combined fresh weight of leaves, stems and roots for four mustards in Trial 2, 2014. There is a significant effect of seed rate for all cultivars with a linear fit for three cultivars (P<0.05) but a quadratic curve was required for a significant fit for cv Vitasso (P=0.001). Ida Gold biomass declines slightly with seed rate, whereas Scala and ISCI99 increase.

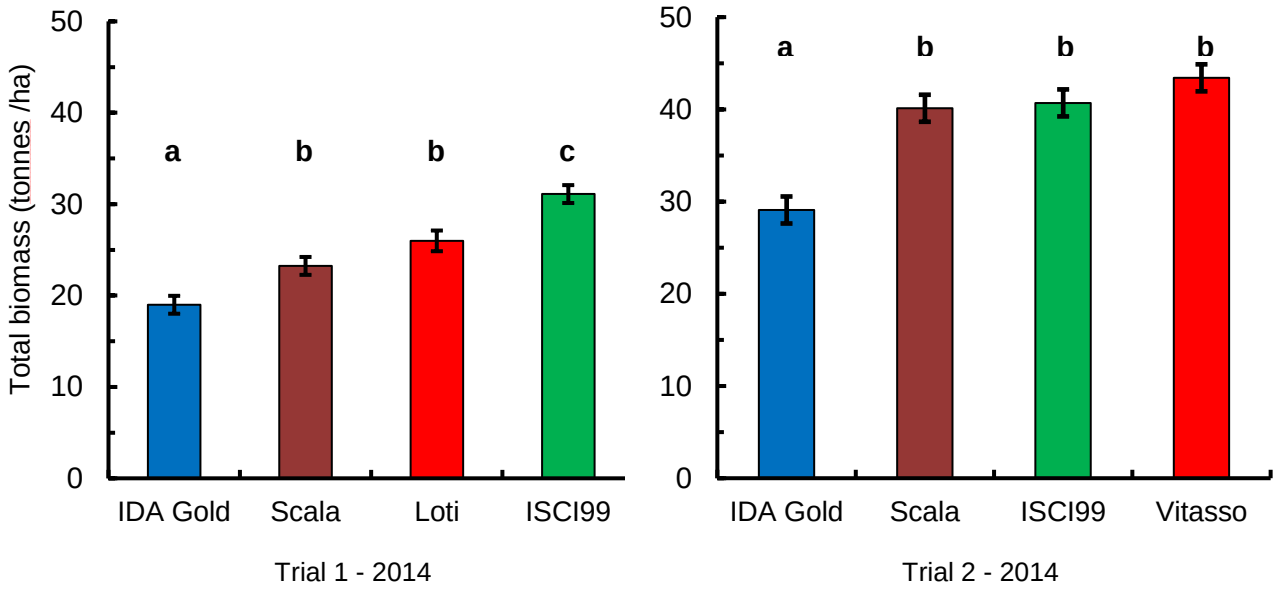


Figure 6: Total production of combined fresh weight of leaves, stems and roots for four mustard cultivars in each of two trials. Values are means ± SEM across all seed rates. For Trial 1 there are three statistically significant groups (Oneway ANOVA, P<0.05 ,a b and c). For Trial 2 Ida Gold was less productive than the other three cultivars (P = 0.001). For each cultivar included in both trials, biomass was significantly higher in Trial 2.

The number of stems produced per sq metre in each trial at the thousand grain weight seed rate is shown in Figure 7. Partitioning of biomass between leaves, stems and roots was analysed for the plants grown in each trial at this seed rate (Fig. 8). Leaf biomass generally contributed less to the differences in total biomass between cultivars than did stem or root

weight. The difficulties inherent in recovering intact root systems from the field should be considered when interpreting the root biomass data.

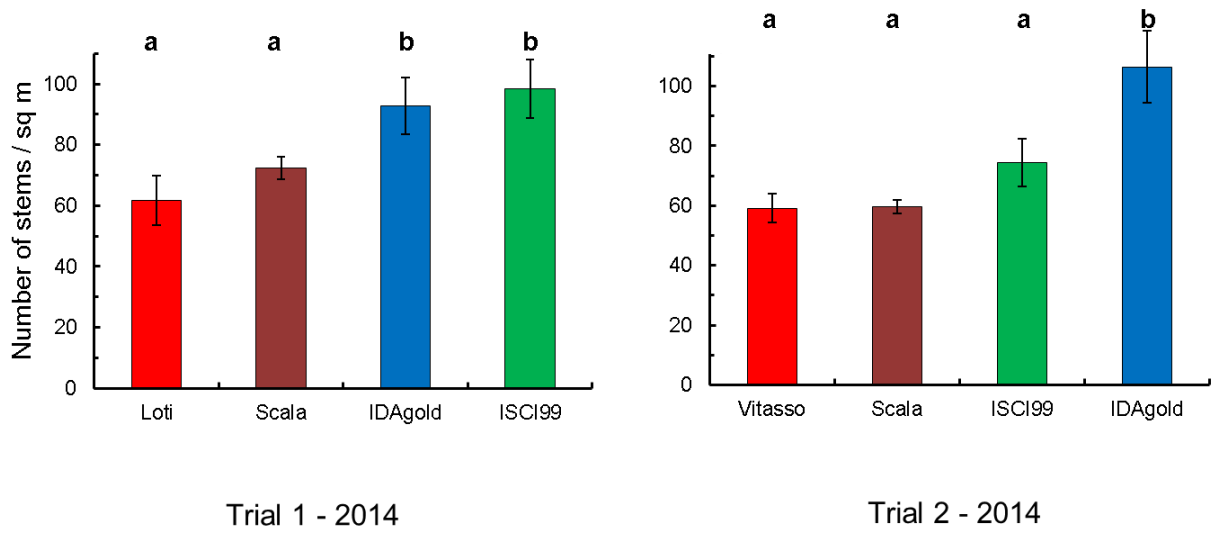


Figure 7: Number of stems produced in each of the two trials for mustard cultivars sown at thousand grain weight aimed at providing 100 plants/m^2. Values are means ± SEM with different letters for cultivars that differ in each trial ($P < 0.05$; SNK, Oneway ANOVA). Germination and/or post emergence survival was significantly higher for Scala and ISCI99 in Trial 1.

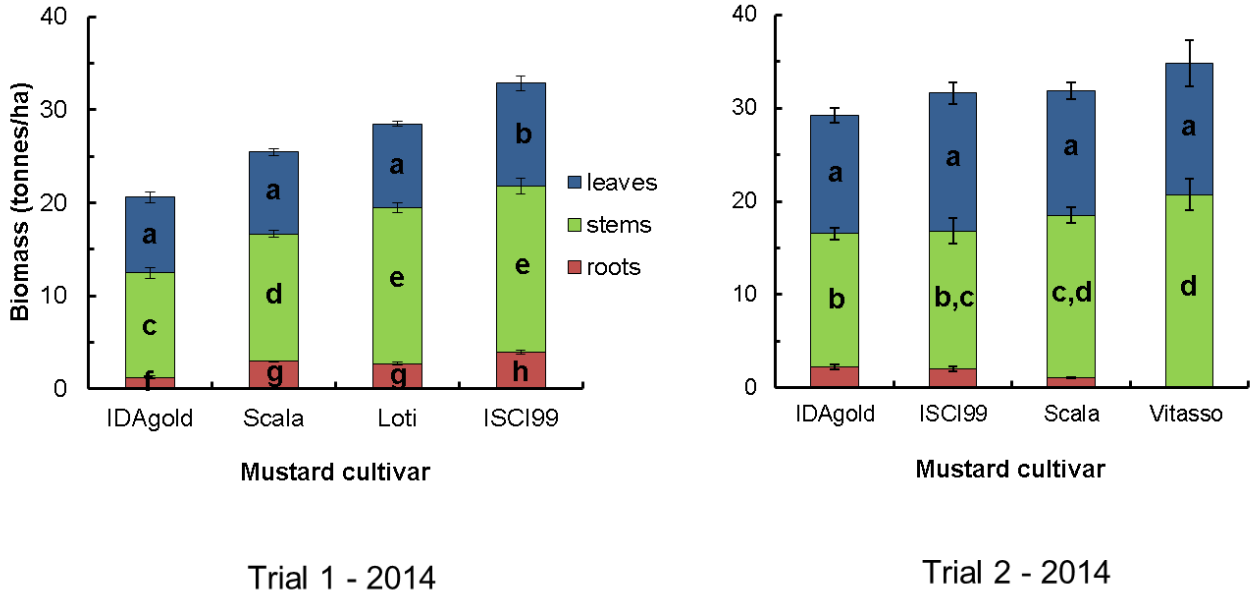


Figure 8: Yield of roots, stems and leaves for mustard cultivars sown at thousand grain weight aimed at providing 100 plants/m^2. Values are means ± SEM with different letters when they differ from the same part of the yield in other cultivars in that trial ($P < 0.05$; SNK, Oneway ANOVA). At this single seed rate, the increased total biomass for each cultivar in Trial 2 does not hold for ISCI99. NB. Root biomass was not collected from Vitasso plants in Trial 2.

Glucosinolate analysis

Separate leaf and stem samples were collected at three developmental stages from the radish trial and the 2nd mustard trial for all seed rates. Samples were frozen then subsequently freeze-dried, weighed and ground prior to extraction of glucosinolates and analysis by HPLC. Due to the very large number of samples collected and the limited capacity for HPLC analysis and in particular freeze-drying, it was collectively agreed to analyse only samples from the two extreme seed rates of 6 and 12 kg/ha for the mustards or 10 and 20 kg/ha for the radishes. At these seed rates, samples were analysed from all three developmental stages. The major glucosinolates characteristic of each species were quantified and their concentrations in dry mass of leaves and stems are presented in Figs. 9-11. Dry biomass weights were not available for these trials and so extrapolation to glucosinolate content/ha was comparative and based on differences in fresh weight between cultivars and seed rates.

Brown mustards: Sinigrin made up more than 90% of total glucosinolate content in green tissues of *B. juncea* cultivars, with higher concentrations found in the leaves (Fig. 9). There was a significant effect from the cultivar, and there was a significant combined effect from the cultivar and tissue type on the sinigrin concentration: cultivars differed significantly in leaf sinigrin concentrations but not in stem sinigrin concentrations.

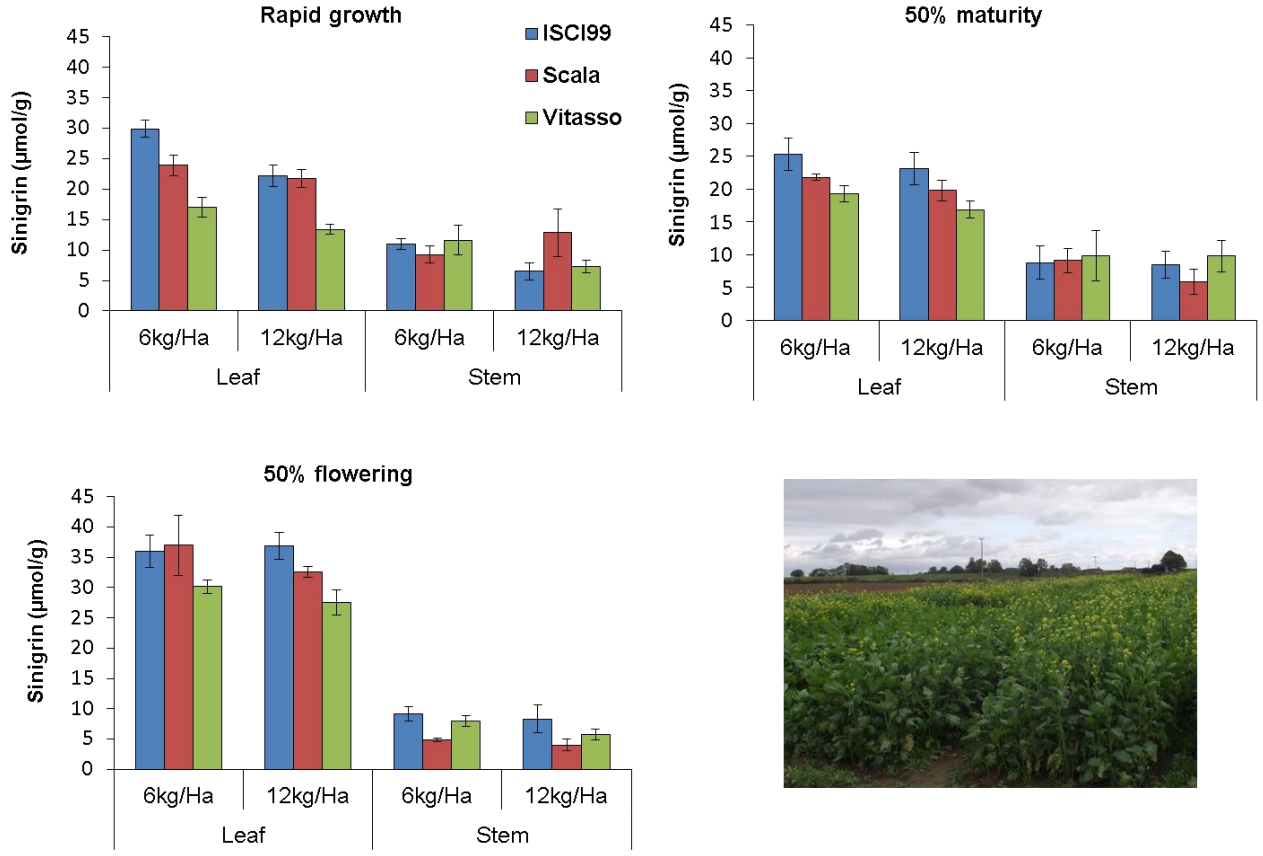


Figure 9: Concentration of sinigrin (the major glucosinolate of brown mustards) in leaf and stem samples of three brown mustard cultivars (ISCI99, Scala, Vitasso). Samples were analysed at three different growth stages and those from plants drilled at 6 kg/ha and 12 kg/ha seed rates were compared. Error bars are standard errors of the mean (n=3-6 biological repeats).

Sinigrin concentrations in ISCI99 leaves were on average ~12% and ~44% higher than in Scala and Vitasso leaves respectively. There was a significant effect of seeding rate on sinigrin concentrations which were on average 20% higher at the lower rate of 6 kg/ha.

Sinigrin concentrations were modulated by growth stage with mean concentration in leaves significantly higher at 50% flowering relative to both other growth stages and mean concentration in the stems significantly lower at 50% flowering relative to the rapid growth stage

Radish: Glucoraphenin and glucoraphasatin are both aliphatic glucosinolates and account for over 90% of ITC releasing glucosinolates in *R. sativus* (cv. Diablo and cv. Bento) shoots. Variability of glucosinolates within sample sets was much higher than with the mustards (Fig. 10). Concentrations of glucoraphenin were significantly higher in Bento than in Diablo, and in leaves than in stems (ANOVA: $F_{(1, 64)} = 9.143$; $p<0.01$; and ANOVA:$F_{(1,64)} =54.164$; $p<0.001$ respectively). A significant effect of growth stage on glucoraphenin was also identified (ANOVA:$F_{(2,64)}=3.521$; $p=0.035$). There was a three way interactive effect of growth stage, tissue type and seeding rate on glucoraphasatin concentrations (ANOVA:$F_{(2,64)}=3.823$; $p=0.027$). Concentrations were significantly lower in leaves sampled at 50% maturity from plants at 20 kg/ha relative to stems sampled at rapid growth at 10 kg/Ha and stems sampled at 50% flowering at 20 kg/Ha (TukeyHSD, p adj= 0.0145 and p adj=0.039 respectively). No interactive effect of any combination of seeding rate, growth stage, cultivar, and tissue type on glucoraphenin concentration was detected.

Total glucosinolate concentrations were significantly higher in Bento than in Diablo and in leaves than in stems (ANOVA:$F_{(1, 64)}=5.453$; $p=0.023$; and ANOVA:$F_{(1, 64)}=15.05$; $p<0.001$ respectively) and a significant effect of growth stage on total glucosinolate concentrations was also identified (ANOVA:$F_{(2,62)}=4.143$, $p=0.020$). The glucosinolate concentrations from radish plants sampled at the rapid growth stage were significantly higher than total glucosinolate concentrations from radish plants sampled at the 50% maturity stage (Tukey HSD, p adj=0.016). No interactive effect of any combination of seeding rate, growth stage, cultivar and tissue type on total glucosinolate concentration was found.

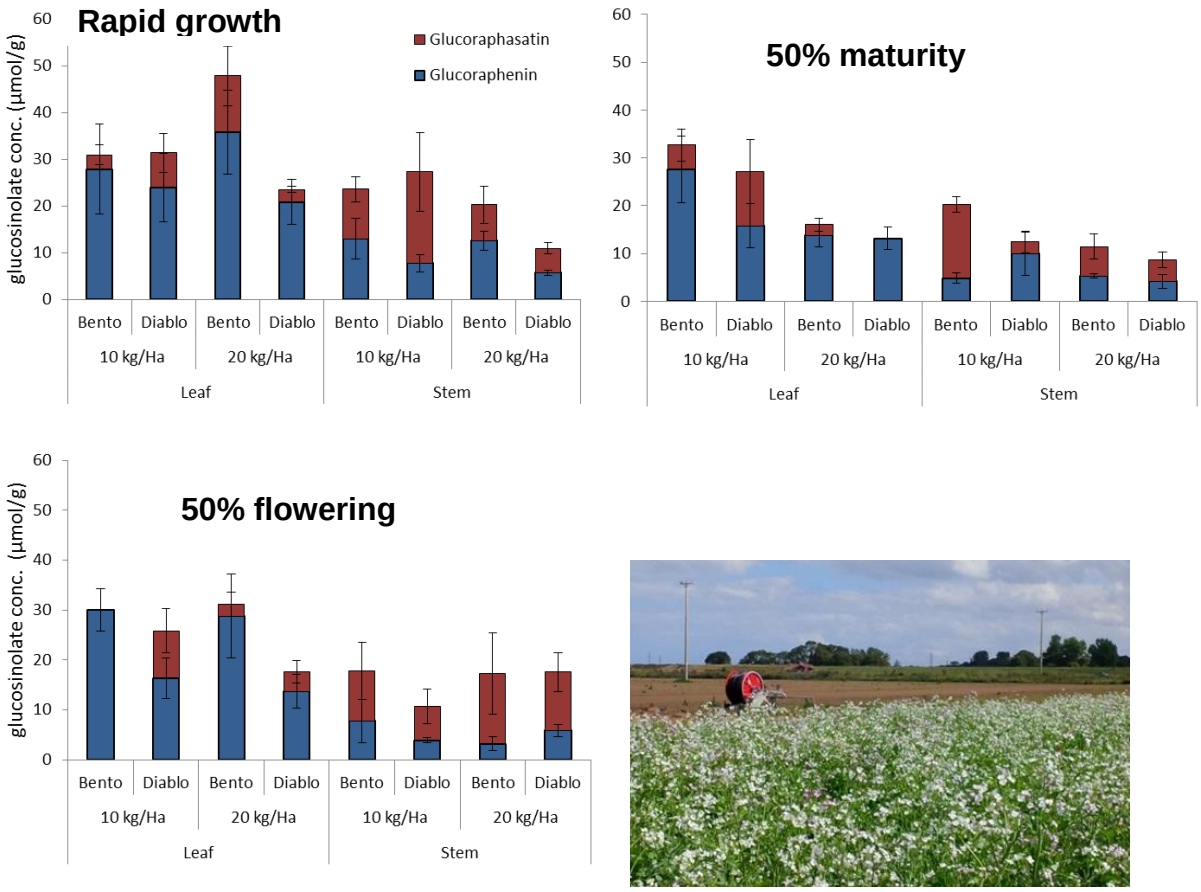


Figure 10: Concentrations of glucoraphenin and glucoraphasatin in leaf and stem samples of two radish cultivars (Bento, Diablo). Samples were analysed at three different growth stages and those from plants drilled at 10 kg/ha and 20 kg/ha seed rates were compared. Error bars are standard errors of the mean (n = 3-4).

Unlike the brown mustards, the white mustard Ida Gold does not produce the aliphatic glucosinolate sinigrin in appreciable amounts. The aromatic glucosinolates glucotropaeolin and sinalbin accounted for over 90% of the total glucosinolate content in the green biomass of this cultivar (Fig. 11). A significant statistical three way interaction was observed between the effects of tissue type, seeding rate and growth stage on total glucosinolate concentration (ANOVA: $F_{(2,\ 32)}$=5.22; p=0.011). Total glucosinolate concentrations were significantly higher in leaves in all conditions. Leaf glucosinolate concentration was higher at the 50% flowering stage (~45 µmol.g^{-1}) than the rapid growth stage (~30 µmol.g^{-1}) and stem glucosinolate concentration decreased with plant growth stage (from approximately 12 µmol.g^{-1} at rapid growth to 5 µmol.g^{-1} at 50% flowering). On average, the differences and relative contributions of glucosinolate concentrations in the leaf and stem increased over time. Glucosinolate concentrations were significantly higher at higher seeding rates only for leaves sampled from plants at 50% maturity.

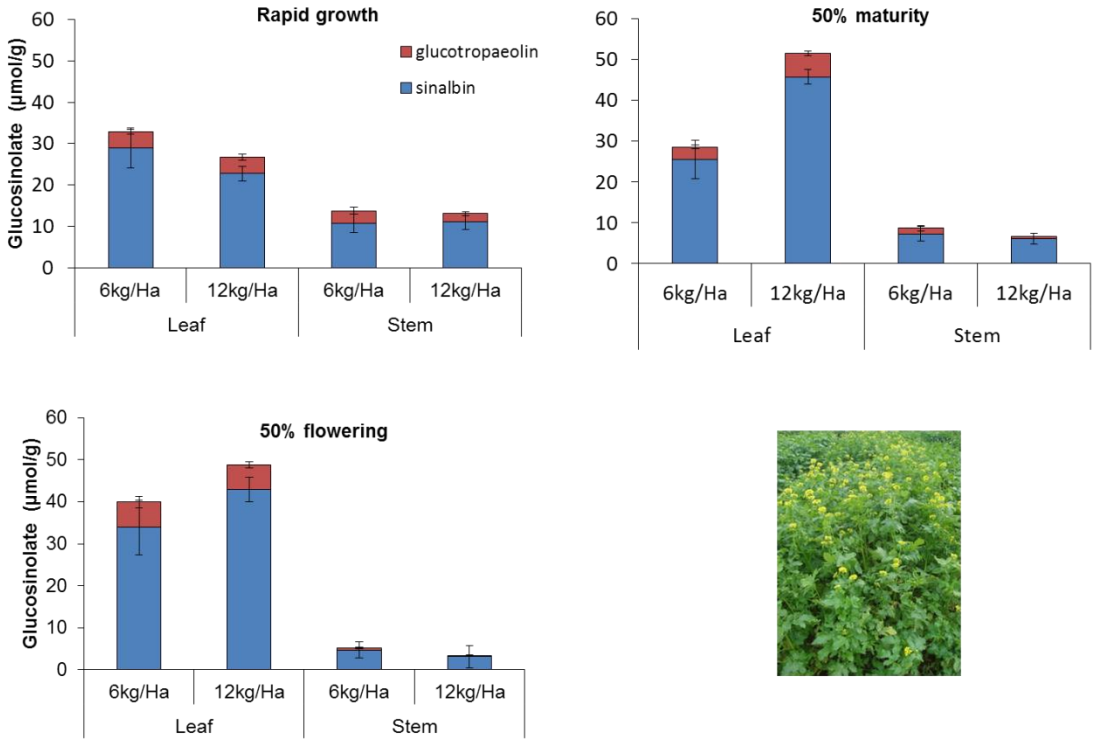


Figure 11: Concentration of sinalbin and glucotropaeolin in leaf and stem samples of the white mustard Ida Gold. Samples were analysed at three different growth stages and those from plants drilled at 6 kg/ha and 12 kg/ha seed rates were compared. Error bars represent standard errors of the mean (n = 3−4).

Glucosinolate concentration in the field

The total glucosinolate concentration expected per area of field (m^2) at 50% flowering (i.e. the typical stage at which the biofumigants are incorporated) was assessed across the biofumigant cultivars and least/greatest experimental seeding rates. For *B. juncea* brown mustard cultivars, total sinigrin concentration in the field ranged from 16 to 24 mmol/m^2. Both cultivar and seeding rate individually had significant effects on the concentration of sinigrin in the field ($p<0.01$ and $p<0.047$ respectively). Fields in which ISCI99 was sown at a seeding rate of 12 kg/ha contained a significantly higher glucosinolate concentration than fields in which Scala and Vitasso were sown at the same rate (Fig. 12). No interactions were found between field concentration, cultivar and seeding rate in *B. juncea* cultivars.

For the *S. alba* white mustard Ida Gold, mean glucosinolate concentrations ranged from 1.4 mmol/m^2 to 1.6 mmol/m^2 and 9.2 mmol/m^2 to 11 mmol/m^2 for glucotropaeolin and sinalbin respectively. Seeding rate had no effect on glucosinolate field concentration, but total glucosinolate concentration per area of field was significantly lower in fields growing Ida Gold than in fields growing the *B. juncea* cultivars.

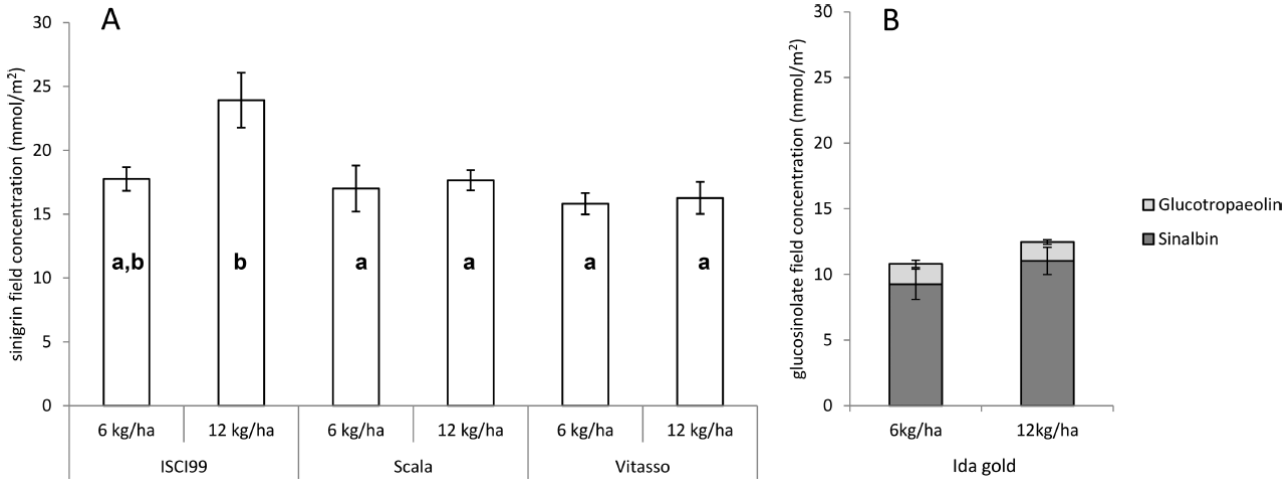


Figure 12: (A) Mean concentrations of sinigrin per area of field growing *B. juncea* (ISCI99, Scala, and Vitasso) seeded at rates of 6 kg/ha or 12 kg/ha and (B) mean concentrations of glucotropaeolin and sinalbin per area of field growing *S. alba* (Ida Gold) seeded at rates of 6 kg/ha or 12 kg/ha. Error bars represent standard errors of the mean (n=3-4).

Mean field glucosinolate concentrations in the radish cultivar Diablo ranged from 13 to 17 mmol/m^2 and 16 to 18 mmol/m^2 for glucoraphasatin and glucoraphenin respectively (Fig. 13). For the radish cultivar Bento, mean glucosinolate concentrations ranged from 5.4 to 14 mmol/m^2 and 28 to 31 mmol/m^2 for glucoraphasatin and glucoraphenin respectively. No significant difference in total glucosinolate concentrations was identified between the cultivars or seeding rates for *R. sativus*, but concentrations of glucoraphenin were signficantly higher in Bento than in Diablo (p=0.042).

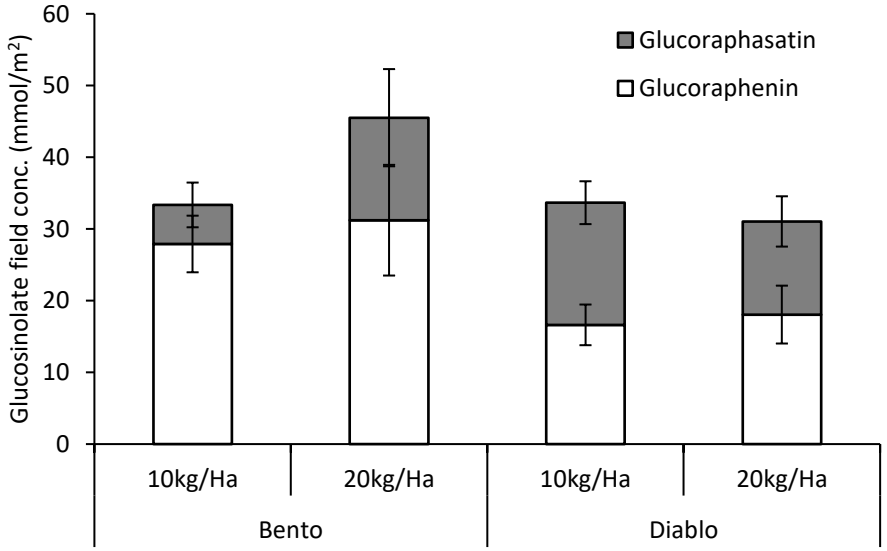


Figure 13: Mean glucosinolate concentrations per area of field growing radish cultivars (Bento and Diablo) drilled at rates of 10 kg/ha or 20 kg/ha. Error bars represent standard errors of the mean (n=3-4).

Conclusions

For the brown mustards, field-grown cultivar ISCI99 produced higher glucosinolate concentrations (24 mmol.m^{-2} glucosinolate at a drilling rate of 12 kg/ha and at 50% flowering) than either Scala or Vitasso (~17 mmol.m^{-2} and ~16 mmol.m^{-2} respectively). The major glucosinolate found in *B. juncea* cultivars, namely sinigrin, has been the subject of many studies relating to biofumigation. Allylisothiocyanate (AITC), a product of sinigrin hydrolysis, is often reported to be one of the most toxic naturally occurring isothiocyanates by virtue of its short side chain and high volatility. Depending on the plant and type of control required, an estimated 517 to 1294 nmol/g soil of methyl ITC is required for soil sterilisation (Brown 1991). Our results indicate a maximum AITC potential of 16-24 mmol/m^2 which, assuming a soil bulk density of 1.4 g cm^{-3} and incorporation to 10 cm, would give a considerably lower maximum of 170 nmol/g. In addition, given that ITC production is dependent on soil conditions, incorporation depths can be substantially deeper, and glucosinolates are unlikely to be fully converted to ITC, true ITC concentrations following incorporation are likely to be even lower.

The white mustard, Ida Gold had the lowest field concentration of glucosinolates in this work. In addition, the majority glucosinolates were the aromatic glucosinolates sinalbin and glucotropaeolin, which are reported to be less effective than the short chain aliphatic sinigrin in field conditions. Therefore, the field toxicity of Ida Gold is likely to be lower than the other brassicas used in this work.

The brassica with the highest overall isothiocyanate release potential was the radish cultivar Bento which produced ~45 mmol.m^{-2} glucosinolate at a drilling rate of 20 kg/ha and at 50% flowering, compared to ~31 mmol. m^{-2} for Diablo. Glucosinolate concentrations in radish were more variable within sample sets than those for the mustards. Variability in the biofumigation potential of *R. sativus* limits its appropriateness as a biofumigant candidate because uniform and replicable outcomes are desirable. In addition, the two major glucosinolates identified in *R. sativus:* glucoraphenin and glucoraphasatin are hydrolysed to isothiocyanates which are reportedly less volatile and toxic than the smaller chain allylisothiocyanate formed from hydrolysis of sinigrin, the primary glucosinolate in *B. juncea.*

The more sparsely sown mustard plants grew larger, fully compensating for their reduced numbers by the time they had developed to flowering. For the radish cultivars, higher seeding rates resulted in larger biomasses, with doubling of seeding rates from 10 to 20 kg/ha resulting in average total-biomass increases of ~40 and ~17% for Bento and Diablo, respectively. However, the higher proportions of stem for the radish cultivars at the higher seeding rates may affect ITC release downstream, as stems are tougher and harder to mulch.

- *B. juncea* cv. ISCI99 and *R. sativus* cv. Bento have the greatest isothiocyanate (ITC) release potential of the commercial cultivars tested.
- Seeding rate had little effect on the final biomass of the biofumigant and the glucosinolate concentration at the time of incorporation. For the mustards *B. juncea* and *S. alba*, lower seeding rates resulted in as much ITC release potential as higher seeding rates.
- Incorporation should occur at 50% flowering for all species when both glucosinolate concentrations and plant biomass are at their highest. For radish, concentration of total glucosinolates was significantly higher at the rapid growth stage but when biomass is taken into account, highest ITC release potential is still at 50% flowering.

- The data suggests that costs can be minimised by reducing some of the recommended seeding rates without negatively affecting biofumigation potential.

Comparison between the biomass data from two separate mustard trials carried out at the same site but drilled a month apart indicates that environmental factors relating to eg. temperature, daylength, soil conditions will probably be more instrumental than seeding rates in determining final biomass yields. This will influence total glucosinolate content and therefore biofumigation potential. Therefore, field trials were set up in 2016 to investigate this with four successive sowings from June to September at the same site.

2.3. 2015 Field Trials

The main aim of the 2015 trials was to assess the impact on PCN populations at two sites with different soil types, using the best performing cultivars (ISCI99, Bento, Ida Gold), optimum seeding rates and best N/S supplementation. Following earlier discussions, bristle oats were included as a non-biofumigant control in addition to fallow to test for the general impact of incorporation of organic matter. The fallow plots were treated in the same manner as the biofumigant/bristle oat plots at incorporation (ie. cultivated to mimic the act of incorporation). No pesticides were used in the trial and no herbicides were applied during crop growth. The plots were hand weeded. Ammonium sulphate fertilisation of the trial (500 kg/ha) provided approximately 105 kg N/ha and 120 kg S/ha.

Detailed glucosinolate analysis was not planned for these trials, but a single combined top growth sample was collected for each biofumigant at each trial site at two time points during the growth phase to confirm the expected levels of glucosinolates. Four or five replicate combined top growth samples for each biofumigant at each trial site were collected at incorporation to provide a measure of the final biofumigation potential. Unfortunately, these samples could not be analysed due to breakdown of the freeze drier during processing on two separate occasions.

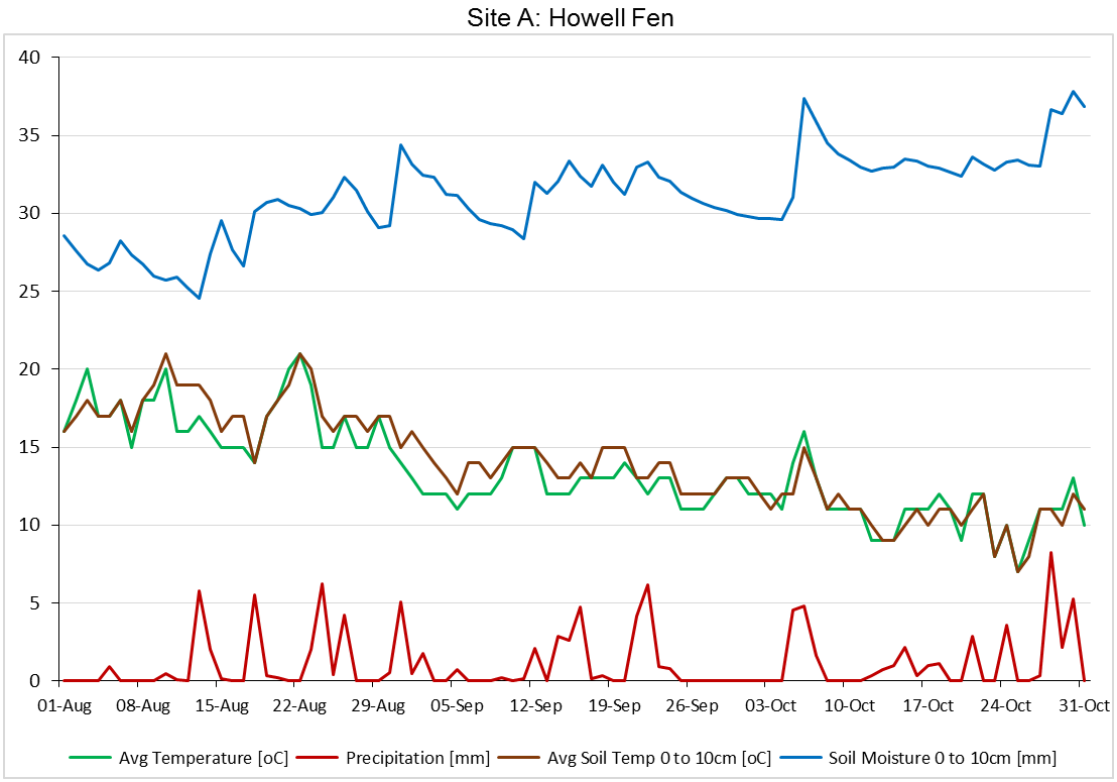
The details of the two sites are below:

Site A. Howell Fen (Reservoir) 53° 00' 04.8" *N*; 0° 17' 12.5" *W* Sandy loam soil.

Site B. Holbeach Marsh (28 Acre) 52° 50' 54.7" *N*; 0° 02' 17.34" *E* Silty loam soil.

	P		K		Mg		pH
Field Name	mg/l	index	mg/l	index	mg/l	index	
Howell Fen (Reservoir)	53	4.2	265	3.1	62	2.2	7.5
Holbeach Marsh (28 Acre)	25	2.9	129	2.0	55	2.0	8.0

Figure 17: Average air temperature, soil temperature to 10 cm depth, daily precipitation and soil moisture to 10 cm depth for the Trial A site over the time period from drilling to incorporation.



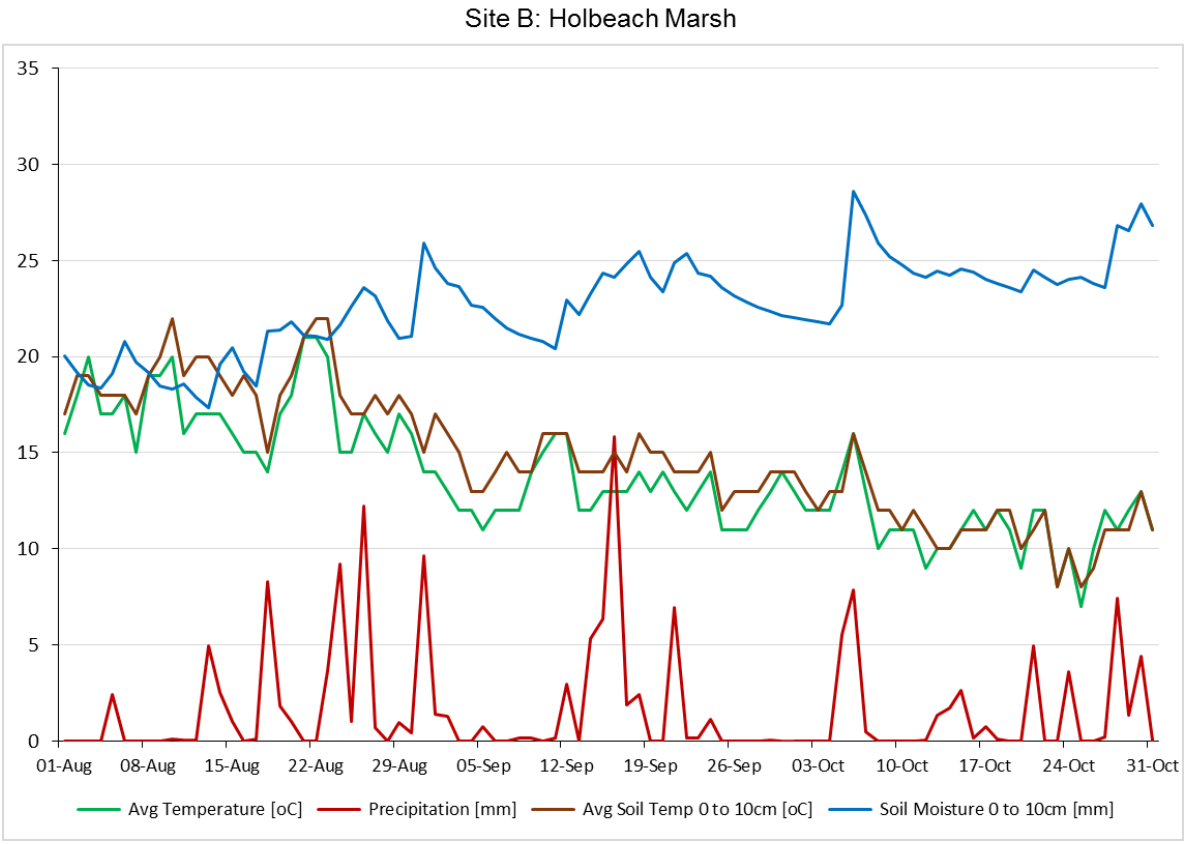


Figure 18: Average air temperature, soil temperature to 10 cm depth, daily precipitation and soil moisture to 10 cm depth for the Trial B site over the time period from drilling to incorporation.

Including the controls, there were five treatments with five reps at each of two sites, drilled in August. Seed rates for ISCI99 and Ida Gold were 8 kg/ha with Bento at 15 kg/ha. Soil samples were taken for PCN analysis pre-trial, pre-incorporation and approx.12 weeks post-incorporation, giving a total of 75 soil samples for each of the two trials. Cyst bags each containing 50 *G. pallida* cysts were inserted in each plot at sowing and removed at incorporation (late October). A second set of bags was buried at incorporation and retrieved approximately 12 weeks post-incorporation, giving a total of 50 cyst bags per trial. The cysts used for these bags were from the same stock as those used for the soil moisture and soil temperature trials.

All soil samples were sent to Leeds for drying and cyst extraction. After drying, field samples were mixed using a soil sample divider and cysts were extracted by the Fenwick can method from 100 g sub-samples. Duplicate sub-samples were extracted from the pre-trial soil. Samples from Trial Site A yielded consistently more cysts than those from Trial B – the average cysts per 100g for all Trial A samples was 49 compared with 24 cysts per 100g for Trial B. All the cysts from each extraction were rehydrated, sterilised and used in hatching assays.

The mean number of viable J2s per cyst was similar for both sites before the trial started, with a high level of variability between samples. There were no significant changes in viability during the growth of the biofumigant crops (ie. between sowing and pre-incorporation sampling) (Figs. 19 & 20). Similarly, although there was a general trend for a decline in viable J2 per cyst between pre- and post-incorporation of the biofumigants, which appeared to be more marked in Trial B where very low and consistent numbers of viable J2s remained post-incorporation (Fig. 20), none of the differences were significant (One-way ANOVA with Tukey's multiple comparisons test for each trial separately). The only significant differences were in Trial A between the fallow sample at sowing and both the fallow and oats treatment post-incorporation (Fig. 19).

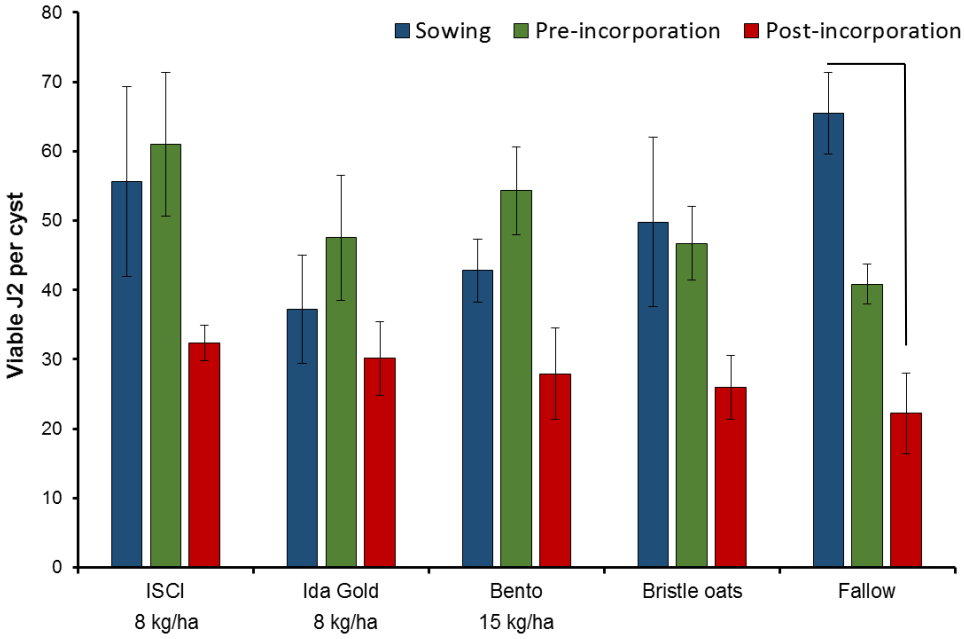


Figure 19: HAPI trial A: Comparison of mean viable J2 per cyst extracted from field soil samples collected at sowing, pre-incorporation of biofumigant and at 12 weeks post-incorporation. Control plots were either fallow or contained bristle oats 'Exito'. n = 5; * = P<0.05

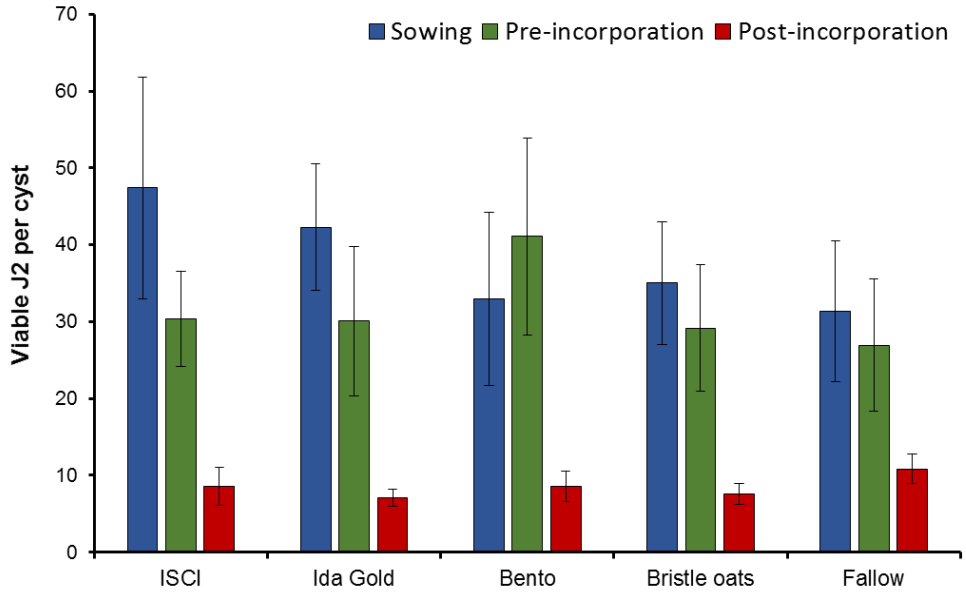
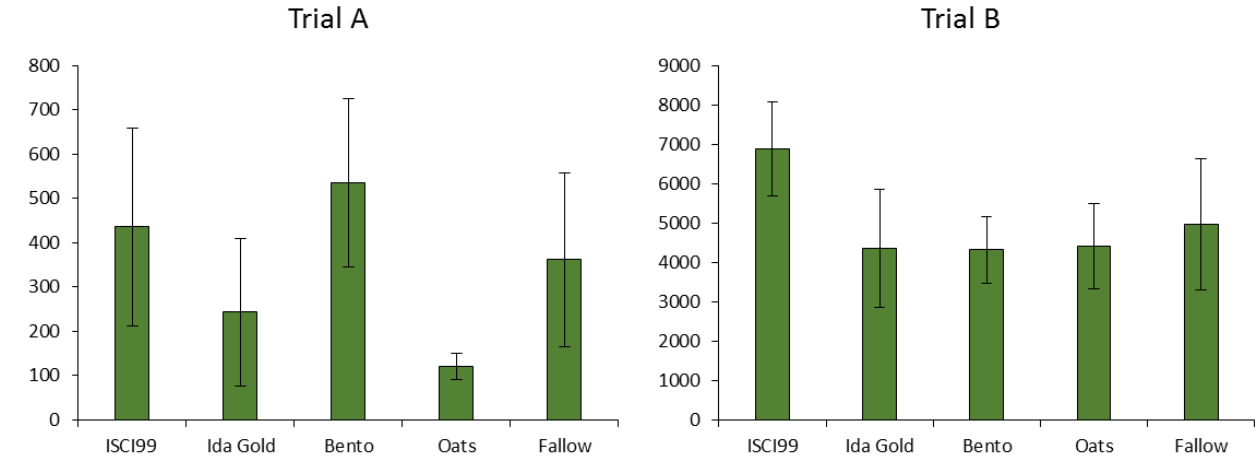


Figure 20: HAPI trial B: Comparison of mean viable J2 per cyst extracted from field soil samples collected at sowing, pre-incorporation of biofumigant and at 12 weeks post-incorporation. Control plots were either fallow or contained bristle oats 'Exito'. n = 5

These results, analysing effects on the pre-existing population of *Globodera* cysts in the field sites, suggest that there is a general decline in viable J2s over time, which may be enhanced by the agronomic practices associated with incorporation of a biofumigant/cover crop. It was observed that those cysts recovered at the later sampling times appeared more damaged than those in the pre-sowing samples. The large variation between the different samples from the same treatments made it difficult to detect any significant effects, even when numbers of viable J2s were reduced.

When the bagged cysts from pre- and post-incorporation were analysed there were similarly variable results. There was a large apparent difference in numbers of viable J2s between the two trials at the pre-incorporation stage. However, there were no significant differences between any treatments within either trial, with large variability between replicates (Fig. 21). Similarly, there were no significant differences between treatments for the cysts that had been inserted at incorporation and then subsequently removed (Fig. 22).

Figure 21: Comparison of total mean viable J2 per bagged cyst sample collected



immediately pre-incorporation of biofumigant. Control plots were either fallow or contained bristle oats 'Exito'. n = 5. There were no significant differences between treatments for either trial (One-way ANOVA with Dunnett's post hoc test for comparison with fallow).

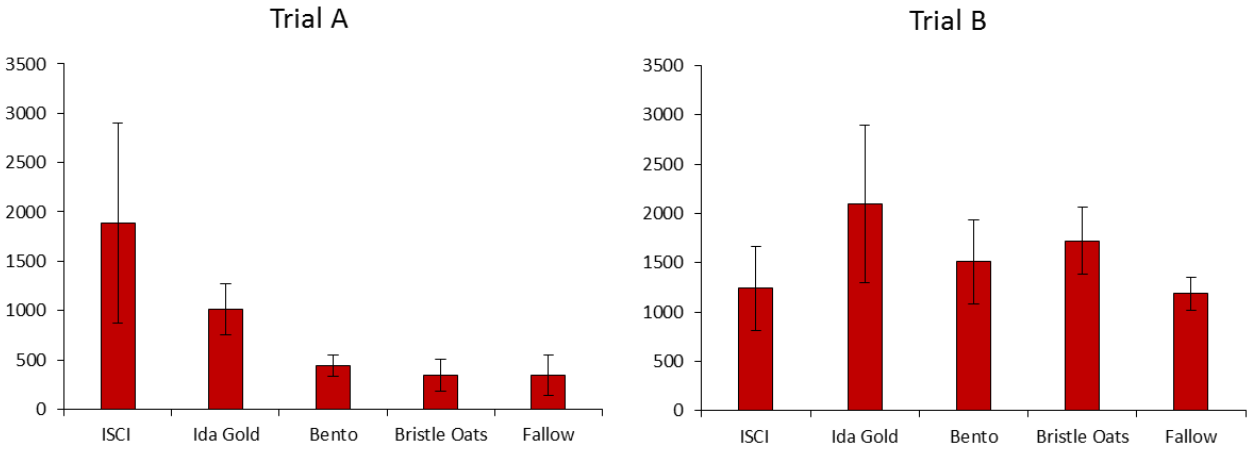


Figure 22: Comparison of total mean viable J2 per bagged cyst sample collected post-incorporation of biofumigant. Control plots were either fallow or contained bristle oats 'Exito'. n = 5. There were no significant differences between treatments for either trial (One-way ANOVA with Dunnett's post hoc test for comparison with fallow).

2.4. 2016 Field Trials – Drilling Date

The aim of the field trials carried out in 2016 was to assess the effect of biofumigant drilling date on growth, biomass at incorporation and glucosinolate levels. The rationale for these trials was based on analysis of biomass data from the two trials in 2014, where drilling date had a large effect on biomass of mustards at incorporation. The difference in biomass was potentially a more important factor in determining overall "biofumigation potential" than other environmental/agronomic aspects.

The trial encompassed four replicate plots for each of three biofumigants (*B. juncea* "ISCI99", *S. alba* "Ida Gold" and *R. sativus* "Bento") with the whole design replicated on each of four drilling dates: Early June (11/06/2016), early July (07/07/2016), early August (13/08/2016), early September (10/09/2016). Total above-ground biomass was measured and separate leaf, stem and root samples were taken for glucosinolate analysis at both the budding/early flowering stage and at incorporation. The time taken to incorporation increased from 1st-3rd drilling (6, 7 & 10 weeks). The plants from the last drilling date did

not reach flowering stage by late autumn so were left to overwinter. Unfortunately these plants did not survive the winter and so no biomass or glucosinolate samples were taken. The trial was carried out at the Elsoms site (52° 48' 50.4" *N*; 0° 07' 44.4" *W*) with sandy loam soil. The trial had additional N and S inputs (100 kg/ha N and 20 kg/ha S) provided as granular fertiliser.

The logistics and feasibility of glucosinolate analysis were discussed at the annual group meeting in January 2017. The total possible number of samples if all had been collected would have been 288: 4 drilling dates, 3 cultivars, 4 replicates, 3 tissue types and 2 sampling points. Given the resources then available, there was a need to reduce the number of samples for analysis. The most stringent prioritisation would be 3/3 drilling dates, 4/4 reps, 2/3 biofumigants, 1/2 sample points (prioritising the incorporation time point), 2/3 tissue types (leaf and stem). This would reduce the workload to a more manageable 48 samples, prioritising *B. juncea* “ISCI99” and *R. sativus* “Bento”. All frozen samples were delivered to Leeds, freeze-dried and ground for analysis of the priority samples at York.

The total above-ground fresh biomass was measured at incorporation and calculated as tonnes/ha. The final biomass of the brown mustard ISCI99 more than doubled between the June and July drilling dates ($p=0.009$; One-way ANOVA with Tukey’s post hoc test), but there was no significant difference in biomass at incorporation between the July and August drillings. This trial therefore confirmed the observation from the seeding rate trials in 2014, that a later summer sowing of brown mustard produced greater biomass at incorporation (Fig. 23).

The final fresh biomass of radish Bento was higher than for ISCI99, as seen in previous trials, but in contrast to ISCI99 there was no significant difference in biomass between the three drilling dates (Fig. 23).

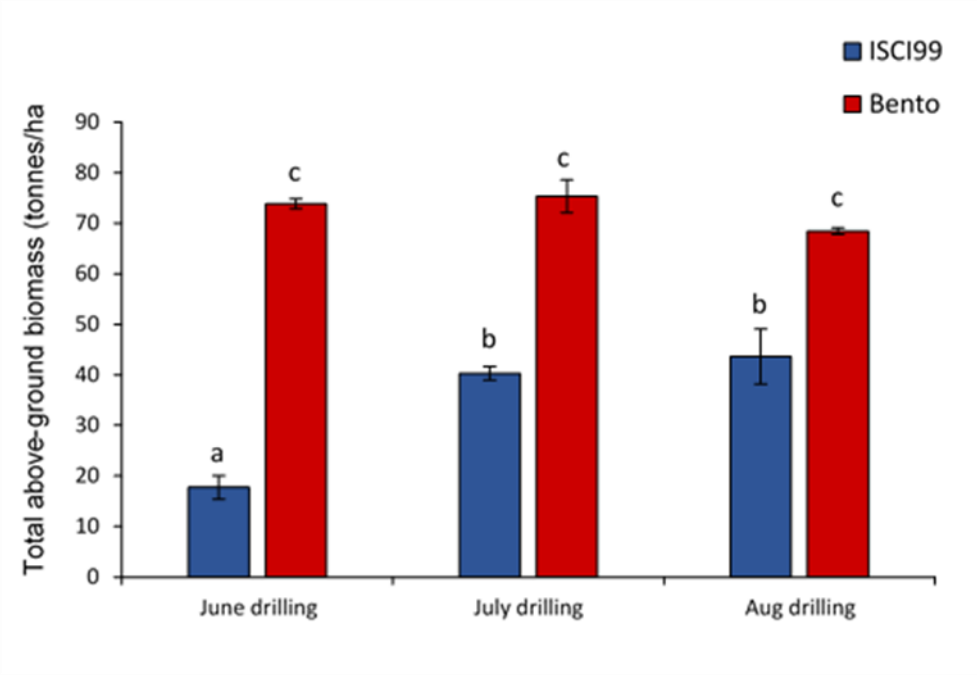


Figure 23: Total above-ground fresh biomass at incorporation for *B. juncea* 'ISCI99' and *R. sativus* 'Bento' drilled at three different dates in 2016. Biomass for the July and August drillings of ISCI99 was significantly higher than that for June (n=3; One-way ANOVA with Tukey's post hoc test). Biomass of Bento did not differ between drilling dates, but was significantly higher than ISCI99 for each sampling.

There were no significant differences between drilling dates in concentration of the major glucosinolate, sinigrin, in dried leaf tissue of the brown mustard ISCI99 (One-way ANOVA with Tukey's post hoc test). Sinigrin concentration was significantly lower in stems than leaves for all drilling dates, but also differed significantly for stems between drilling dates (One-way ANOVA with Tukey's post hoc test; 1st drilling vs. 2nd drilling p=0.018; 1st drilling vs. 3rd drilling p=0.0003; 2nd drilling vs. 3rd drilling p=0.028) (Fig. 24).

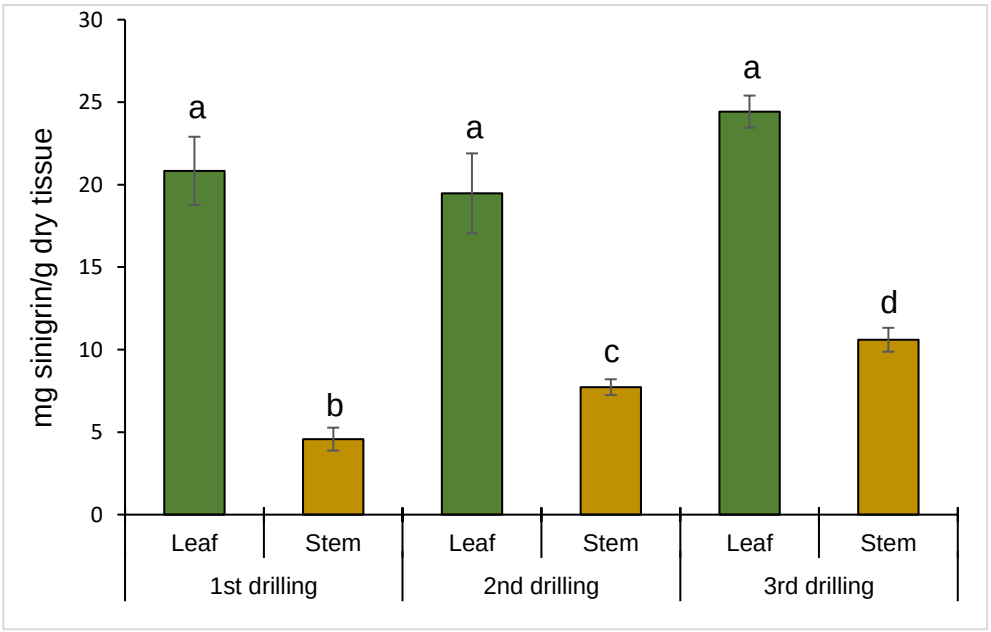


Figure 24: Concentration of sinigrin at incorporation for *B. juncea* 'ISCI99' drilled at three different dates in 2016. Sinigrin concentration does not differ for leaf samples between

drilling dates (n=4; One-way ANOVA with Tukey's post hoc test). Sinigrin concentration of stems increases with later drilling date but is always less than for leaves.

This suggests that a later drilling date of brown mustard ISCI99 would be beneficial in terms of biofumigation potential, largely due to the increased biomass, which would lead to a higher level of glucosinolate per hectare at incorporation. A later date may also fit better in the cropping schedule, although it should be noted that the August drilling required an additional three (for Bento) or four (for ISCI99) weeks in the field before the biofumigant crops were ready for incorporation.

Two glucosinolates were analysed in leaf and stem tissue of the radish 'Bento': glucoraphenin, which dominates in stems, and glucoraphasitin, which in leaves is present in similar amounts to glucoraphenin. There were no significant differences between drilling dates for total glucosinolate concentration in dried leaf tissue of 'Bento' (One-way ANOVA with Tukey's post hoc test). Total glucosinolate concentration was significantly lower in stems than leaves for all drilling dates as found previously. There was no difference in total stem glucosinolate concentration between the 1st and 2nd drilling, but the concentration in stem tissue from the 3rd drilling was significantly lower than either of the earlier drillings. (One-way ANOVA with Tukey's post hoc test; 1st drilling vs. 2nd drilling p=0.546; 1st drilling vs. 3rd drilling p= 0.0285; 2nd drilling vs. 3rd drilling p= 0.0056) (Fig. 25).

When analysing the levels of the two individual glucosinolates, leaf glucoraphenin concentration was not significantly different between drilling dates. In stems, glucoraphenin concentration was significantly lower for the 3rd drilling date than the 2nd (P=0.0322), but was not different from the 1st drilling. Glucoraphasatin increased significantly in leaves between the 2nd & 3rd drillings (P=0.0065), but there were no significant differences between drilling dates for stems (Fig. 25).

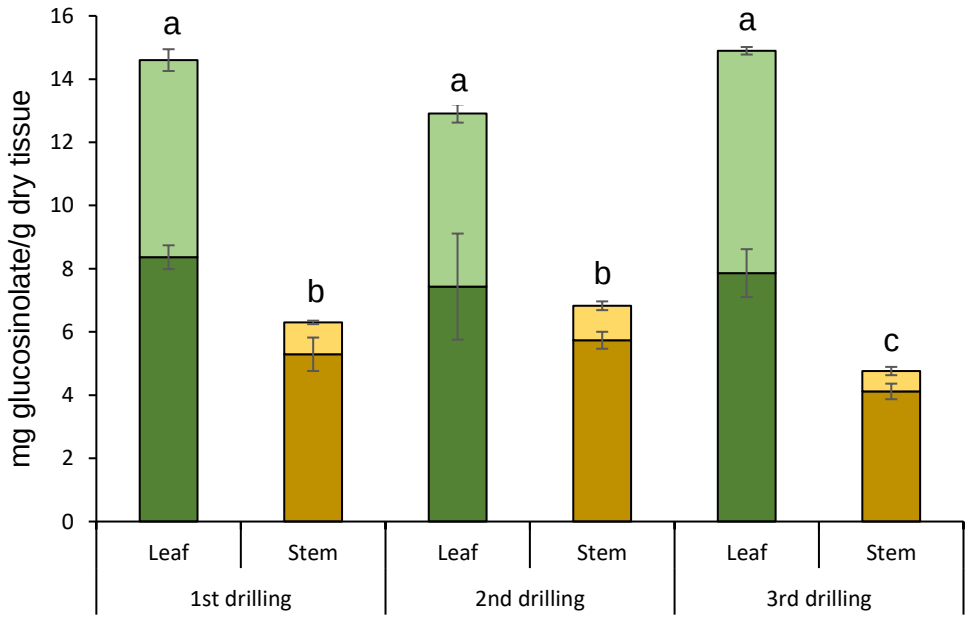


Figure 25: Concentration of the glucosinolates glucoraphenin and glucoraphasitin at incorporation for *R. sativus* 'Bento' drilled at three different dates in 2016. Total glucosinolate concentration does not differ for leaf samples between drilling dates (n=4; One-way ANOVA with Tukey's post hoc test). Total glucosinolate concentration of stems does not differ

between the 1st and 2nd drilling, but the concentration in stem tissue from the 3rd drilling is significantly lower than either of the earlier drillings.

Unlike for the mustard 'ISCI99', there was no beneficial effect on biomass from later drilling of the radish 'Bento'. This, combined with stable glucosinolate concentration in leaves and a slight decline in stem glucosinolate for the August drilling, suggests that if radish was the biofumigant of choice, then an earlier June or July drilling would provide the benefit of a shorter growing time to incorporation (7 weeks vs 10 weeks) with no reduction in biofumigation potential.

Soil temperature was monitored at the trial site. This varied largely between 15-20 ºC from mid-June to mid-September, but then fell steadily and remained below 10 ºC from the beginning of November to the end of February. This may affect the efficacy of biofumigation if incorporation is at the end of October, as required with an early August drilling. The timing may not be a concern however, as biofumigation was effective for PCN in a clay loam soil at 30% soil moisture in the raised bed trial when incorporation took place in late November (see Raised bed trials).

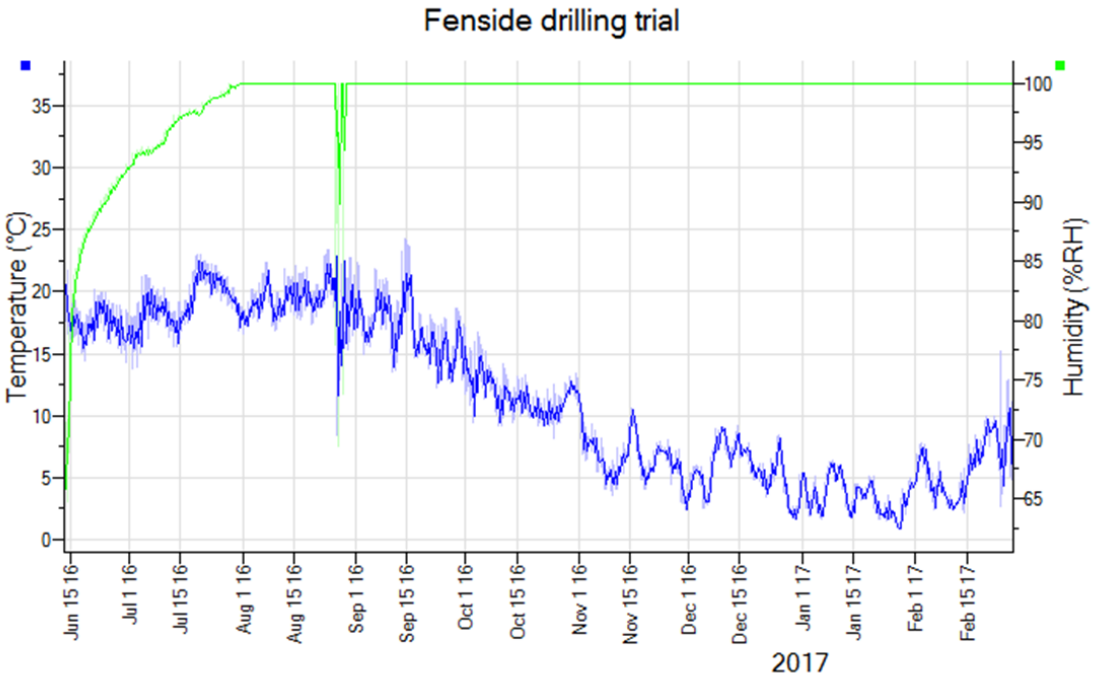


Figure 26: Soil temperature (blue) during the drilling date trial from June 2016 – March 2017.

2.5. Glasshouse trial to assess nutrition effects - 2015

Large pot trials in a glasshouse were carried out to assess the effect of nitrogen and sulphur soil amendments on biomass and levels of glucosinolates in five biofumigants: the white mustard Ida Gold, the brown mustards Scala and ISCI99, the radish Bento and *Eruca sativa* Nemat (rocket) (Fig. 27). There were four treatments with four reps each, comprising:

(i) Control

(ii) High nitrogen, 100 kg/ha Nitram

(iii) High nitrogen + high sulphur 100 kg/ha N/30 kg/ha S (Sulphomex + Nitram)

(iv) Ammonium sulphate 400 kg/ha to provide 80 kg N/100 kg S

The planting density was 100 plants/m^2 in large, 12 litre pots containing a mix of loam top soil:peat:sand in the proportions 7:3:2. One plant per pot was collected at bolting, non-flowering and one plant per pot at flowering for separate analysis of leaf, stem and root. Additional plants were sampled for fresh/dry biomass. Chlorophyll fluorescence (Fv/Fm) measurements were taken at sampling for each plant. Problems with freeze-drier failures at both Leeds and York resulted in the trial being repeated for three of the biofumigants with a third trial required for Ida Gold.

Figure 27: Glasshouse trial set up to assess effects of nitrogen and sulphur supplementation on plant growth and glucosinolate content.

- With the exception of the radish, Bento, supplemented plants generally had higher Fv/Fm values (indicative of less stressed plants; Fig. 28) but this was only significant for the white mustard Ida Gold for the N/S supplementation ($p=0.0168$; One-way ANOVA with Dunnett's multiple comparison test).
- Glucosinolate concentration in the leaf, stem and root tissues of ISCI99 was determined for plants at both first bolting and at flowering (typical incorporation point). None of the nutritional supplements significantly changed the concentrations of sinigrin in any tissue, or the concentration of the more root-specific gluconasturtiin in roots, compared with the control plants ($n=4$; One-way ANOVA with Dunnett's post hoc test).
- Sinigrin concentration was consistently higher in the young stems of the early bolting plants than the older stems of the flowering plants.
- Subsequent analysis focused for this report on the flowering stage, given that our other data has confirmed this to be the optimum stage for incorporation.

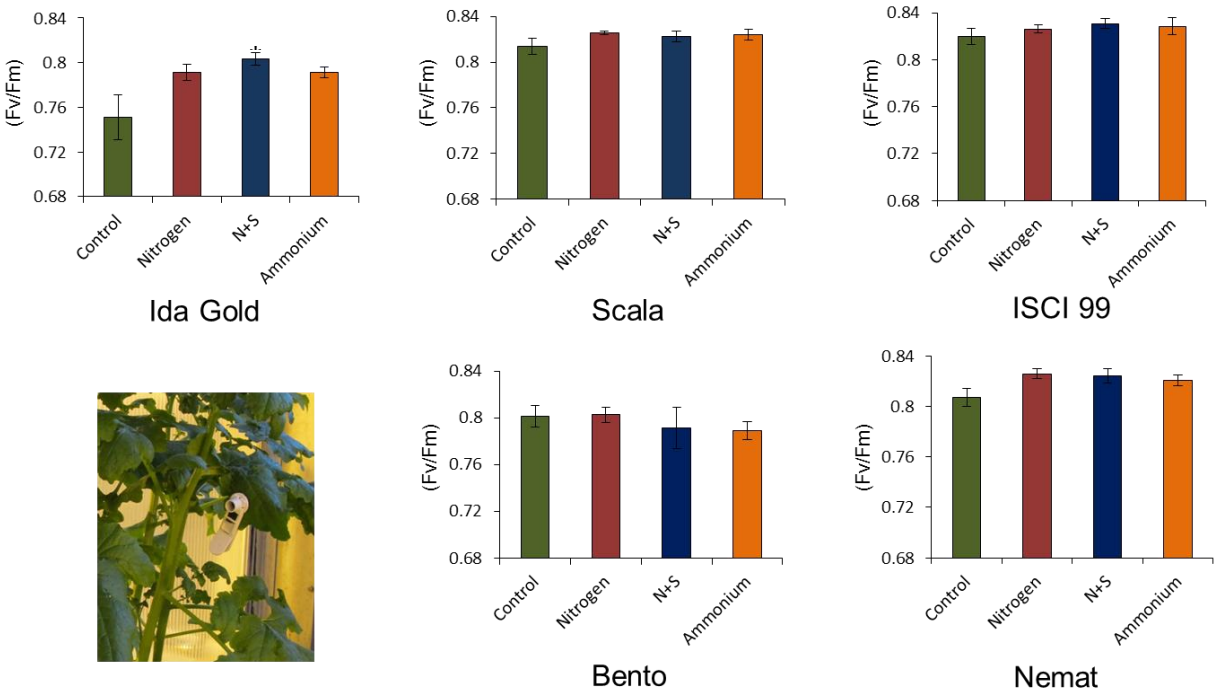


Figure 28: Chlorophyll fluorescence measurements (Fv/Fm) taken from leaves of brassica plants prior to first sampling. A lower value indicates less efficient photosynthesis and is generally taken to be an indicator of a more stressed plant. Despite clear differences in appearance between the control and supplemented plants (Fig. 29), there was little difference in chlorophyll fluorescence and the only significant difference between control and supplemented plants is indicated for Ida Gold (* $p<0.05$; One way ANOVA with Dunnett's multiple comparison test).

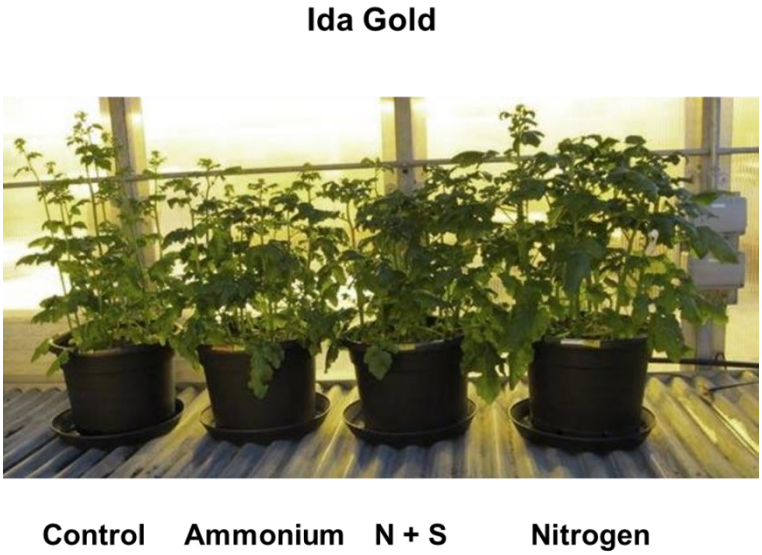


Figure 29: Representative plants of the white mustard **"**Ida Gold" growing in each of four nutrient conditions. The control plants had smaller leaves, thinner stems and flowered earlier.

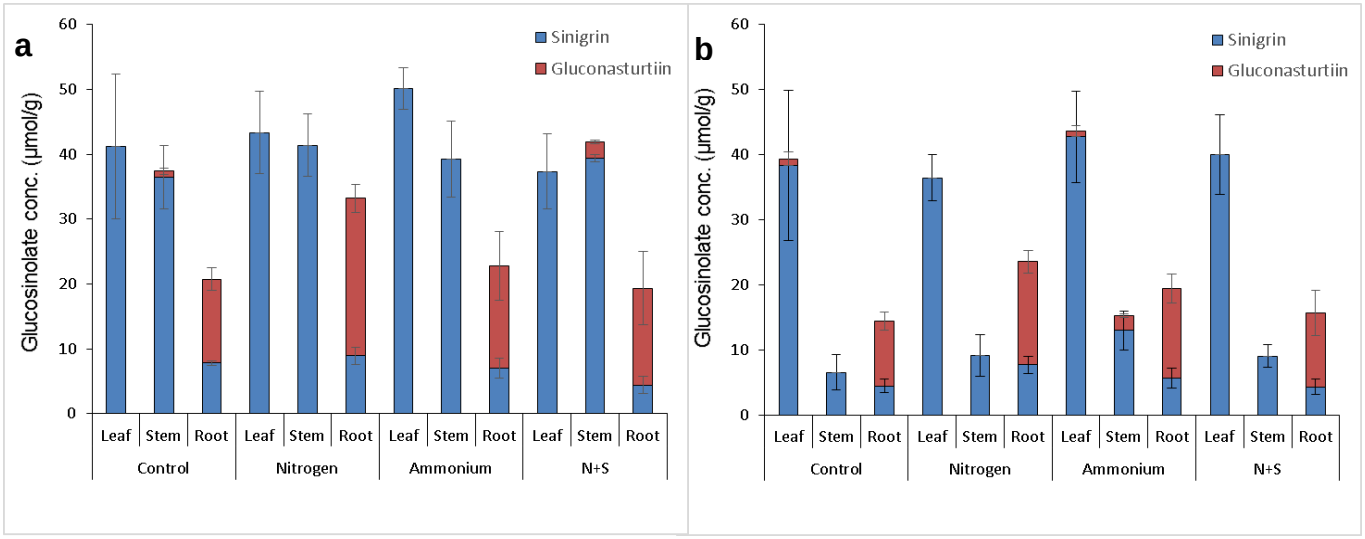


Figure 30: Concentration of sinigrin and gluconasturtiin in leaf, stem and root tissue of *B. juncea* ISCI99 grown in each of four nutrient conditions **a)** Plants sampled at early bolting **b)** Plants sampled at flowering. Comparing each tissue type to its control, there are no significant differences between concentration of sinigrin in leaf, stem or root or for gluconasturtiin in roots. (n=4; One-way ANOVA with Dunnett's post hoc test).

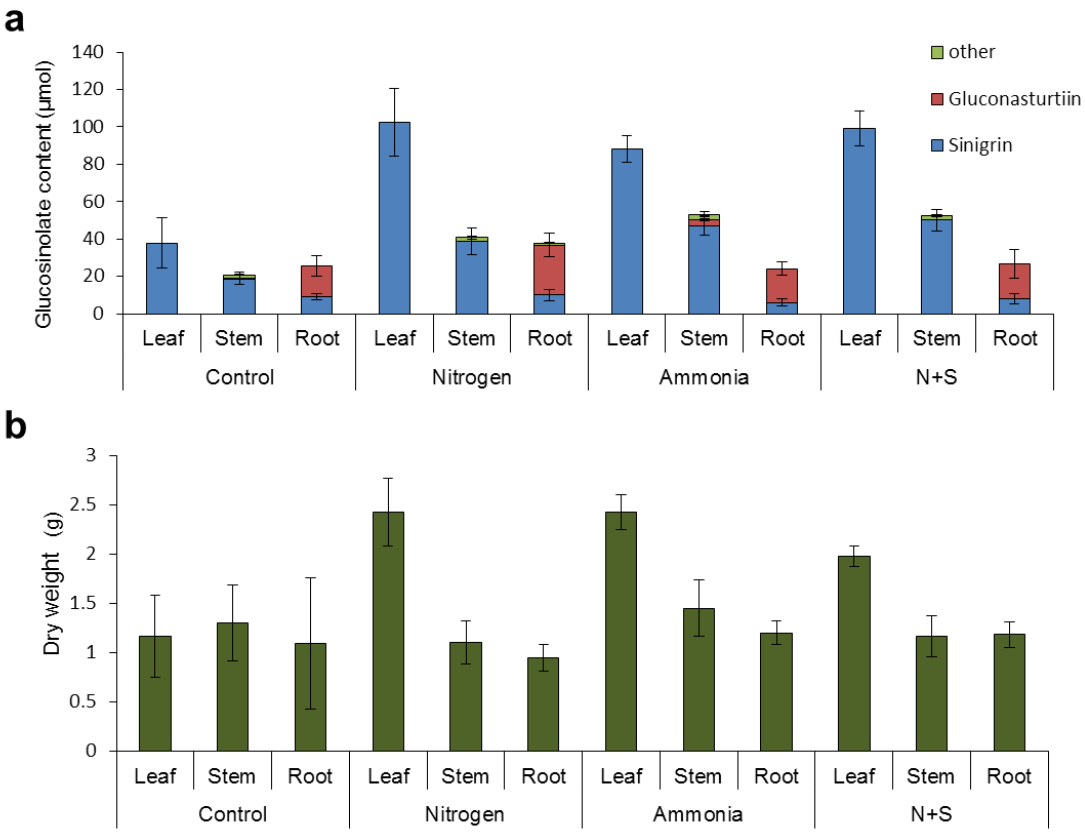


Figure 31: a) Total glucosinolate content per plant of leaf, stem and root (n=4) for *B. juncea* ISCI99 grown in each of four nutrient conditions, taking into account the dry weight of each

plant. **b)** The mean dry weight of ISCI99 plants grown in each of four nutrient conditions (n = 4). Leaf biomass is therefore a good indicator of total glucosinolate content.

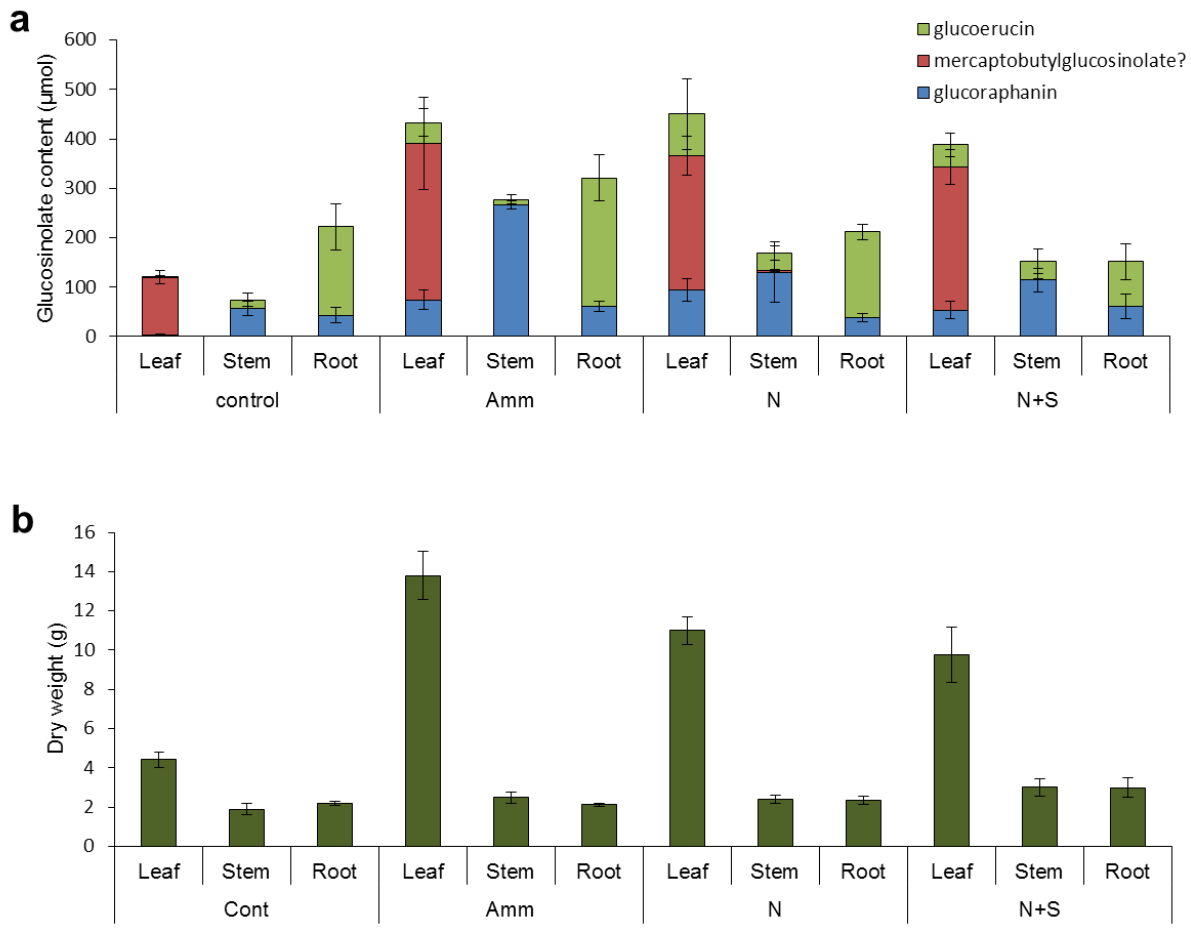


Figure 32: a) Total glucosinolate content per plant of leaf, stem and root (n=4) for *E. sativa* Nemat grown in each of four nutrient conditions, taking into account the dry weight of each plant. **b)** The mean dry weight of Nemat plants grown in each of four nutrient conditions. Leaf biomass is again a good indicator of total glucosinolate content.

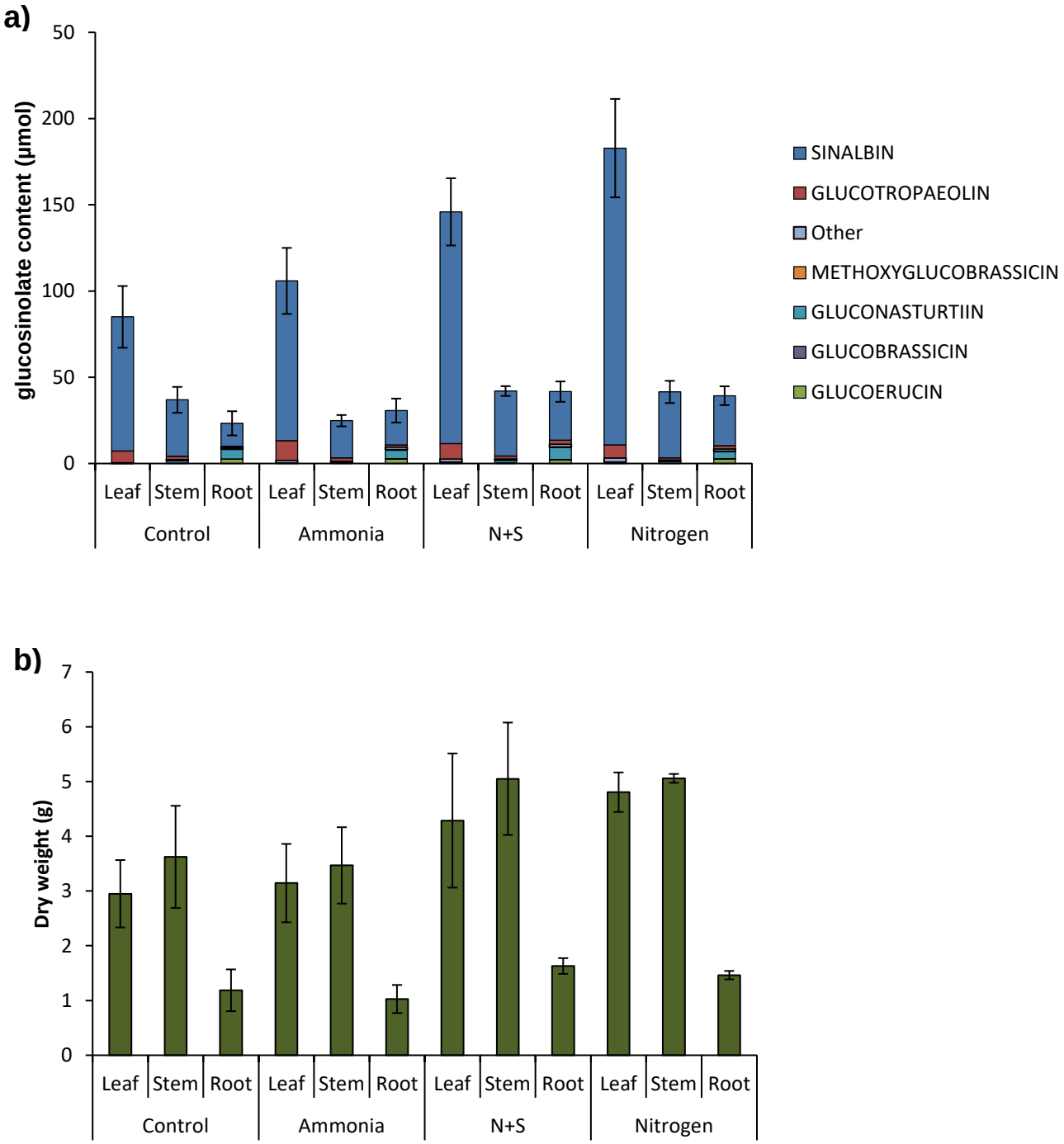


Figure 33: a) Total glucosinolate content per plant of leaf, stem and root (n=4) for *S. alba* Ida Gold grown in each of four nutrient conditions, taking into account the dry weight of each plant. **b)** The mean dry weight of Ida Gold plants grown in each of four nutrient conditions. Leaf biomass is a general indicator of total glucosinolate content. The increase in total leaf glucosinolate in the presence of nitrogen supplementation reflects an increase in the major glucosinolate sinalbin (significant only for the single nitrogen treatment relative to control, p=0.0287; One-way ANOVA with Dunnett's post hoc test), with the minor glucosinolates not increasing proportionally.

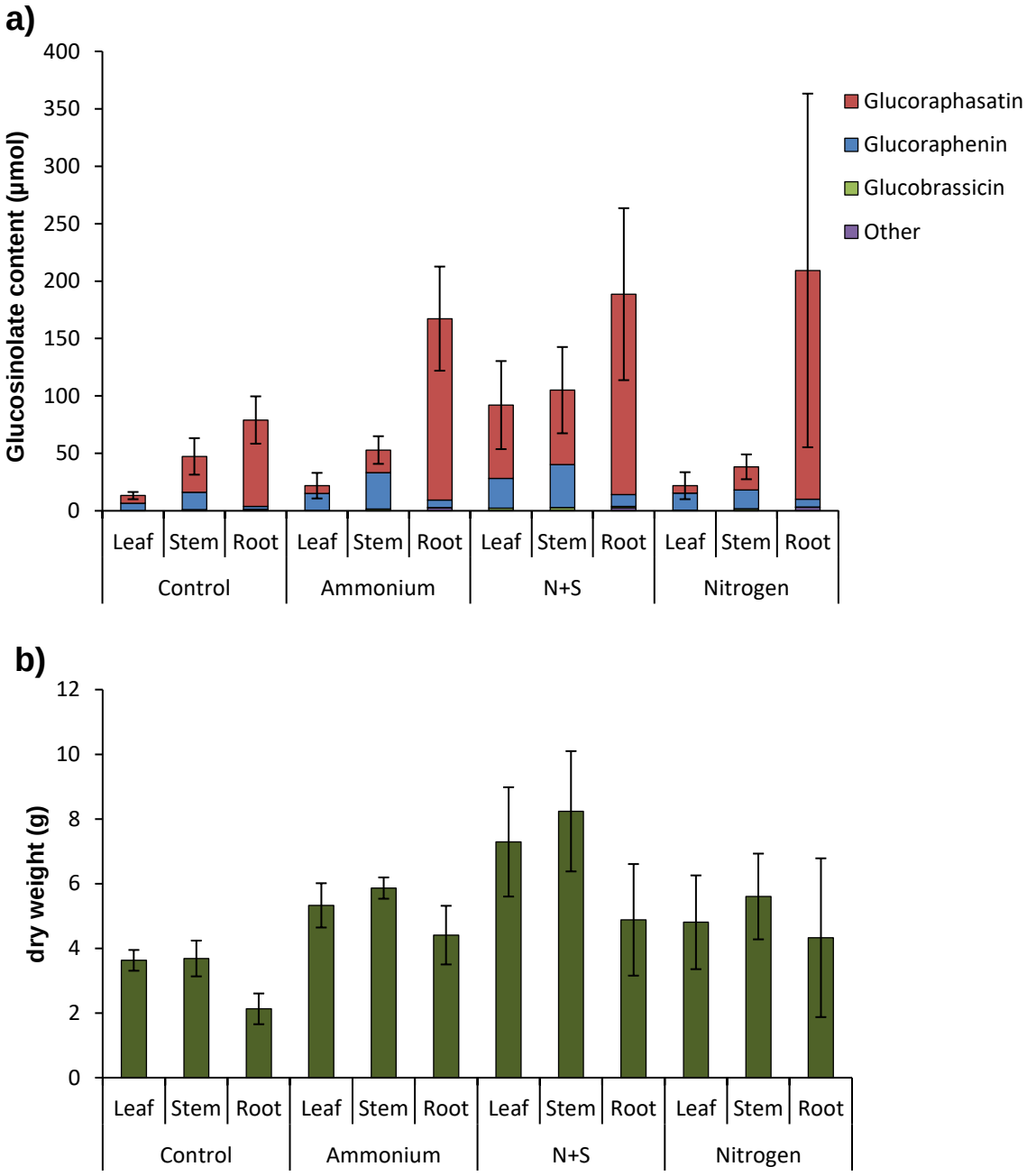


Figure 34: a) Total glucosinolate content per plant of leaf, stem and root (n=4) for *R. sativus* 'Bento' grown in each of four nutrient conditions, taking into account the dry weight of each plant. **b)** The mean dry weight of Bento plants grown in each of four nutrient conditions. For this cultivar, biomass does not appear to correlate so clearly to glucosinolate content.

Interestingly, although for most of the biofumigants the leaf tissue contributed the greatest proportion of the total plant glucosinolate, for *R. sativus* roots made the largest contribution, despite not providing more biomass than either stems or leaves in each condition. Despite optimisation and improvement of the extraction technique for radish to reduce variability between samples, there was still large variation in total glucosinolate between plants, particularly for the roots, although this largely reflects the variation in root weight.

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2.6. Effect of soil type and moisture - Raised bed trial

A raised bed trial was carried out at the University of Leeds farm to investigate the effects of soil type, and moisture level at incorporation, on the efficacy of biofumigation for control of *G. pallida*. Soil moisture may affect breakdown of glucosinolates and the permeation of isothiocyanate through the soil matrix, whilst soil type may affect levels of glucosinolates and/or biofumigation efficacy.

There are two raised beds each 15m x 2m. Each bed can be divided into 2 for different treatments and has an automated rain shelter that can be activated upon sensing rain. For this trial, each bed had a different soil type in the two halves and the two beds were used for the two different soil moisture regimes (Fig. 35).

B. juncea ISCI99 was planted in two soil types, A (Clay Loam; 27% sand, organic matter 6%, total N = 0.33% and B (Sandy Loam; 73% sand, organic matter 12%, total N = 0.31%) at a density of 100 plants/sq metre. The trial was set up mid-August and incorporation occurred late November/early December. Incorporation took place at each of two soil moisture levels, 10% and 30%. *G. pallida* cysts (50) in mesh bags were inserted at planting (six locations in each bed; 30 cm depth) and removed at incorporation. Additional bags of cysts (4 for each condition) were inserted into large pots containing the same soil and placed alongside each bed to act as controls. A second set of cysts was put in the soil at incorporation and removed for analysis after 5 months. Control cysts were again buried in large pots of soil that did not have plant material added but were at the appropriate soil moistures.

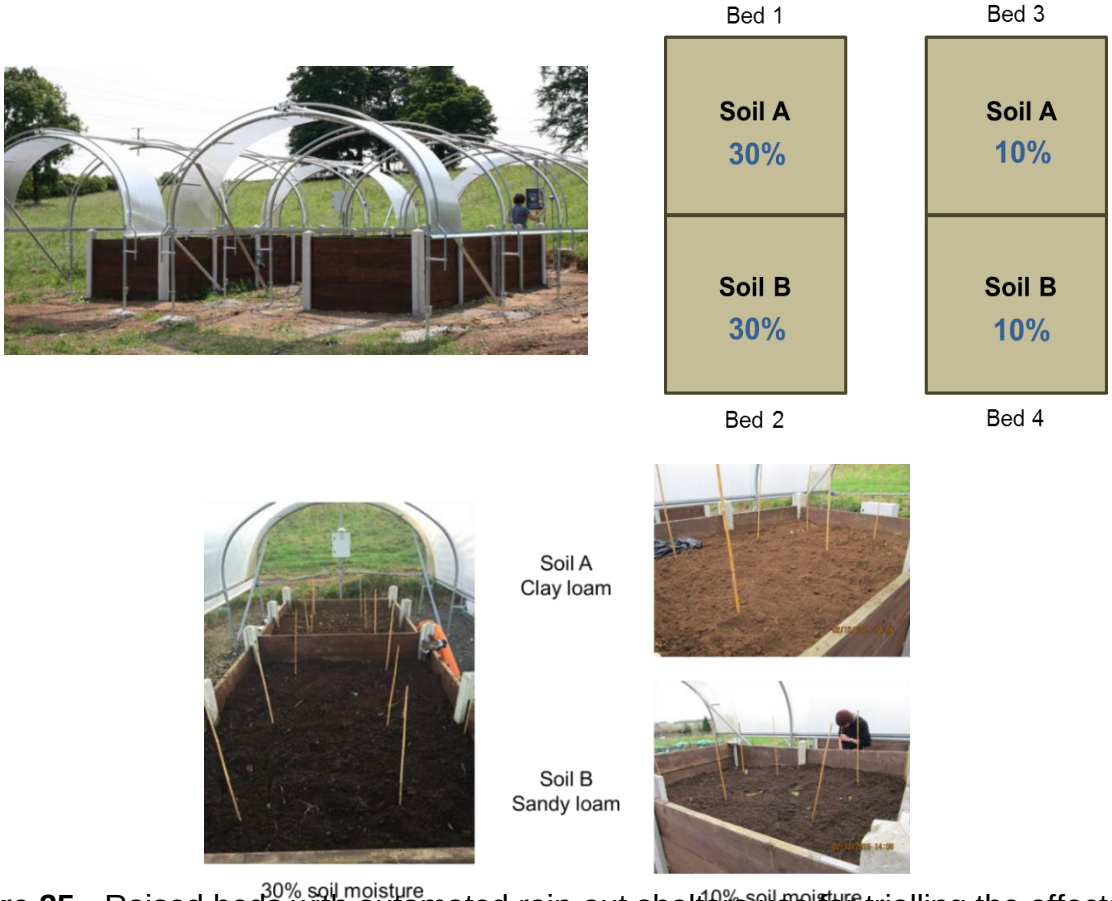


Figure 35: Raised beds with automated rain-out shelters use for trialling the effects of soil moisture (10% or 30%) and soil type on biofumigation efficacy of *B. juncea* ISCI99. Each full bed contained the two soil types (A-Clay loam and B-Sandy loam) and was maintained at one of the two soil moistures for incorporation.

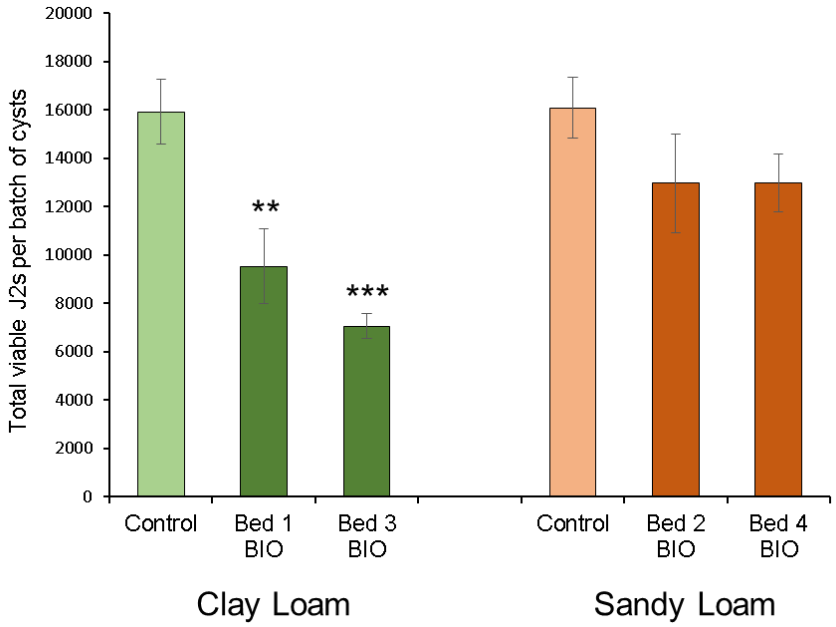


Figure 36: Total viable J2s per batch of 50 cysts that had been present in bags in the soil during growth of the biofumigant ISCI99. BIO = cysts in presence of the biofumigant. Control = cysts buried in the same soil type with no plants. n = 6; ** = P<0.01, *** = P<0.001 in comparison to the control for the same soil type.

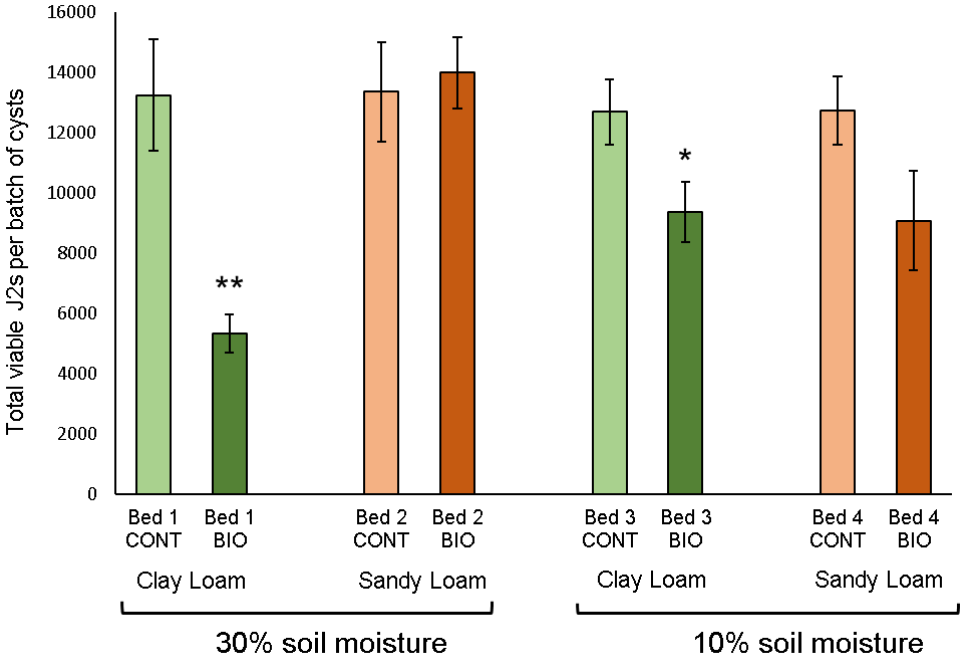


Figure 37: Total viable J2s per batch of 50 cysts that were buried in the soil at the point of biofumigant incorporation and removed 5 months later. BIO = cysts in presence of the biofumigant. Control = cysts buried in the same soil type with no plants. n = 6 (treatment) and 4 (control); * = P<0.05, ** = P<0.01 in comparison to the control for the same soil type and soil moisture.

There was a significant decline in viable J2 for those cysts present in the clay loam soil during growth of the ISCI99 plants, compared to the cysts in non-planted soil and this was observed for both beds containing that soil type (Fig. 36). However, the small reduction in viability for cysts in the sandy loam soil was not significant. When the cysts that had been buried in the soil at incorporation were analysed, a significant effect was again seen in the clay loam soil, but not in the sandy loam soil (Fig. 37). This effect was greater when incorporation took place at 30% soil moisture than at 10 % soil moisture.

2.7. Effect of soil temperature

The soil temperature at time of biofumigant incorporation may potentially influence the observed efficacy by affecting the breakdown of plant material, hydrolysis of glucosinolates by myrosinase, or the subsequent diffusion and degradation of isothiocyanates. The plan in the original proposal was to assess this using the raised beds that have the option of undersoil heating.

Before the trials could be started, the performance of the undersoil heating system was evaluated. i-button data loggers were inserted into the soil at 2 depths (12 cm and 35 cm) and five bed locations to monitor soil temperature, based on the assumption that temperature may be more stable in the centre of a bed than near the edge. Temperature was measured every hour from 19th August – 29th October and the under soil heating, set to 18 ºC or 12 ºC, was started on 17th September. Figure 38 shows typical data for one position in the bed set at 18 ºC, covering the period when the heating was switched on. Application of heating clearly altered the temperature profiles and raised the soil temperature at 35 cm depth, but also caused a more pronounced diurnal fluctuation in temperature. The temperature at 12 cm did not show a sustained increase and also became less stable.

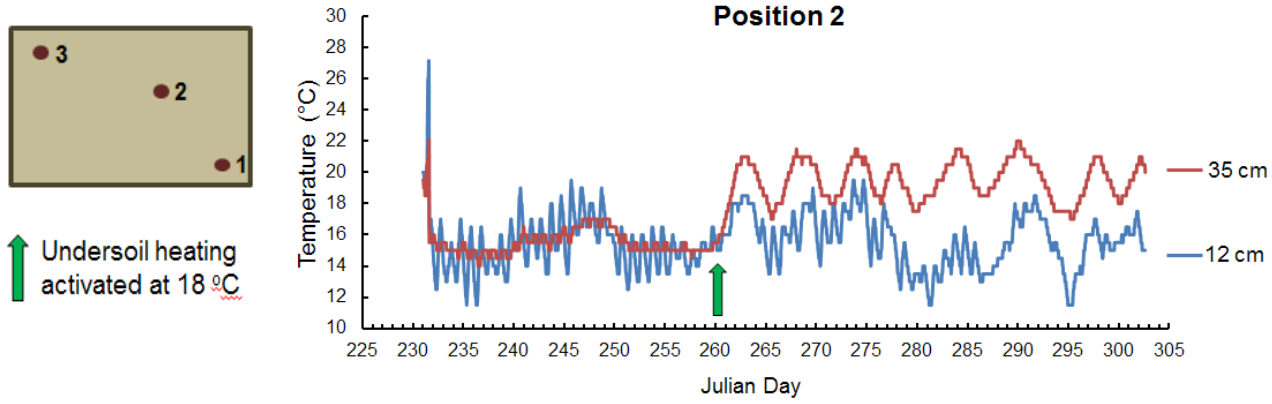


Figure 38: Hourly temperature recorded at 12 cm and 35 cm soil depth in a raised bed with under-soil heating set to maintain 18 ºC from Julian Day 260 (indicated with green arrow). The data was recorded in Position 2 of the bed as indicated on the schematic diagram on the left.

The temperature difference between the two soil depths also generally displayed a diurnal rhythm. Position in the raised bed also affected the temperature difference between the two soil depths as shown in Figure 39.

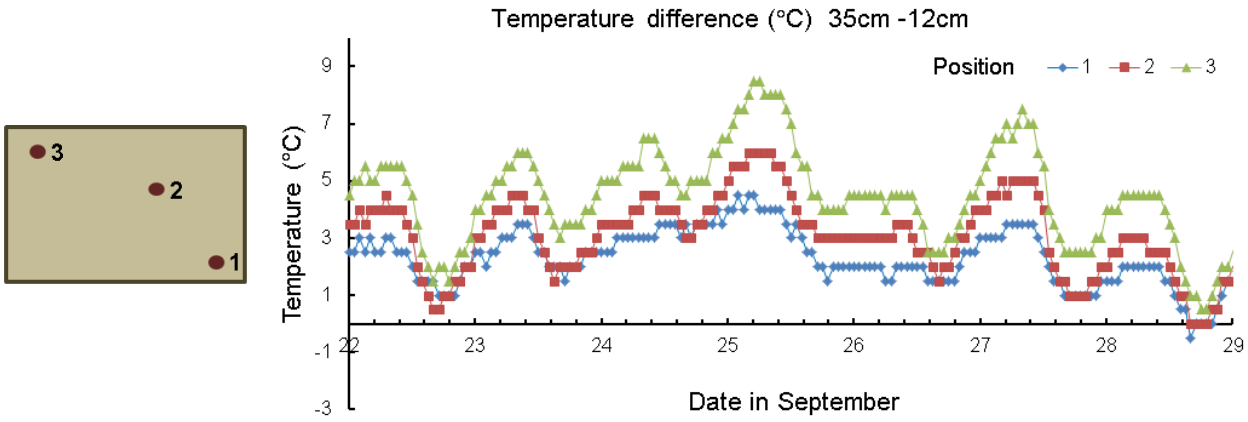


Figure 39: Temperature difference between 12 cm and 35 cm soil depth in a raised bed with under-soil heating set to maintain 18 ºC. The three lines on the graph represent hourly data recorded in the three different positions of the bed as indicated on the schematic diagram on the left.

It was concluded that the beds did not provide the required level of control for carrying out the soil temperature experiments over the desired range of temperatures. Therefore a glasshouse/pot assay trial was carried out using plant growth cabinets to achieve five consistent soil temperatures (10, 12.5, 15, 17.5, 20 ºC). This had the additional advantage of allowing us to test a wider range of temperatures than would have been possible in the raised beds.

B. juncea ISCI99 was grown in large pots in the glasshouse as for the nutrition trial with 100 plants/m^2 and N/S amendment of 100 kg/ha N/30 kg/ha S (Sulphomex + Nitram). At flowering, all green tissue (approx. 500g) and roots from each pot were chopped and mixed into the soil from that pot which was watered to approximately 15% soil moisture prior to incorporation. The amended soil was then used to fill 6 x 7" pots that were watered to 30% soil moisture and maintained at that level for the remainder of the experiment. A cyst bag containing 40 cysts of *G. pallida* was buried in each pot during filling. Six replicate, uncovered pots were set up for each of the five temperatures and placed in plant growth cabinets. Six controls were set up for each temperature in pots of soil that had not been amended with biofumigant plant tissue. All cyst bags were removed after 12 weeks, the cysts retrieved and hatching assays carried out to assess relative J2 viability.

Viability of J2s in the control treatments showed a significant linear trend with increasing temperature ($P<0.0001$) (Fig. 40). As the soil temperature increased, there was a drop in viable J2s. Possibly there had been spontaneous hatch of J2s during the 12 weeks buried in soil at the higher temperatures. A similar, but less significant ($P=0.002$) trend was observed for the cysts that had been exposed to the incorporated mustard tissue. There was generally high variability between replicates of the same treatment. Therefore when comparisons between control and biofumigant treatment were compared for each temperature, the only significant differences were at 17.5 ºC and 20 ºC, when the number of viable J2s in the control samples was already low. However, the results at the two temperature were contrasting, with a negative effect on viability only observed at 20 ºC.

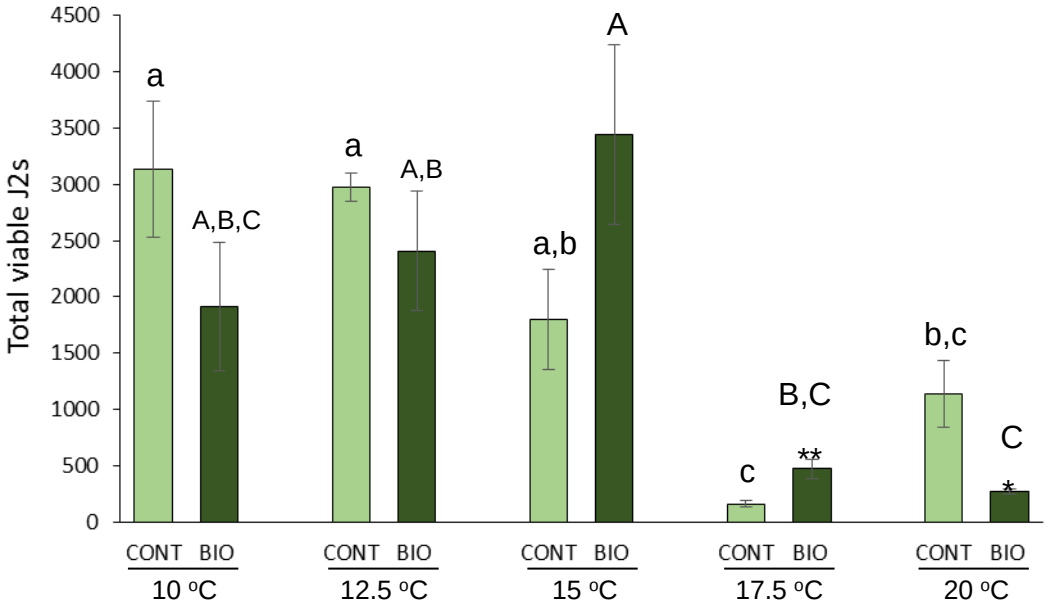


Figure 40: Total viable J2s per batch of 40 cysts that were buried in the soil at the point of biofumigant incorporation and removed 12 weeks later. BIO = cysts in presence of the incorporated biofumigant. CONT = cysts buried in pots of soil with no biofumigant incorporation at the same temperature. n = 5-6; * = P<0.05, ** = P<0.01 in comparison to the control for the same temperature (t-test). Different letters indicate significant differences between the same treatments at different temperatures (One-way ANOVA with Tukey's post hoc test).

2.8. Objective 3: Characterise novel compounds in plant accessions shown to be effective against pests and pathogens.

Isothiocyanates (ITCs) have generally been regarded as the most toxic glucosinolate breakdown products from brassicaceous plant tissues and are considered to be the active agents in biofumigation-mediated pest suppression. However, recent field trials have demonstrated that allyl ITC does not affect nematode assemblages (Vervoort *et al*. 2014). Other work has highlighted that pest suppression arising from mulching brassicaceae may be influenced by changes in the soil microbial community rather than direct ITC toxicity (Weerakoon *et al*. 2012).

Our own work has suggested that some brassica accessions (e.g. *E. sativa* cv Nemat and *R. sativus* cv Weedcheck) contain as yet unidentified, potent compounds that adversely affect nematodes (Fig. 41). In addition, it is probable that brassicas release volatile halogen (methyl iodide, bromide and choride; collectively methyl halides) and sulphur-containing compounds, such as dimethyl sulphide (DMS) and dimethyl disulphide (DMDS), all of which are known to be toxic and may also play a role in pest suppression.

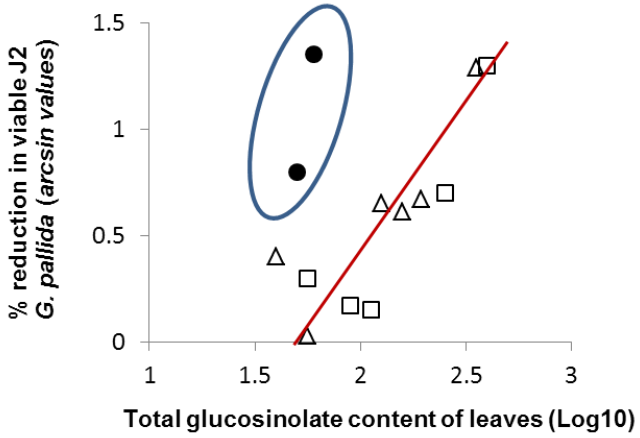


Figure 41: Correlation between total glucosinolate content of leaves and viability of *G. pallida* J2s after exposure to a range of plant extracts. For most plants there is a linear relationship between glucosinolate content and efficacy (red line) but two brassica cultivars (circled in blue) have efficacy greater than would be expected from their glucosinolate content.

Outside of the brassicas, a number of plant species are purported to have nematicidal effects, including marigolds, *Tagetes* spp. and lupins, *Lupinus* spp. Given the time, effort, and cost associated with testing the ability of green manures to suppress plant-parasitic nematodes, development of a simple method to assess the potential of a plant to act as a biofumigant would allow for the rapid screening of various plant accessions for their biofumigation potential. A quick, affordable assessment of biofumigation potential could hasten and reduce wastage in the process of trialling crop species for biofumigation. Reporter organisms that respond to stimuli associated with biofumigation, such as the presence of bioactive isothiocyanates, can be used as a marker for biofumigant activity.

The use of *C. elegans* reporter strains that respond to Dazomet and isothiocyanates was therefore investigated as a rapid screening tool for a wide range of non-standard, potential biofumigant plants. These strains made use of the xenobiotic metabolic elements involved in detoxification of isothiocyanates. The enzymes most closely associated with detoxification

of isothiocyanates are glutathione S-transferases: for example exposure to Dazomet induced the specific up-regulation of a *C. elegans* glutathione S-transferase, GST-31. The response of ITC-specific reporter lines to exposure to a given plant extract can therefore be used as an indicator of the presence or absence of ITCs in that extract.

A reporter strain that utilised the promoter from the *C. elegans gst-31* gene linked to green fluorescent protein responded to Dazomet which releases methyl-ITC, and a range of pure isothiocyanates.

Having confirmed the response of the reporter line to pure isothiocyanates, nematodes were exposed to a range of plant leaf extracts. Response was specific to brassicaceous leaf extracts, including the common biofumigants *B. juncea* and *S. alba* as well as more obscure members of the brassicaceae such as *Biscutella didyma* (shield mustard) and *Erucastrum gallicum* (dog mustard), which all induced up-regulation of the *gst-31*::GFP reporter (Fig. 39). However, no response was seen to non-glucosinolate containing plants such as tomato (*S. lycopersicum*), cotton (*Gossypium hirsutum*) or wheat (*Triticum aestivum*) (Fig. 42). Induction of GFP was dose-dependent, indicating that the intensity of fluorescence observed may be indicative of the isothiocyanate concentration of the extract and therefore glucosinolate content of the extract (Fig. 43). Differential responses to leaf extracts of the same concentration from different brassicas could therefore be indicative of the total glucosinolate content of the leaves of tested plants.

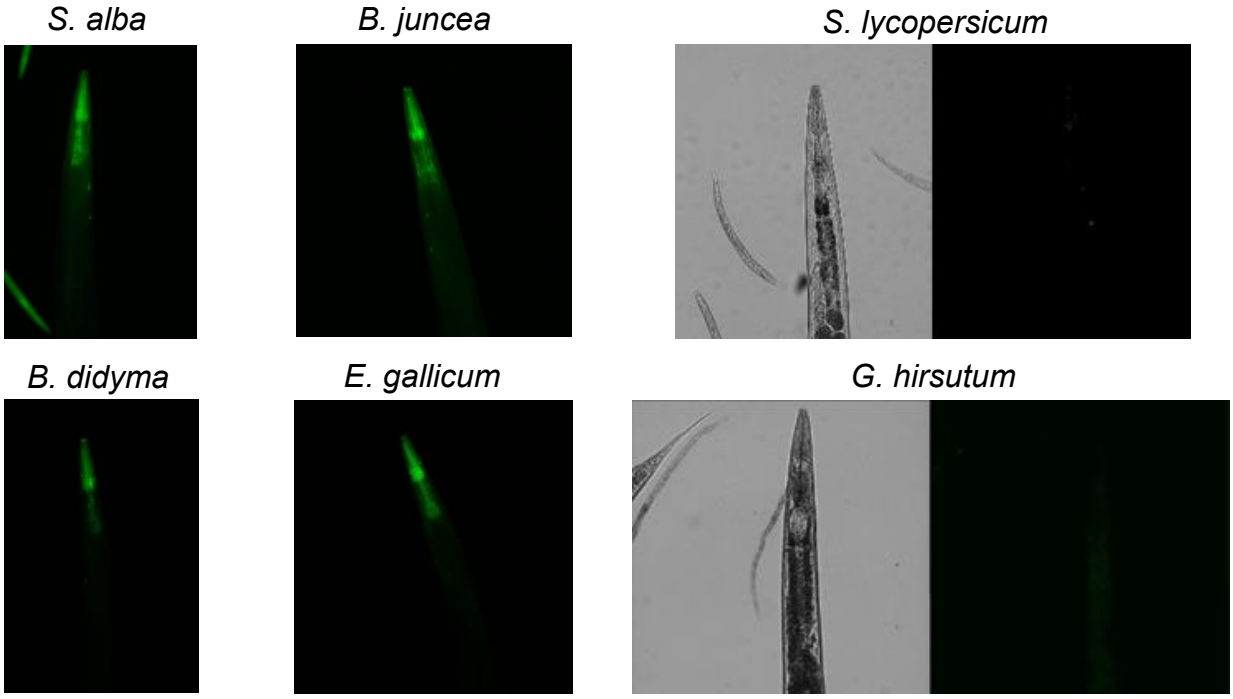


Figure 42: *C. elegans gst-31-GFP* reporter strain responds specifically to Brassicaceous green tissue extracts with the green fluorescence apparent mainly in the nematode pharynx. No fluorescence is observed upon exposure to extracts of tomato and cotton. All nematodes were exposed for 8 hours to 1.8 mg/ml plant extracts prepared from freeze-dried green tissues that were then extracted in sterile water to the appropriate concentration and filtered before use.

The relative level of induced GFP expression in the *C. elegans* reporter strains was determined by measuring pixel intensity of microscope images captured using the same

exposure settings. It was found that the GFP induction response correlated well with concentration of the plant extract (Fig. 43).

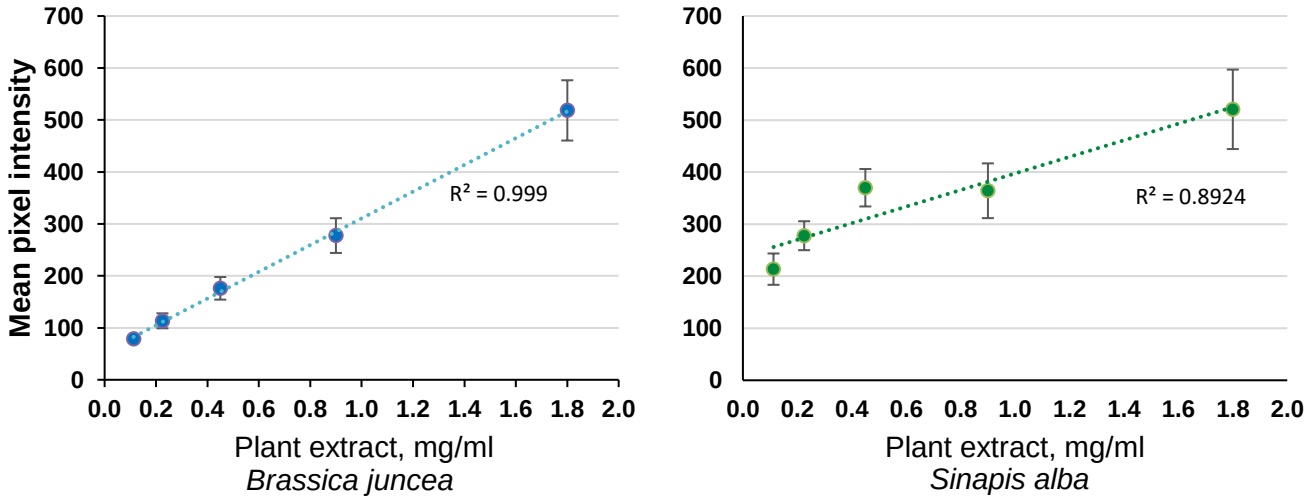


Figure 43: Induction of GFP expression in the *C. elegans gst31::GFP* reporter strain as measured by pixel intensity correlates significantly with the concentration of Brassicaceous leaf extract applied to the nematodes after 16h incubation. Exposures were performed in triplicate and at least 5 representative images from each repeat were measured for pixel intensity.

ITC release from biofumigants at incorporation

The ability to estimate ITC release from biofumigation and its impact on soil pests in a field setting is hampered by complex soil and gas diffusion dynamics. Typically, ITC release from biofumigation is directly measured from soil extracts using ethylacetate or dichloromethane. ITC values can range from less than 1% to more than 50% of what is expected based on the GSL concentration of the biofumigant and are reported to persist from less than 24 hours to 5 days. The half-life of methyl isothiocyanate, a related synthetic compound, is also highly variable, ranging from 0.8d to 12d depending on soil characteristics.

In collaboration with Harper Adams, we carried out the first flux measurements of isothiocyanates from soils following incorporation of a biofumigant. Volatile flux samples from soils incorporated with *B. juncea* tissue were taken 2-8 minutes, 1 hour, 4 hours and 24 hours post mulching. Fluxes of allyl isothiocyanate (AITC), dimethyl sulphide (DMS), dimethyl disulphide (DMDS), methanethiol, isoprene, and a number of methyl halides were looked for but only AITC, DMS and DMDS showed any significant effect and are shown in Figure 44.

Previously published results from ITC extractions from soil using ethylacetate have demonstrated that the highest proportion of ITCs is released shortly after mulching and decreases thereafter. We were unable to detect any AITC in our soil samples frozen 12 and 24 h post mulching, and this pattern is reflected in the AITC volatilising from the soil (Fig. 44a) which reduces quickly and is undetected at 24 h post mulching. The area below the curve represents the total volatilised AITC. At four hours more than half the total measured AITC has volatilised and at 24 hours 2 of the 4 plots analysed have slightly negative fluxes ($-2x10^{-3}$ to $-4x10^{-3}$ g/m^2/day). DMS and DMDS fluxes, on the other hand, increase over time with the highest fluxes measured at 24 hours (Fig. 44 b and c). Total dose of DMS and DMDS is unknown as measurements past 24 h were not taken due to technical limitations.

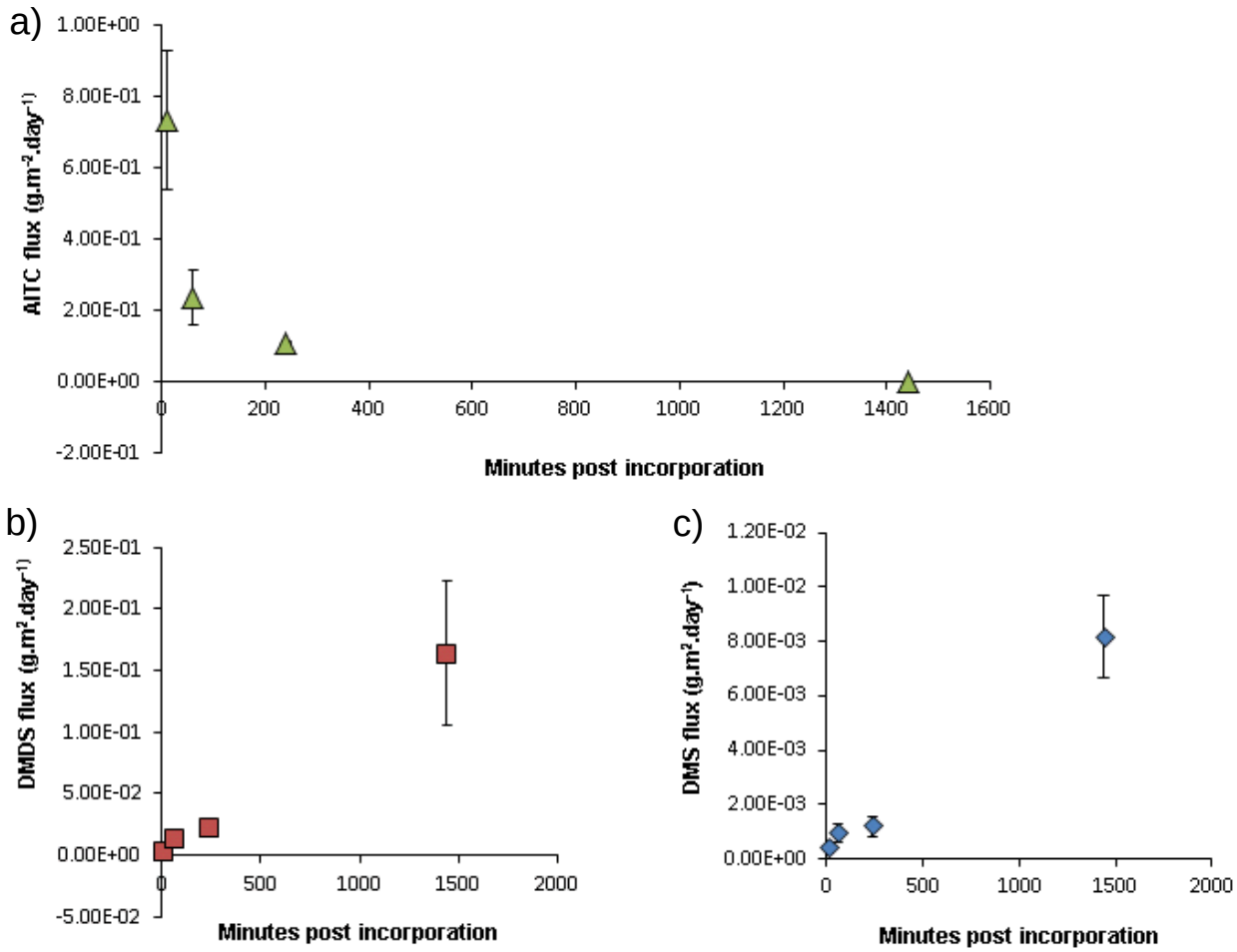


Figure 44: Soil flux measurements of a) Allyl isothiocyanate (AITC; sinigrin); b) Dimethyl disulfide (DMDS); c) Dimethyl sulfide (DMS)from a field at 5, 60, 240 and 1440 minutes after incorporation of *B. juncea* ISCI99.

Given the rapid loss of ITCs from the soil after biofumigant incorporation, it is possible that compounds such as DMS and DMDS, which increase over time, make an equal or greater contribution to the observed toxicity to nematodes and/or other pests and pathogens.

It was unlikely that a “broad scan” analysis would identify compounds of interest. Also, given the “biofumigation effect” that has been detected during growth of Brassicas before mulching and incorporation, we decided to carry out a targeted analysis of likely compounds that are released from growing roots and leaves, focusing on those such as DMS/DMDS together with a range of volatile halide compounds that may have pesticidal activity. A range of both Brassica and non-Brassica (lupin, marigold) plant species were analysed, based on previous reports of their bioactivity. *Eruca sativa* “Nemat” and *R. sativus* “Weedcheck” were included in the analysis, based on our previous work.

Plants were grown in a 9:1 sand:compost mix throughout their lifecycle and watered three times a week with a modified Hoagland’s No.2 Solution (6.5 mM potassium nitrate, 4.0 mM calcium nitrate, 2.0 mM ammonium dihydrogen phosphate, 2.0 mM magnesium sulphate, 4.6 µM boric acid, 0.5 µM manganese chloride, 0.2 µM zinc sulphate, 0.1 µM ammonium heptamolybdate, 0.2 µM copper sulphate, and 45 µM iron(III) chloride; supplemented with 50 mM sodium chloride, 0.5 mM sodium bromide, and 0.05 mM potassium iodide). The

addition of chloride, bromide, and iodide salts was intended to prevent depletion of the halides from the medium in the pots.

In order to sample the airspaces of the above and below-ground parts of each plant, a two-chambered sampling box was used (Fig. 45). The lower chamber was formed of an opaque PVC base with a clear acrylic lid, with a total volume of 10.01 L; the clear acrylic upper chamber gave a sampling volume of 27.25 L. Plants were checked for physical damage or visible signs of disease or malnourishment, and only those plants that were outwardly healthy were sampled. Plants were carefully removed from pots and the roots were washed of soil, taking care not to damage root tissue. The roots were then patted dry and the fresh weight of each plant was recorded.

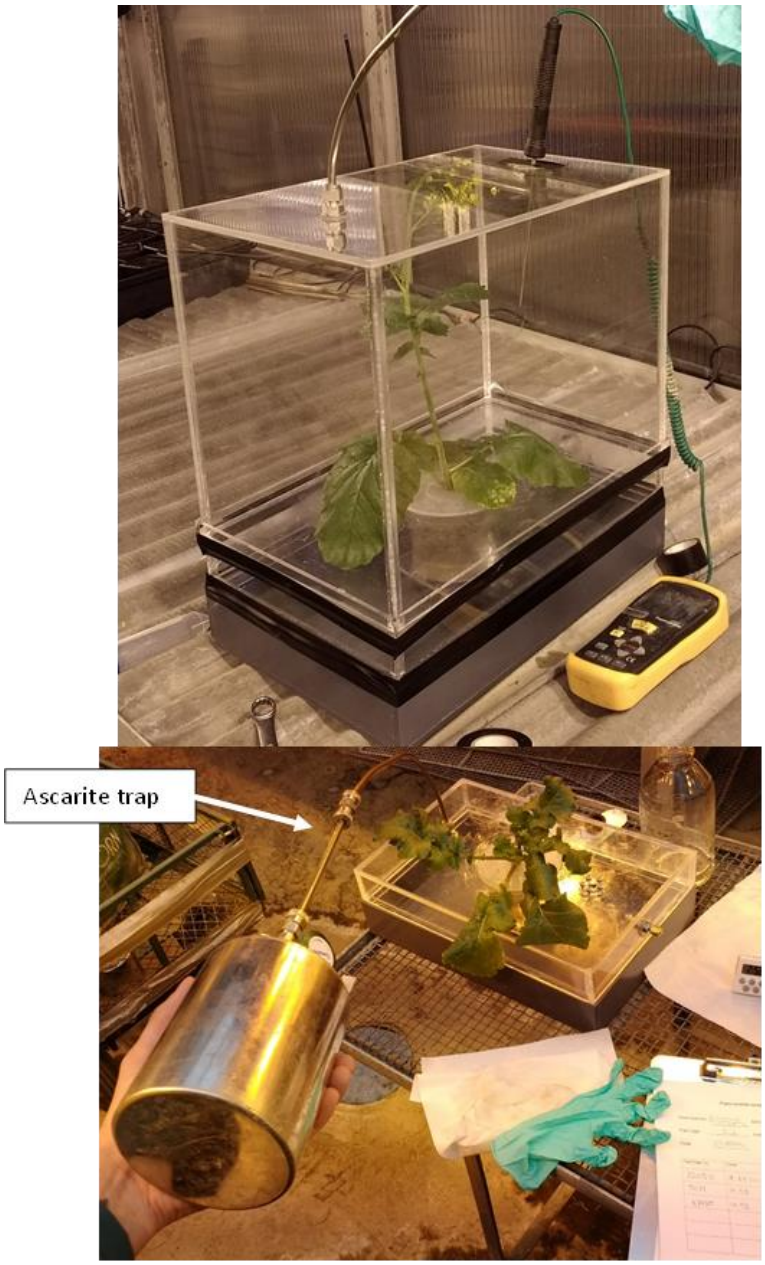


Figure 45: Plant volatile sampling chamber. The sampling chamber is depicted in use: **Top,** the assembled sampling chamber set up to sample volatiles from the aboveground biomass of the plant; **Bottom,** sampling volatiles from the belowground biomass, with the canister and Ascarite trap in place. The hole through which the roots of the plant are separated from

the aboveground biomass is covered with a silicon pad and sealed with silicone vacuum grease, to ensure separation of sampling.

Plants were first placed into the root sampling chamber, with the roots kept in a pot of water to prevent wilting. A silicone septum was placed around the root-shoot junction and silicone vacuum grease was used to create an airtight seal. The temperature within the chamber was recorded, using a probe inserted through a hole in the lid of the chamber. Samples were taken 2 minutes, 11 minutes, and 20 minutes after placing the plant into the root chamber by affixing a canister to the glass-coated, stainless steel sampling line, via a length of sampling line filled with Ascarite, and turning the valve to open the canister, allowing air within the chamber to be drawn by pressure differential. The Ascarite trap was constructed using a short length of glass-lined steel tubing filled with Ascarite II Adsorbent, 20-30 mesh sodium hydroxide-coated silica granules with a piece of coiled silver wire blocking each end, and served to remove moisture and carbon dioxide from the sampled gas as it was drawn into the sampling canister.

The upper chamber was then placed on top of the root chamber, enclosing the aboveground biomass of the plant within. The glass-coated sampling line and Ascarite trap were once more affixed to the sampling port and a temperature probe was inserted through a separate hole on the top of the chamber. Sampling followed the same procedure as with the root chamber, with the chamber temperature recorded at the time of each sampling. The ambient temperature was also noted throughout sampling. The concentrations of gases in the canisters were analysed on an HP GC/MSD fitted with a PoraPlot Q column (25 m, 0.32 ID, 5 µm thickness).

After sampling, the plants were dried and the root and aboveground biomass components were separated and placed in paper bags in a 65°C incubator to dry. After 4 days in the incubator, plant roots and shoots were separately weighed and returned to the incubator. Samples were weighed again after 24 hours, and if the mass had not changed through subsequent drying, this was recorded as the dry biomass of that sample.

Both roots and shoots of the Brassicas emitted volatile compounds with potential nematicidal activity such as methyl iodide, methyl bromide, chloroform and dimethyl sulphide, although the compounds tested for were not detected at significant levels from lupin and marigold. The rate of volatile emission was calculated as the amount produced per unit tissue biomass per day. Methyl iodide was the most abundant compound detected, present at 10-100-fold the level of methyl bromide. In general, volatile emission was higher from shoot tissue than from root tissue, with the exception of *E. sativa* ‘Nemat’ that emitted very low levels of both methyl iodide and methyl bromide.

Volatile production by B. juncea ‘ISCI99’

The profile of gases emitted by the brown mustard cultivar ‘ISCI99’ was found to change with the growth stage of the plant (Fig. 46). The flux of methyl chloride from the roots decreased over successive growth stages, while from the aboveground biomass it went from a negative flux during vegetative growth to a positive flux during flowering, then returned to a negative flux in senescence. Methane thiol fluxes varied from sample to sample but were generally positive, and increased from the roots in senescent plants. The output of methyl bromide was generally positive, decreasing over successive growth stages from the roots, and peaking in production from the aboveground biomass in the flowering stage, at 0.51 ± 0.02 µg/g dry biomass/day. Methyl iodide production from the roots fell after the vegetative stage from 2.63 ± 0.82 µg/g biomass/day to 71.61 ± 0.37 ng/g biomass/day in flowering and 154.67 ± 69.31 ng/g biomass/day in senescence; flux from the aboveground

biomass was highest in the vegetative stage (12.48 ± 0.68 µg/g biomass/day), falling to less than half that value when flowering (5.73 ± 0.45 µg/g biomass/day), and then reducing further in senescence (79.42 ± 28.3 ng/g biomass/day). Dimethyl sulphide fluxes were negative in the vegetative plant stage, showed high variation in flowering plants, and became positive in senescent roots but with high variability (1.06 ± 0.76 µg/g biomass/day). Output of chloroform appeared high from vegetative stage roots, but with high variation (39.40 ± 74.15 µg/g biomass/day), falling and stabilising in flowering (8.09 ± 4.20 µg/g biomass/day) and senescent roots (11.89 ± 2.04 µg/g biomass/day).

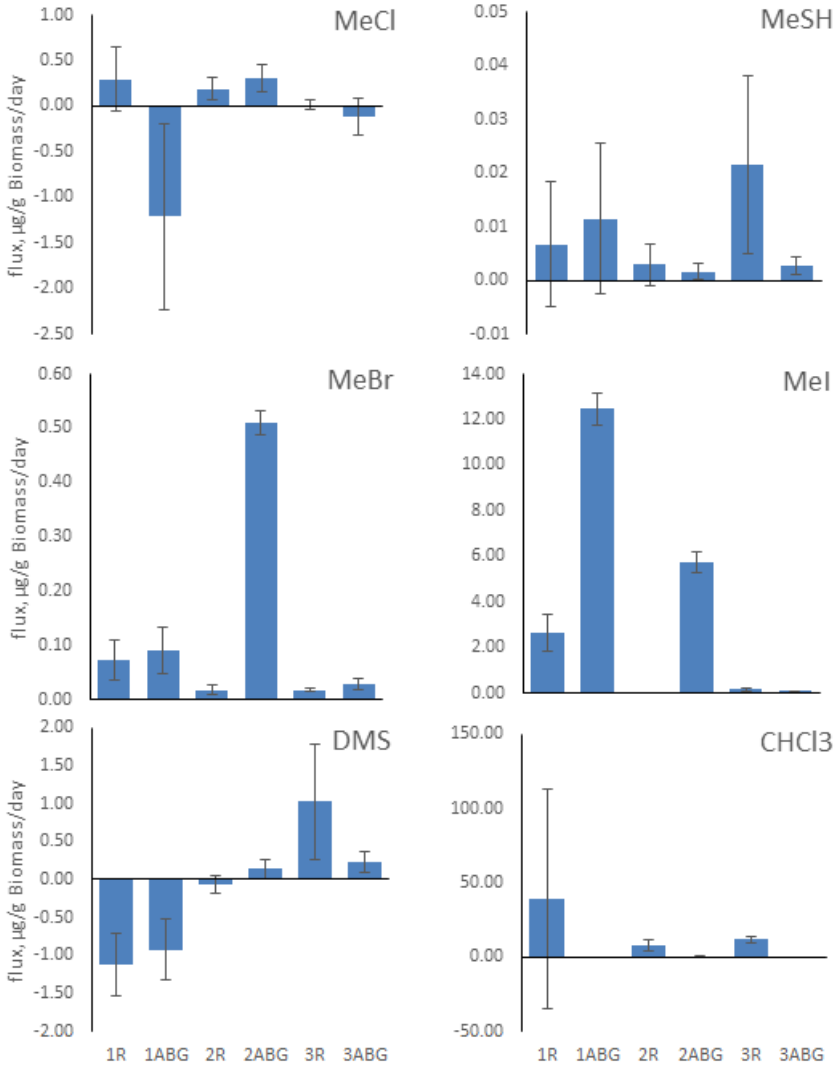


Figure 46: Gas fluxes from *Brassica juncea* cv. 'ISCI99' roots and aboveground biomass over 3 growth stages The change in concentration of gases over a 20 minute period was extrapolated to give a value of micrograms flux per gram plant dry biomass per day. Horizontal axis markers indicate growth stage (1 = vegetative growth, 6 weeks; 2 = flowering, 12 weeks; 3 = senescence, 18 weeks) and section of plant (R = roots; ABG = aboveground biomass). Error bars represent standard errors of the mean.

Volatile production by R. sativus 'Weedcheck'

Production of methyl chloride by the radish cultivar WeedCheck was variable in the roots of vegetative stage plants (0.31 ± 0.64 µg/g dry biomass/day) but stable in the aboveground biomass (Fig. 47). Methane thiol emissions were on the nanogram scale in all samples apart from vegetative stage roots, where there was high variation. Methyl bromide production in the aboveground biomass peaked in flowering plants (0.49 ± 0.04 µg/g dry biomass/day); root production peaked in senescence (0.08 ± 0.07 µg/g dry biomass/day). Methyl iodide production was highest per gram biomass in the vegetative stage and declined thereafter. A similar trend was observed in the production of DMS from roots, whereas a negative flux observed in the aboveground biomass of vegetative plants became positive in later stages. Production of chloroform was principally observed in the roots of vegetative plants (59.87 ± 14.05 µg/g dry biomass/day).

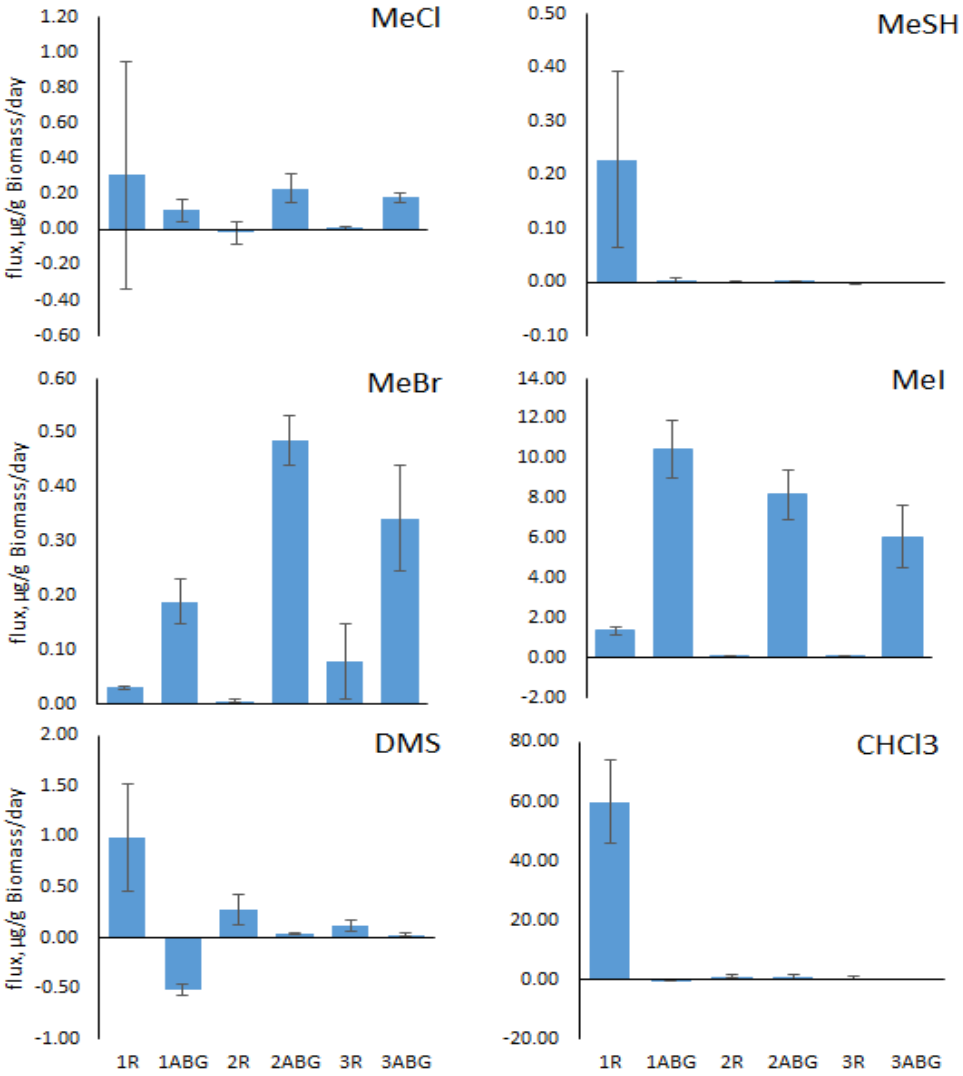


Figure 47: Gas fluxes from *Raphanus sativus* cv. 'WeedCheck' roots and aboveground biomass over 3 growth stages. The change in concentration of gases over a 20 minute period was extrapolated to give a value of micrograms flux per gram plant dry biomass per day. Horizontal axis markers indicate growth stage (1 = vegetative growth; 2 = flowering; 3 = senescence) and section of plant (R = roots; ABG = aboveground biomass).

Error bars represent standard errors of the mean. n = 3 for growth stages 1 and 3; n = 5 for the flowering stage.

Volatile production by R. sativus 'Diablo'

The trends observed in *R. sativus* cv. 'Diablo' were similar to those in 'WeedCheck,' with some notable differences (Fig. 48). A negative methyl chloride flux was observed in vegetative stage roots, while the aboveground biomass reported positive fluxes at all stages. Methane thiol levels were low in all stages, with high variability. Methyl bromide production peaked in flowering stage plants at nearly double the value observed in 'WeedCheck,' but with high variability (1.16 ± 0.43 µg/g dry biomass/day). Methyl iodide release followed a similar trend to methyl bromide, aboveground biomass emissions peaking in flowering plants at 30.97 ± 11.14 µg/g dry biomass/day; production in the roots was highest per gram biomass in vegetative stage plants (1.15 ± 0.69 µg/g dry biomass/day). DMS emissions were negative in vegetative roots but positive in all other samples, with high variability. Chloroform production was again highest in the roots of vegetative stage plants (53.69 ± 28.31 µg/g dry biomass/day).

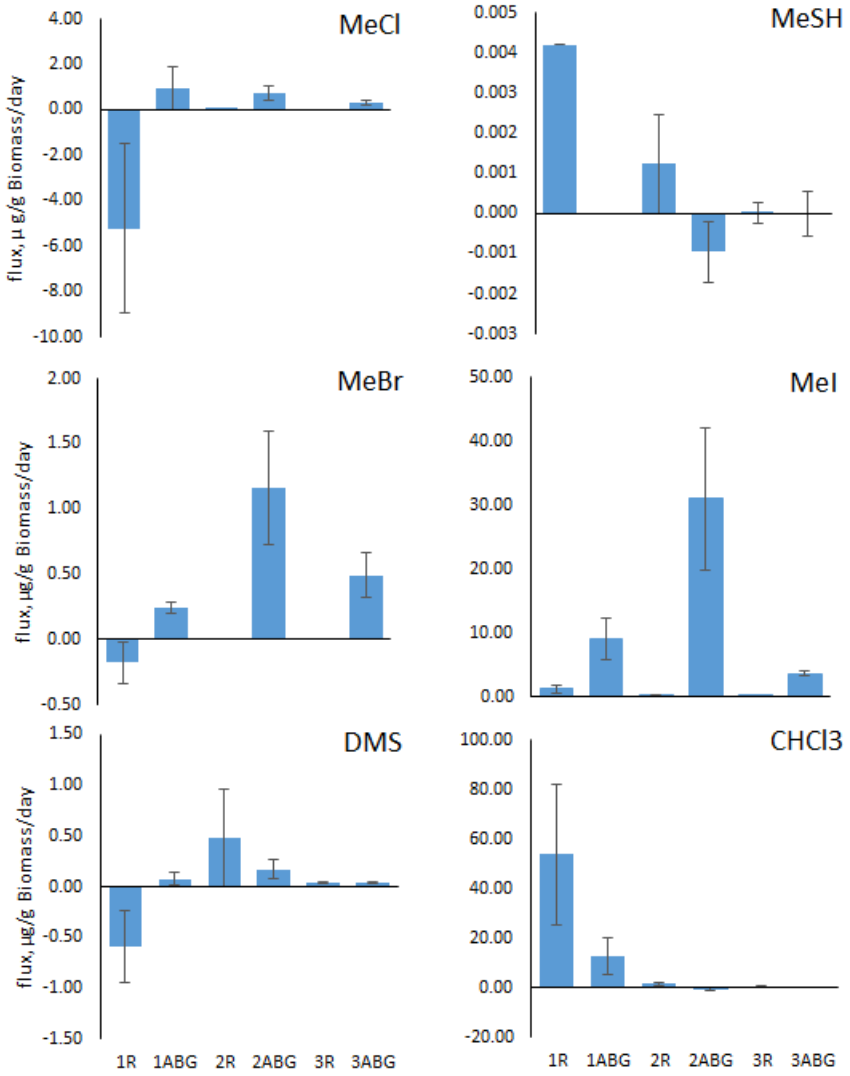


Figure 48: Gas fluxes from *Raphanus sativus* cv. 'Diablo' roots and aboveground biomass over 3 growth stages. The change in concentration of gases over a 20 minute period was extrapolated to give a value of micrograms flux per gram plant dry biomass per day. Horizontal axis markers indicate growth stage (1 = vegetative growth; 2 = flowering; 3

= senescence) and section of plant (R = roots; ABG = aboveground biomass). Error bars represent standard errors of the mean. n = 3 for all growth stages.

Volatile production by E. sativa 'Nemat'

The emissions profiles from *Eruca sativa* cv. 'Nemat' at different growth stages varied from those observed in *B. juncea* and *R. sativus* (Fig. 49). Methyl chloride flux was highly variable, giving error greater than the average value in vegetative and senescent plants, as well as in the roots of flowering plants. Methane thiol fluxes were in the nanogram range with high error (a single reading is given for vegetative stage above and belowground biomass, resulting in no error bars). Methyl bromide production peaked in both roots and the aboveground biomass at flowering (roots: 0.019 ± 0.009 µg/g dry biomass/day; ABG: 0.29 ± 0.18 µg/g dry biomass/day). Methyl iodide production from the roots was the highest in flowering stage plants of the cultivars tested, at 0.24 ± 0.07 µg/g dry biomass/day; production in the aboveground biomass peaked at flowering (10.02 ± 4.61 µg/g dry biomass/day). DMS fluxes were generally positive in the roots, and negative or near zero in the aboveground biomass. Production of chloroform followed a similar pattern to that observed in the other tested species: *E. sativa* vegetative stage roots gave the highest flux recorded, at 153.68 ± 84.74 µg/g dry biomass/day.

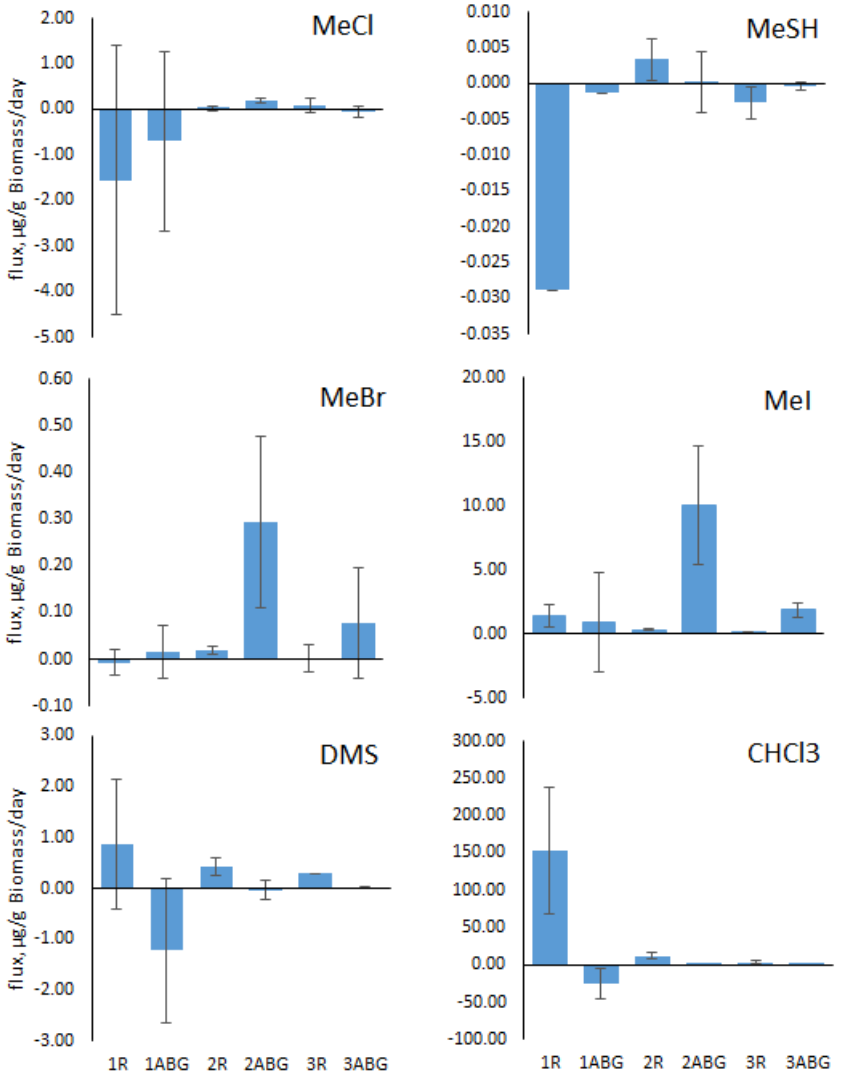


Figure 49: Gas fluxes from *Eruca sativa* cv. 'Nemat' roots and aboveground biomass over 3 growth stages. The change in concentration of gases over a 20 minute period was

extrapolated to give a value of micrograms flux per gram plant dry biomass per day. Horizontal axis markers indicate growth stage (1 = vegetative growth; 2 = flowering; 3 = senescence) and section of plant (R = roots; ABG = aboveground biomass). Error bars represent standard errors of the mean. n = 3 for all growth stages.

The fluxes of each compound from the roots and aboveground biomass at each growth stage were multiplied by the average dry biomass of those sections, to give an estimate of the average per-plant flux (Table 1).

Table 1 Total flux per plant, based on average dry biomass

Species and		Stage	Biomass	Average mass, g	Flux, µg/day per plant											
					MeCl	±	MeSH	±	MeBr	±	MeI	±	DMS	±	CHCl3	±
Brassica juncea	ISCI99	V	BBG	0.147	0.043	0.052	0.001	0.002	0.011	0.005	0.385	0.120	-0.165	0.060	5.778	10.876
			ABG	0.847	-1.027	0.869	0.010	0.012	0.077	0.036	10.562	0.580	-0.784	0.339	0.051	0.090
		F	BBG	0.963	0.182	0.117	0.003	0.004	0.017	0.009	0.069	0.004	-0.067	0.116	7.793	4.042
			ABG	3.420	1.036	0.519	0.006	0.005	1.742	0.077	19.583	1.547	0.469	0.446	0.742	0.805
		S	BBG	0.763	0.012	0.046	0.016	0.013	0.014	0.002	0.118	0.053	0.783	0.578	9.077	1.556
			ABG	8.150	-0.874	1.654	0.022	0.014	0.229	0.091	0.647	0.231	1.856	1.117	0.052	0.335
Raphanus sativus	WeedCheck	V	BBG	0.233	0.072	0.150	0.053	0.038	0.007	0.001	0.314	0.050	0.231	0.124	13.969	3.279
			ABG	1.453	0.155	0.098	0.004	0.007	0.274	0.060	15.198	2.078	-0.748	0.081	-0.540	0.112
		F	BBG	7.142	-0.141	0.469	-0.001	0.016	0.044	0.020	0.452	0.098	1.988	1.098	7.926	3.517
			ABG	16.034	3.680	1.292	0.010	0.007	7.803	0.721	131.230	20.018	0.615	0.204	13.441	11.719
		S	BBG	7.783	0.057	0.057	-0.018	0.017	0.616	0.544	0.299	0.345	0.923	0.399	4.824	3.422
			ABG	17.903	3.241	0.479	-0.003	0.002	6.132	1.745	108.639	27.902	0.556	0.184	1.474	0.280
	Diablo	V	BBG	0.423	-2.214	1.579	0.002	0.000	-0.076	0.066	0.487	0.291	-0.248	0.150	22.729	11.984
			ABG	2.533	2.353	2.434	0.000	0.000	0.618	0.105	22.987	8.106	0.199	0.176	32.614	18.598
		F	BBG	11.013	0.146	0.027	0.013	0.014	0.021	0.031	1.480	0.581	5.318	5.283	16.024	7.304
			ABG	14.783	11.018	4.337	-0.014	0.011	17.213	6.430	457.801	164.669	2.531	1.373	-9.513	8.716
		S	BBG	10.773	-0.121	0.065	0.000	0.003	0.052	0.026	0.367	0.058	0.414	0.069	7.048	1.664
			ABG	13.317	4.211	1.005	0.000	0.007	6.528	2.291	48.397	6.405	0.460	0.127	1.431	0.982
Eruca sativa	Nemat	V	BBG	0.157	-0.244	0.462	-0.005	0.000	-0.001	0.004	0.220	0.134	0.136	0.200	24.076	13.276
			ABG	0.883	-0.621	1.733	-0.001	0.000	0.014	0.049	0.766	3.417	-1.076	1.253	-21.890	18.098
		F	BBG	1.773	0.009	0.102	0.006	0.005	0.033	0.017	0.507	0.116	0.735	0.309	20.741	7.522
			ABG	6.110	1.216	0.288	0.001	0.026	1.790	1.115	61.230	28.152	-0.176	1.063	0.664	0.888
		S	BBG	2.180	0.190	0.323	-0.006	0.005	0.003	0.066	0.198	0.227	0.650	0.004	6.966	3.357
			ABG	7.273	-0.297	0.897	-0.003	0.004	0.558	0.859	13.248	3.853	0.097	0.168	1.698	0.418

Stage: V = vegetative, F = flowering, S = senescent; Biomass: BBG = belowground biomass (roots), ABG = aboveground biomass. n = 3 for all sampled plants expect WeedCheck F stage, where n = 5

Toxicity of volatile emitted compounds to nematodes

A nested-dish assay was developed to test the toxicity of emitted compounds to nematodes in a closed environment when in a volatile form (Fig. 50). The activity of the individual volatile compounds was tested against J2s of potato cyst nematode and the model nematode *Caenorhabditis elegans*. The possibility of synergistic effects between compounds was also tested. The assays were carried out with methyl iodide, dimethyl sulphide and chloroform as the other compounds are gaseous at room temperature so were considered unsuited to the assay.

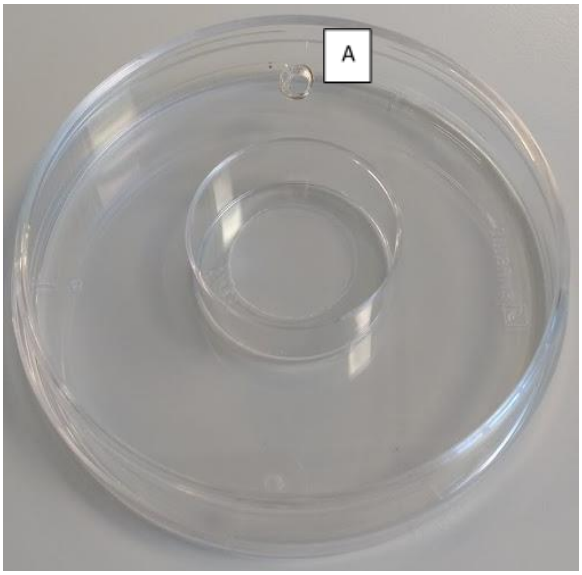


Figure 50: The nested dish system used to assay toxicity of volatile compounds. The lower half of a 35 mm petri dish is sealed to the inside of the lid of a 90 mm petri dish using vacuum grease and a hole (A) is burned into the upper section of the larger, outer dish. A known quantity of nematodes is pipetted into the smaller, central dish in a 2 ml aliquot of sterile water, and the larger dish is closed and sealed with vacuum grease. The compound to be tested is pipetted through the hole (A), which is quickly sealed with vacuum grease.

Initial experiments with *C. elegans* adults assessed the difference in toxicity of methyl iodide applied directly in solution with the nematodes to that of methyl iodide pipetted into the surrounding dish, so that only volatilised compound would come into contact with the nematodes. Subsequent assays applied compounds only to the outer dish. Compounds were pipetted into the dish in 3 µl aliquots, diluted where necessary with absolute ethanol, and with control treatments of 3 µl absolute ethanol and with no addition of any compound. Where two compounds were applied to one dish, this was applied as two 3 µl aliquots, diluted as above, with a 6 µl absolute ethanol control treatment.

Before application of compounds, both the total number of nematodes in each dish and the number of those nematodes that were dead or inactive was counted in triplicate. Following application of the compounds, the number of inactive or dead nematodes was counted at set timepoints. From these numbers, the percentage lethality or inactivity was calculated for each dish.

A further nested-plate assay was conducted with cysts. The values from the single cultivar that had the highest methyl iodide and dimethyl sulphide flux from the belowground biomass were used, and applied at 1x, 7x and 49x doses from the equivalent of 1 g dry biomass, to give values for 1 day, 7 days, and 7 weeks exposure. After 24 h incubation, cysts were

crushed and the eggs were collected and stained with 0.001 % Meldola's blue dye to assess viability.

Initial testing compared the toxicity of compounds applied in solution to nematodes suspended in buffer with compounds applied separately to the buffer and allowed to volatilise. Following 18h incubation with methyl iodide, it was found that both treatments resulted in high *C. elegans* mortality (97.38 ± 1.27 % in solution, 99.38 ± 0.33 % in the outer dish), with no significant difference between the two (P>0.05); both treatments were distinct from the untreated control (P<0.001) (Fig. 51). The initial volume of methyl iodide was explorative, informing subsequent experiments.

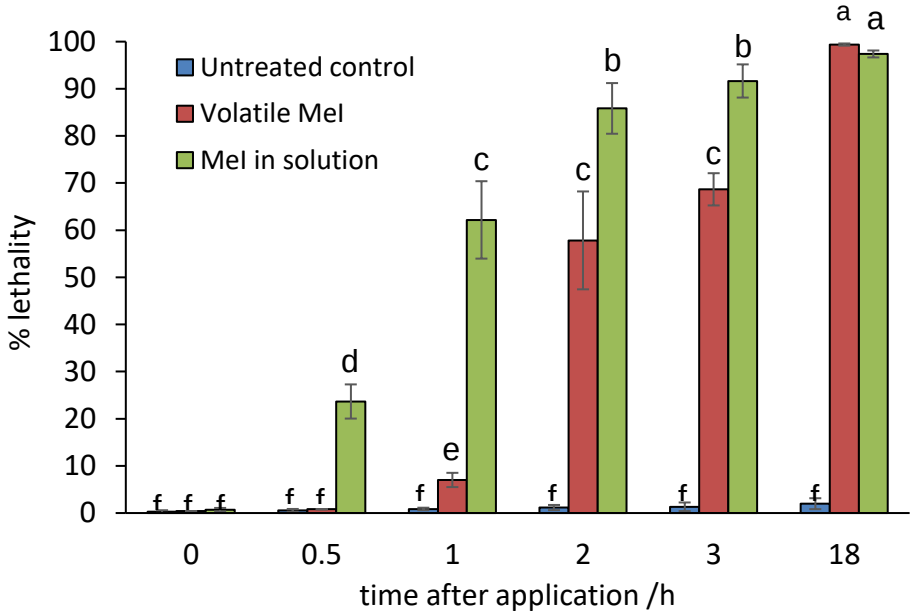


Figure 51: Toxicity of methyl iodide to *C. elegans* N2 adults in applied solution and in a volatile form. An aliquot of 3 µl (6.84 mg) methyl iodide was applied to each of the treatments, and the number of dead nematodes counted before and at 0.5, 1, 2, 3 and 18 hours after application. Methyl iodide appears to act more quickly when applied in solution, but this difference disappeared after overnight incubation. N = 226 ± 42; groups are based on a Student-Newman-Keuls analysis, P < 0.05. A t-test indicated P < 0.001 for the treatments at 18h versus the non-treatment control.

To test toxicity for plant-parasitic nematodes, and to investigate any synergistic effect between compounds, *G. pallida* J2s were incubated with methyl iodide diluted in ethanol, with or without an additional aliquot of chloroform (Fig. 52). No significant difference between treatments at the same concentration of methyl iodide was observed. Chloroform tested alone appeared to have no impact on nematode viability.

The toxicity of dimethyl sulphide (DMS) was then assayed with *G. pallida* J2s (Fig. 53). All concentrations of DMS appeared to result in near 100 % mortality for nematodes initially, but some nematodes were able to recover after overnight incubation, suggesting that some individuals experienced a nematostatic rather than nematicidal effect. Volumes of DMS applied to nematodes were made to match those of methyl iodide previously trialled.

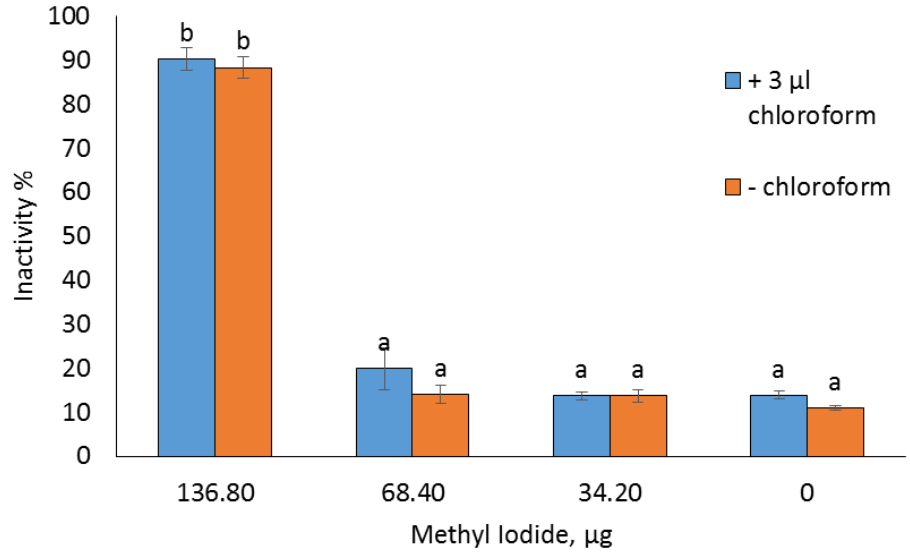


Figure 52: Inactivity in *Globodera pallida* J2s after 18h incubation with volatile methyl iodide, with or without additional chloroform. The *x*-axis displays the total amount of methyl iodide solution in each treatment, applied diluted with absolute ethanol in a 3 µl aliquot. No statistical difference due to addition of chloroform was detected. Exposures were performed in triplicate, n=151 ± 28. Labels give groups based on Student-Newman-Keuls analysis.

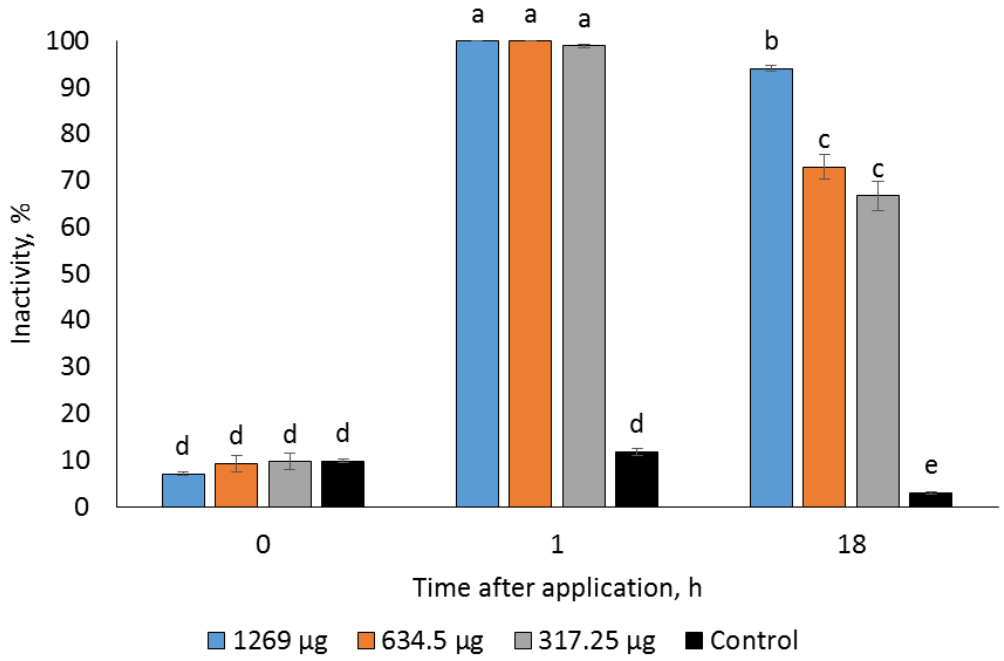


Figure 53: Inactivity in *Globodera pallida* J2s after incubation with volatile dimethyl sulphide. DMS was applied diluted in ethanol in 3 µl aliquots. Nematodes initially appeared to have been killed outright at all concentrations of applied DMS, but there was a dose-dependent recovery of some nematodes after overnight incubation. Exposures were performed in triplicate, n=271 ± 8. Groupings are based on a Student-Newman-Keuls analysis, $P < 0.05$.

The effects of methyl iodide and DMS on encysted eggs were investigated at concentrations estimated to be of biological relevance. Using a dosage based on the maximum output from 1 g dry belowground biomass, toxicity was observed in eggs exposed to 49 times daily flux (11.91 µg of methyl iodide and 32.49 µg of dimethyl sulphide, equivalent to 7 weeks

cumulative exposure). The effects of DMS and methyl iodide were additive, such that combined application of a 7-week flux of dimethyl sulphide and methyl iodide resulted in significantly higher mortality in eggs than application of dimethyl sulphide alone (Fig. 54).

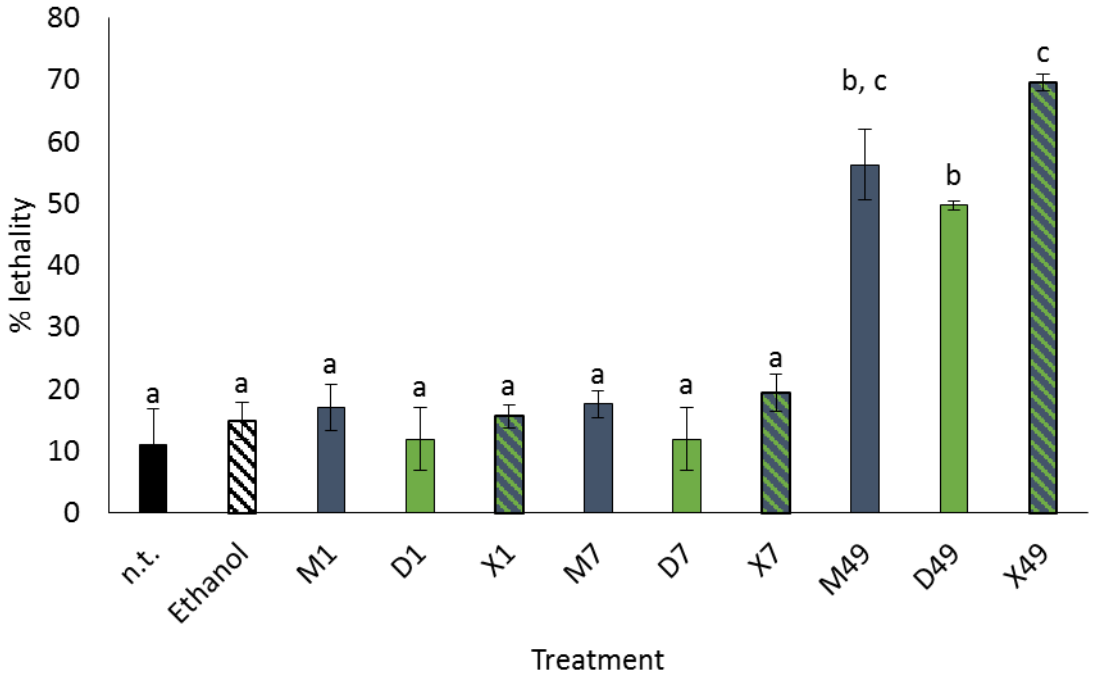


Figure 54: Toxicity of methyl iodide and dimethyl sulphide to *Globodera pallida* encysted eggs. Cysts suspended in 2 ml sterile distilled water were exposed to volatilised methyl iodide and dimethyl sulphide, applied in a 14 µl aliquot diluted in ethanol. Doses were based on the 24h flux from 1 g dry root biomass from flowering stage *R. sativus* cv. 'Diablo': 1 = 1 times the daily flux (methyl iodide = 0.243 µg; DMS = 0.663 µg); 7 = 7 times the daily flux; and 49 = 49 times the daily flux; M = methyl iodide in ethanol alone; D = dimethyl sulphide in ethanol alone; X = combined doses of methyl iodide and dimethyl sulphide in ethanol. Data labels give groupings based on a Student-Newman-Keuls analysis, $P \leq 0.05$. Exposures were set up with 3 cysts per plate in triplicate, n = 433 ± 92 eggs per exposure.

2.9. Objective 4: Determine the effectiveness of biofumigation against trichodorids and longidorids on carrot and raspberry where applicable.

The impact (e.g. influence on feeding behaviour, killing potential or paralysis of nematodes) of specific compounds released during biofumigation has been little studied for the free-living plant parasitic nematodes (FLN). Study *in situ* of the direct effect of these chemicals on feeding nematodes is greatly complicated by the surrounding soil matrix, making real time observation of feeding nematodes almost impossible. To provide robust information of the mode of action of biofumigant chemicals against FLN, we developed and used refractive index matching technologies that involved so called “transparent soils”, that are able to support plants in a medium that allows natural root growth through a pore matrix similar to the complex nature of soils.

Visualisation of nematode activity in situ

The group at the James Hutton Institute (Felicity O’ Callaghan, Lionel Dupuy, Roy Nielson, Stuart MacFarlane) developed an experimental system that allows visualisation of nematode activity live *in situ* and in 3D. Purpose-built growth chambers allowed both plants and nematodes to be cultured within a transparent soil microcosm, in which liquids containing nutrients or stains can be easily introduced. The first part of the work involved optimisation of the refractive index matching solution in which the plants and nematodes are co-cultured, with the best matching being achieved using trehalose solutions of 12 degrees Brix (sugar content). However, while providing the best optical clarity, the trehalose solutions were found to be unsuitable for culturing nematodes. Subsequent work with refractive index matching silica suspensions such as Percoll (GE Healthcare) and Ludox TMA (Sigma) allowed plant and nematode survival for several days, enabling plant- nematode interactions to be monitored over an extended period of time.

Micro-scale imaging of the growth chambers was carried out using a confocal laser scanning microscope (CLSM; Nikon A1R). This allowed high resolution imaging of plant structures and nematode behaviour (Fig. 55). Video footage of plant nematode interactions and 3D volume reconstructions of root growth within transparent soil were also obtained. Within the system, both the plant roots and the nematodes are transparent to some degree so that efforts were made to make these structures more visible during image capture. A strong contrast between root structures and their environment was achieved by staining root cell walls with calcofluor. Transparent soil particles could be dyed with a range of dyes such as sulforhodamine B (Fig. 55a & b), methylene blue and food colouring. However, autofluorescence of roots and particles in response to light of different wavelengths was often found to provide sufficient contrast without the need for exogenous stains. Live nematodes were difficult to detect due to their high transparency; some contrast was achieved by autofluorescence, calcofluor staining or by encouraging the nematodes to ingest fluorescein.

The high resolution provided by CLSM made it possible to observe searching and feeding behaviours and the displacement of the transparent soil particles. It was also possible to resolve anatomical features of both roots and nematodes such as root hairs of between 10 to 1000 µm in length and around 10 µm in width and nematode stylets of 20–30 µm in length. It was therefore possible to identify nematodes to functional group and potentially genus and the sites of preferred feeding on or grazing of the plant root. For example, plant parasitic FLNs could be distinguished from bacterivore nematodes (Fig. 55c & d).

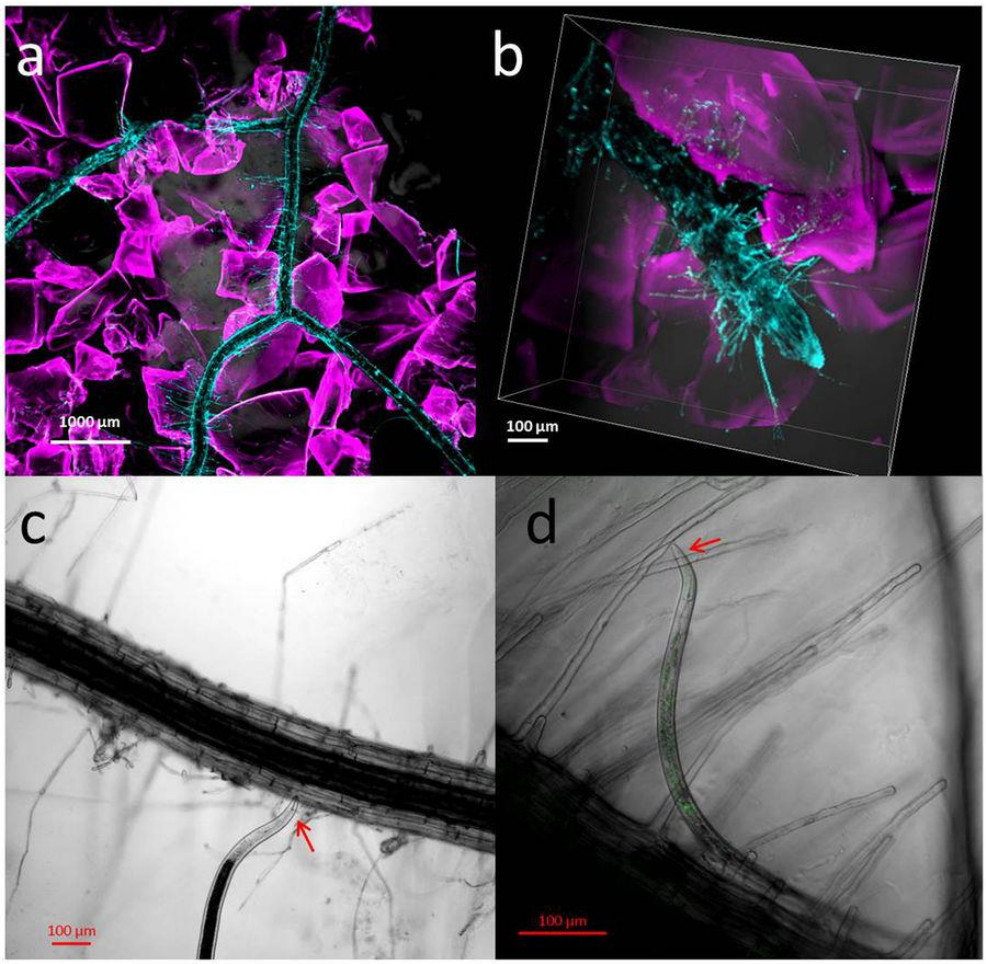


Figure 55: CLSM imaging of plants and nematodes cultured in transparent soil saturated in Ludox® TMA. *Nicotiana tabacum* (**a**) and *Petunia multiflora* (**b**) stained with calcofluor and growing among transparent soil particles stained with sulforhodamine B. A plant-feeding nematode (**c**) and a bacterivore (**d**) associated with roots of *N. tabacum*. Intestinal autofluorescence is visible in green in the bacterivore. Nematode head regions are indicated by red arrows and allow the two species to be distinguished by the presence/absence of a stylet.

For macro-scale imaging, SPIM (Selective Plane Illumination Microscopy) was used to create 3D reconstructions of root architecture (Fig. 56). This imaging technique has the advantage of being able to scan wide sections of the root system at high speed and could therefore provide a useful tool in examining overall levels of nematode infection in an entire root system. Although nematodes were clearly visible among the roots and soil particles, their transparency and small size made their detection difficult.

Nematode detection was subsequently greatly enhanced through the incorporation of the biospeckle technique. This technique, which is based on the interference patterns that living organisms create within a laser light had, to our knowledge, not previously been used in the study of nematodes. Capture of the speckle produced by biological material allows the visualisation of both plant and nematode in Transparent Soil and provides a contrast from the abiotic soil environment (Fig. 57a). Trials conducted in a gel system further showed that increased exposure of the root to the laser light allowed feeding nematodes to be detected, which were only barely visible under bright field illumination (Fig. 57b).

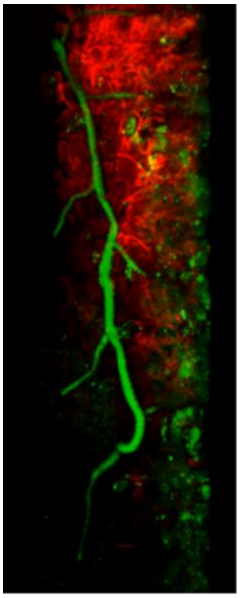


Figure 56: SPIM of lettuce root among Nafion particles dyed with sulforhodamine B.

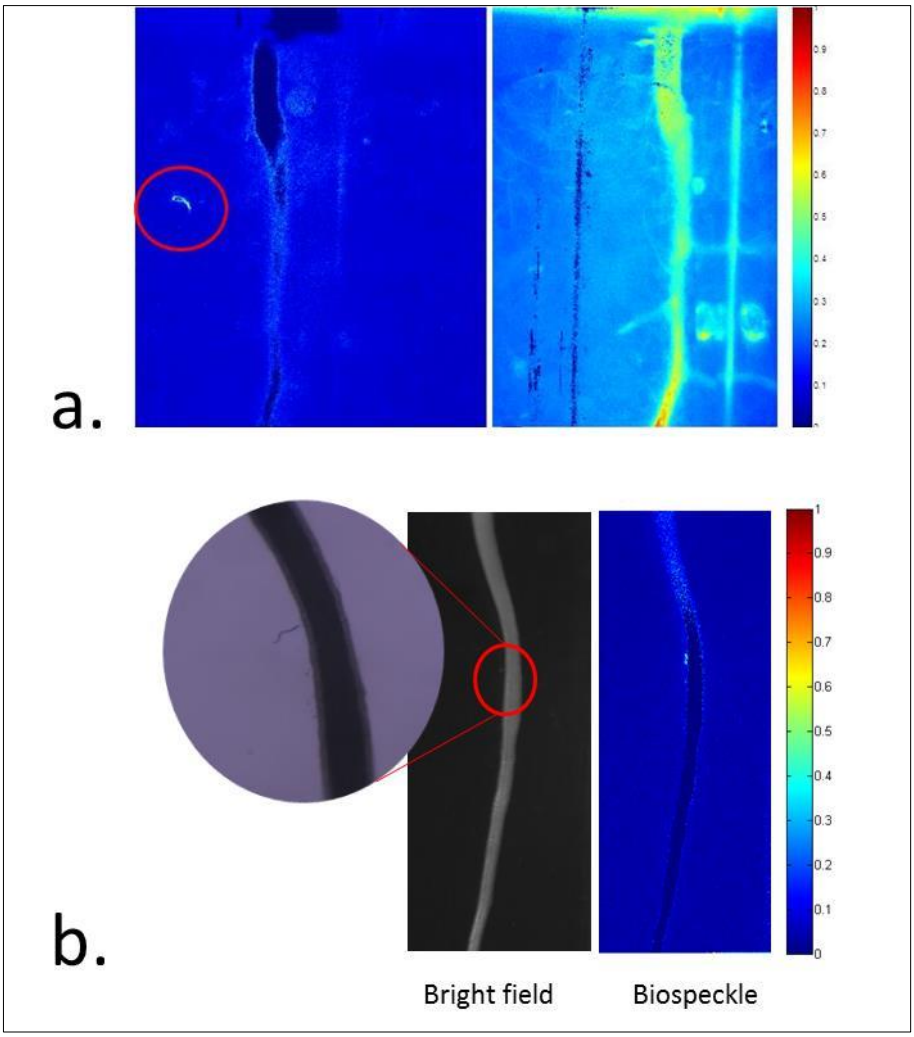


Figure 57: Examples of biospeckle imaging of *in situ* lettuce roots and FLN. **a.** biospeckle patterns of nematode (circled red, left) and root (right) co-cultured in Transparent Soil. **b.** Feeding nematode in phytagel hardly visible under bright field illumination (left) but clearly visible with biospeckle.

Automatic nematode detection

Experiments were carried out to test if the biospeckle technique is suitable for rapid detection of FLN. A procedure has been developed to test and calibrate biospeckle for the precise assessment of nematode numbers and their level of activity. This consists of:

1. Extraction of free living nematodes from soil
2. Preparation of samples by inserting a known abundance of nematodes into spectrophotometer cuvettes. Nematodes are held in suspension using a mixture of water and colloid (Ludox TMA, Sigma).
3. Image acquisition
4. Calibration of image analysis for counting the abundance of nematodes present in the sample
5. Assessing the precision of detection by comparing the number of nematodes inserted with the abundance of detected nematodes

Results showed that laser scans of known abundance (0 to 12) of mixed FLN species and biomass suspended in liquid, but without Transparent Soil, provided accurate estimates of live nematode abundance (Fig. 58a). Abundances of live nematodes inserted were correlated with the number of detected speckling objects (Spearman rank-order correlation p = 0.000). The biospeckle technique was also used to estimate live nematode abundance in Transparent Soil (Fig. 58b).

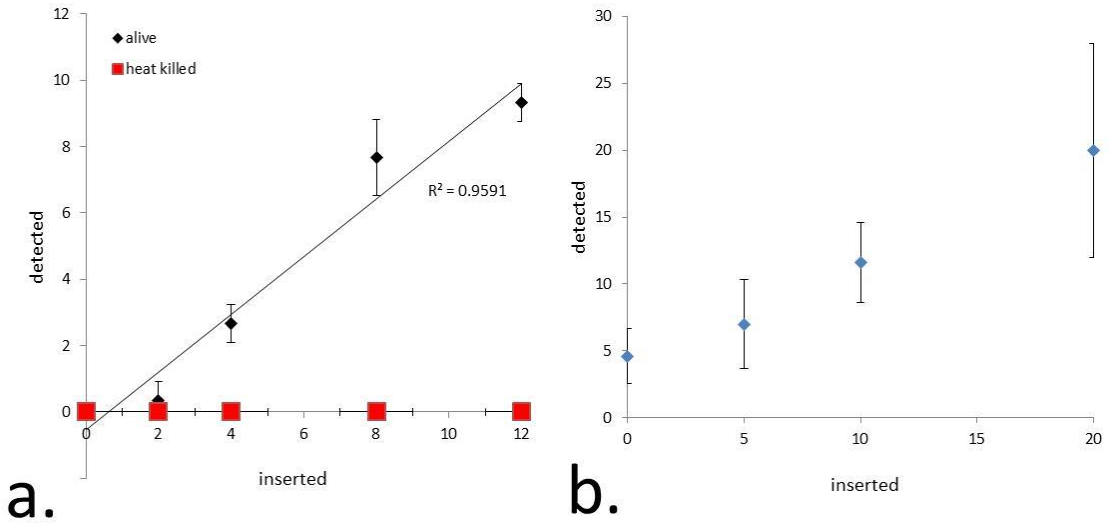


Figure 58: Nematode counting using biospeckle. a. Live nematodes (black markers) inserted into Ludox TMA are detected by biospeckle while heat killed nematodes (red markers) remain undetected. b. Numbers of live nematodes inserted in Transparent soil and Ludox microcosm detected by biospeckle.

Laboratory nematicide tests

Live microscale plant pests such as nematodes create optical interference created through movement as well as cellular processes within the nematode. The resulting signal, termed biospeckle, is quantified by tracking changes in the intensity of a series of brightfield images. The use of biospeckle as a tool in the discovery of novel crop protection products relies on the fact that any compound which affects cellular processes and possibly the movement of

the target organism creates a measurable change in biospeckle. The approach is based on capturing the characteristic biospeckle patterns arising from nematode behaviour and examining how these are affected by chemical treatment. Once robust systems had been developed for the visualisation of nematode activity in Transparent Soil, using biospeckle, the set-up was used to deliver pesticides into the microcosms. Dazomet was chosen as a synthetic, commercially available ITC-releasing pesticide (also known as Basamid) against which the biofumigant compounds could be compared. Preliminary tests compared the biospeckle patterns of *Trichodorus* nematodes which were either heat-killed, un-treated, immersed for 24 h in solutions of 100 mg L^{-1} and 200 mg L^{-1} Dazomet or in 0.06 mM BITC. These first experiments were carried out with nematodes suspended in liquid alone and were then followed by experiments in Transparent Soil.

Dazomet was found to have an effect on the nematode response to touch at concentrations above 100 mg L^{-1}; with a rapid decline in the numbers of responding nematodes occurring at a concentration of 200 mg L^{-1}. In order to become optically transparent, Transparent Soil has to be immersed in a liquid of matching refractive index. This was used as a means of introducing pesticides to the TS microcosm by dissolving them within a matching liquid (Ludox). Assemblages of mixed, locally extracted nematodes were introduced into Transparent Soil microcosms with 100 mg L^{-1} Dazomet dissolved in Ludox TMA and observed for spontaneous movement. As shown in Figure 59, a steep decline in spontaneous activity was apparent within 24 h in Dazomet with no movement visible after 2 weeks. By contrast after two weeks, 80% of microcosms with TS with Ludox alone had detectable nematode movement (25 nematodes per treatment split into 5 replicates; presence/absence of movement per sample was shown to differ significantly, p=0.0476 with Fisher's exact test, after 2 weeks where Dazomet had been applied).

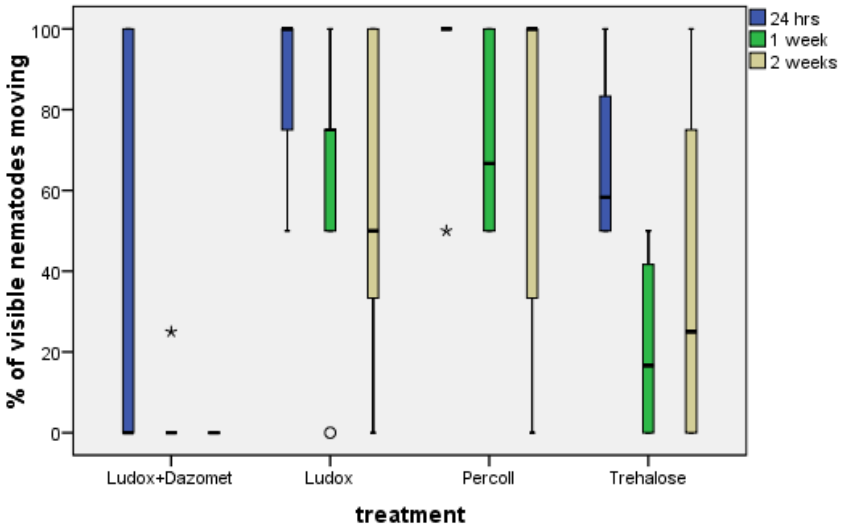


Figure 59: Spontaneous movement of soil FLN in transparent soil with the refractive index matching liquids Percoll and Ludox, besides Ludox with 100mg L^{-1} dazomet. 5 nematodes were inserted per microcosm with each microcosm being replicated 5 times. Data are shown as the percentage of visible nematodes displaying spontaneous movement, with boxes representing 50% of observations and whiskers the upper and lower quartiles. Observations were made 24 h 1 week and 2 weeks after nematode introduction. Spontaneous movement over the first 24 h was visible in all microcosms except in the presence of dazomet.

Following the successful preliminary test of biospeckle as a tool to evaluate pesticide effects, its application was further demonstrated by using it to assess toxicity of chemicals to different trophic groups of nematodes. Free-living nematode populations extracted from soil were exposed to methanol and phenyl isothiocyanate (PEITC). There was insufficient time to

examine the activity of a wider range of ITC compounds in the transparent soil system, but we have demonstrated the feasibility of using this system. Chemicals were applied to two trophic groups of nematode, plant feeders and bacterial feeders, as well as targeting a single plant feeder genus, *Trichodorus*. Bacterial feeding and plant feeding nematodes differ in their behaviour, and this is reflected in their movement. Bacterial feeders are filter feeders, continuously drawing in bacteria with the surrounding liquid by contractions of their oesophageal bulb, then forcing the liquid out while retaining the bacteria. Within liquid, their movement is characterised by high wavelength, low amplitude body movements and oscillation of the head region, typical behaviour when perceiving attractants. By contrast, plant feeder movement proceeds much more slowly. In the absence of plant attractants, plant feeders display sporadic random movements, which are characterised by their short wavelength, high amplitude and the absence of head oscillations.

Nematodes were extracted from field soil by sieving and by the Baermann funnel method. *Trichodorus* species were identified to genus level, while plant and bacterial feeders were mixed, multi-genus groups sampled from Scottish agricultural soils. Plant feeder samples excluded trichodorids (ie. all *Trichodorus* and *Paratrichodorus* species). Each sample for biospeckle analysis contained 5 nematodes and was replicated 5 times for each treatment (except PEITC tests on *Trichodorus* nematodes which had 6 replicates to ensure similar detection rates with nematodes in these samples tending to be smaller in size). Pesticide testing using biospeckle was carried out in polymethacrylate cuvettes. To prepare nematode samples for scanning, 1 mL Ludox® TMA was added to each cuvette and 5 nematodes manually placed on the surface of the colloid. 1 mL of water was then added which resulted in nematodes being suspended in the Ludox® TMA /water mixing layer around the midpoint of the cuvette (Fig. 60). In addition to the samples used in the scanning experiments, 5 replicate samples of 5 nematodes of each target feeding type were kept in Ludox® TMA /water for 1 week after which the presence of spontaneous movement was assessed by light microscopy.

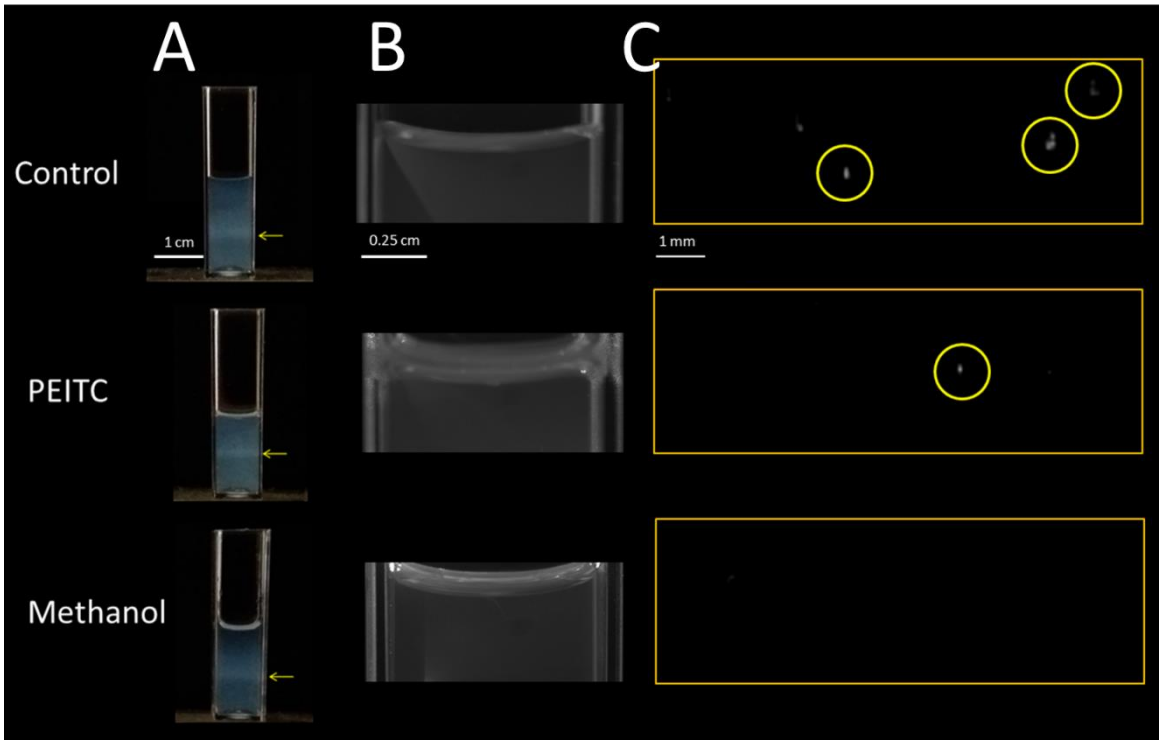


Figure 60: Sample preparation and object detection. **A.** Sample cuvettes containing 1ml Ludox to 1ml water with the Ludox® TMA /water mixing layer containing the nematodes

indicated by yellow arrows. **B.** water-air boundary with PEITC, methanol or water added for controls; PEITC as an oil formed a film on top of the water column, while methanol dissolved. **C.** Examples of bacterial feeder detection within the Ludox® TMA /water mixing layer.

Biospeckle analysis revealed differing behavioural responses as a function of nematode feeding groups. *Trichodorus* nematodes were less sensitive than were bacterial feeding nematodes or non-trichodorid plant feeding nematodes. Following 24 hours of exposure to PEITC, bioactivity significantly decreased for plant and bacterial feeders but not for *Trichodorus* nematodes. Decreases in movement for plant and bacterial feeders in the presence of PEITC also led to measurable changes to the morphology of biospeckle patterns.

Nematode trophic groups are affected differently by methanol and PEITC

Both PEITC and methanol had measurable effects on nematode bioactivity when compared with controls. Here, bioactivity was measured as the Total Biospeckle Intensity, a global indicator determined as total object intensity for each sample of 5 nematodes. Total Biospeckle Intensity varied considerably between the different feeding groups tested (Fig. 61), regardless of the presence or absence of the toxic compounds.

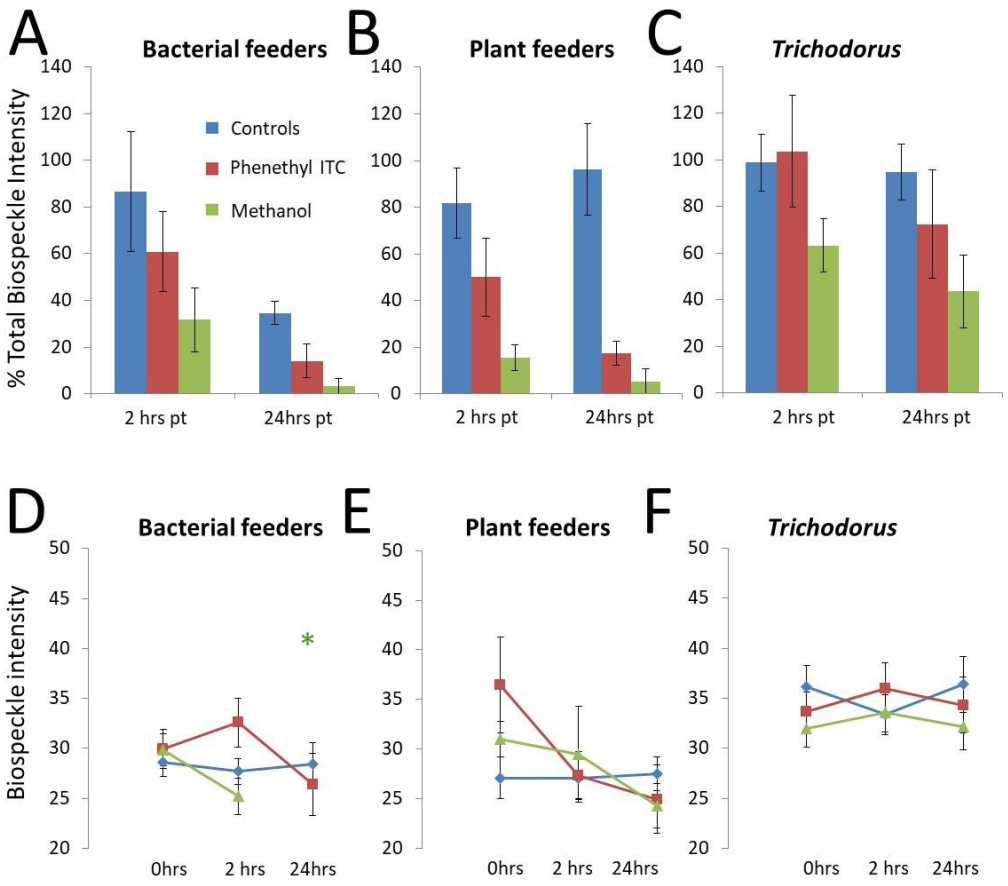


Figure 61: Effects of PEITC and methanol on biospeckle intensity. **A.-C**. Changes in the Total Biospeckle Intensity of bacterial feeder, plant feeder and *Trichodorus* samples 2 and 24 hours after treatment, expressed as the percentage of Total Biospeckle Intensity measured before treatment. Error bars represent the standard error for N=5 (N=6 for *Trichodorus* in PEITC). **D.-F.** Biospeckle intensities for the different feeding groups detected above a threshold of 20. Measurements at 0 hours are prior to the application of chemicals,

while 2 and 24 hours are post-treatment measurements. Biospeckle intensities are shown as the average of all detected objects and error bars represent the standard error. The asterisk denotes the only observation 24 hours after the application of methanol which was not replicated.

For bacterial feeders (Fig. 61A), Total Biospeckle Intensity in the absence of chemical treatment decreased over the 2- day testing period, with levels significantly different after 24 hours ($p=0.283$ after 2 hours and $p=0.013$ after 24 hours). The application of PEITC lead to a decline in the Total Biospeckle Intensity of bacterial feeders which was significant after 24 hours ($p=0.075$ and 0.007 after 2 and 24 hours respectively). The introduction of methanol was immediately followed by a significant decline in Total Biospeckle Intensity ($p=0.026$ and $p=0.003$ after 2 and 24 hours, respectively). For plant feeders (Fig. 61B), Total Biospeckle Intensity did not vary significantly for controls ($p=0.255$ after 2 hours and $p=0.413$ after 24 hours). The application of PEITC on the other hand was followed by a significant decrease in the Total Biospeckle Intensity both 2 and 24 hours after application ($p=0.038$ and $p=0.003$). Similarly, the application of methanol was followed by a significant decline after 2 and 24 hours (both $p<0.001$). *Trichodorus* nematodes (Fig. 61C) were the least affected by either PEITC or methanol. While no significant difference in the Total Biospeckle Intensity over time was noticeable for controls ($p=0.784$ and 0.453 after 2 and 24 hours respectively), the application of PEITC also showed no significant effect after 2 hours or 24 hours ($p=0.355$ and $p=0.072$). No significant change was apparent 2 hours after the application of methanol ($p=0.179$), but a significant decline in the Total Biospeckle Intensity had occurred after 24 hours ($p=0.035$).

Biospeckle intensity is specific to each nematode (unlike Total Biospeckle Intensity, which considers the whole sample). Results showed that biospeckle intensity did not vary significantly for control samples (Fig. 61D-F). Exposure to PEITC and methanol was followed by downward trends in biospeckle intensity for bacterial feeders (Fig. 61D) and plant feeders at 2 and 24 hours (Fig. 61E), though not for *Trichodorus* nematodes (Fig. 61F). For nematodes recovered after 1 week in the Ludox® TMA /water mixing layer, spontaneous movement was observed in 15 out of 17 bacterial feeders, 11 out of 20 plant feeders, and 6 out of 23 *Trichodorus* nematodes. This confirmed *Trichodorus* nematodes to have the lowest motility even in the absence of toxins while bacterial feeders were the most motile, albeit displaying greater variability in velocity and distance travelled.

Trichodorus nematodes were the least susceptible to PEITC and less affected than the non-target bacterial feeder group. Application of 2-propenyl ITC in the field has previously been reported as not significantly affecting the abundance of *Trichodorus* populations, though it probably was deployed at much lower concentrations than in the present study. Plant feeders excluding trichodorid species were the most susceptible to PEITC, being the only group for which there was a significant effect after 2 hours of treatment and showing a prolonged decline in biospeckle intensity. In the presence of methanol, *Trichodorus* nematodes again showed the highest tolerance amongst the tested groups. Methanol had a strong effect on both bacterial and non-trichodorid plant feeders. Overall, bacterial feeder populations are reported to be more tolerant of pollution-induced stress than plant feeders. This, however, appears to be largely due to plant feeders having lower reproductive capacity, while bacterial feeders tend to have high metabolic rates and the ability to re-populate contaminated soil quickly. This work however suggests that susceptibility to toxins can vary significantly within trophic groups of nematodes with *Trichodorus* showing greater resilience than either bacterial or non-trichodorid plant feeders.

In summary, Biospeckle analysis has been refined to provide a fast way of testing the specificity of potential nematicidal compounds. We showed the technique has potential to screen the behavioural responses of diverse trophic communities. The method discriminates both behavioural responses, morphological traits and activity levels and hence could be used to assess the specificity of nematicidal compounds.

Raspberry trials

A polytunnel trial was set up to test whether incorporation of macerated Bento radish and ISCI 99 brown mustard in soil containing FLN was able to suppress these nematodes, and thereby have a beneficial effect on the growth of raspberry plants planted in these soils. Soil was collected from fields known to contain a high concentration of FLN (including Longidorids). The soil was then used to fill 600 L Dolav boxes which were placed in a standard raspberry polytunnel. The boxes were then sown with Bento and ISCI99 seeds in September 2016 at rates provided by consortium partners (100 seeds/m^2). A randomised experimental design was set up consisting of 30 Dolav boxes, consisting of 10 replicates for each for Bento, ISCI99 and untreated (no crop) controls (Fig. 62).

Figure 62: Polytunnel trial of biofumigants growing in 600 L Dolav boxes filled with field soil containing high levels of FLN. Raspberries were planted in the boxes following maceration and incorporation of the biofumigant.

The biofumigant crops were macerated and incorporated into the soil at flowering on April 5th 2017 with nematode samples taken the day before. At the time of maceration Bento had begun to flower 3 weeks before while ISCI99 had started flowering 5 weeks before; the maximum height for ISCI99 shoots was 1.6 m and 1.2 m for Bento. For maceration, plant stalks were cut at soil level and the plants shredded. Soil was loosened with a spade, the shredded mulch spread on top and the soil turned over and then slightly compacted with a spade. Soil in control boxes was turned over and compacted in the same way (Fig 63A). The soil was watered once incorporation was complete. In total the process was completed within 4 hours.

Raspberry canes (Glen Ample; 40 plants/treatment; 4 plants per 600 litre box) were planted 2 weeks afterwards (19 – 20 April 2017) to give sufficient time for the biofumigant chemicals to degrade in the soil and, thereby, prevent potential plant root damage by these chemicals (Fig. 63B). Remaining stumps of the biofumigant crop were removed during the process. There was no statistically significant difference between plant heights per treatment at the time of planting. Raspberry plants carried fruit from June to late October 2017 during which yield was measured. Spawn and root re-enforcement measurements were carried out after fruiting had ceased in October and November 2017 respectively (Fig. 63C). Final nematode sampling was also carried out at the end of the trial.

Root-re-enforcement was measured according to Meijer *et al*. 2015 using a method which consists of measuring the force needed to extract a soil/root core after saturating the soil

with water. The day before samples were taken, soil was saturated by applying 30 litres of water evenly over each plot 24 hours before sampling. This ensured equal soil saturation and soil resistance to the roots. For each plot, one anchorage measurement was carried out at the point of least distance to all four plants in the box. To ensure that water content did not vary significantly between plots, the extracted soil cores were then weighed for wet weight and then dry weight after oven drying at 105 °C for 4 days.

Figure 63: Dolav boxes (**A**) after incorporation of macerated biofumigant, (**B**) after planting with raspberries two weeks later and (**C**) prior to harvest of the

Results

The total raspberry yield from June to October was 4859g, while the maximum height of spawn reached was 312 cm. There was no significant effect of treatment (Bento, ISCI99, untreated) on raspberry yield (Fig. 64A; p=0.288, Kruskal- Wallis test). There was also no significant effect of treatment on spawn height (Fig. 64B) or on the number of spawn (Fig. 64C) (p=0.204 for mean height, p=0.277 for median height, p = 0.473 for maximum height in Kruskal- Wallis tests; p = 0.57 for spawn counts in a Chi Square test). Root-reinforcement was not significantly affected by treatment (Fig. 64D) (p=0.920, Kruskal-Wallis), with similar soil water content (no significant difference between treatments, p=0.606, Kruskal-Wallis).

There was a significant inverse correlation between yield and maximum spawn length (p= 0.006, Spearman's correlation), though not for mean (p=0.054, Spearman) or median (p=0.377, Spearman) spawn length (Fig. 65). Correlations between root reinforcement and spawn mean, medium and maximum height were also significant (p=0.001 for all three, Spearman). There was no significant correlation between root reinforcement and fruit yield (p=0.127, Spearman), nor between root reinforcement and spawn counts (p=0.821, Spearman).

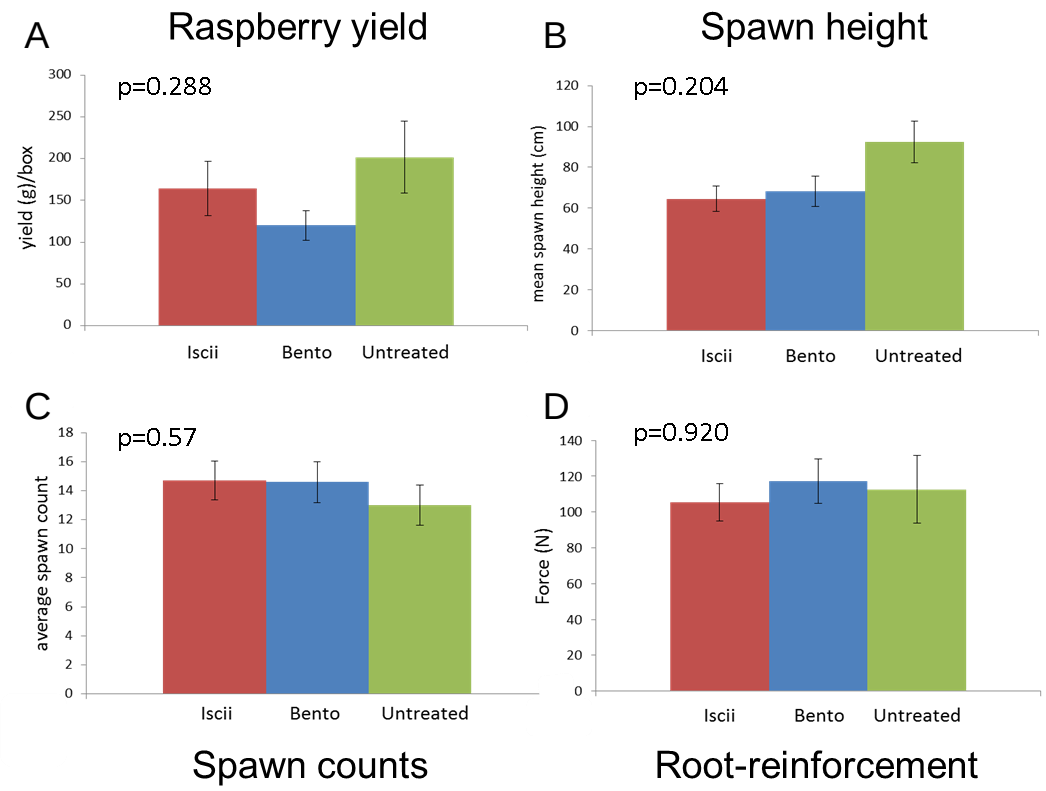


Figure 64: Raspberry yield and growth compared across treatments for ISCI99, Bento and untreated, showing (A) total raspberry yield, (B) mean heights at the end of raspberry fruit production, (C) spawn counts, and (D) root re-enforcement. Error bars represent the standard error.

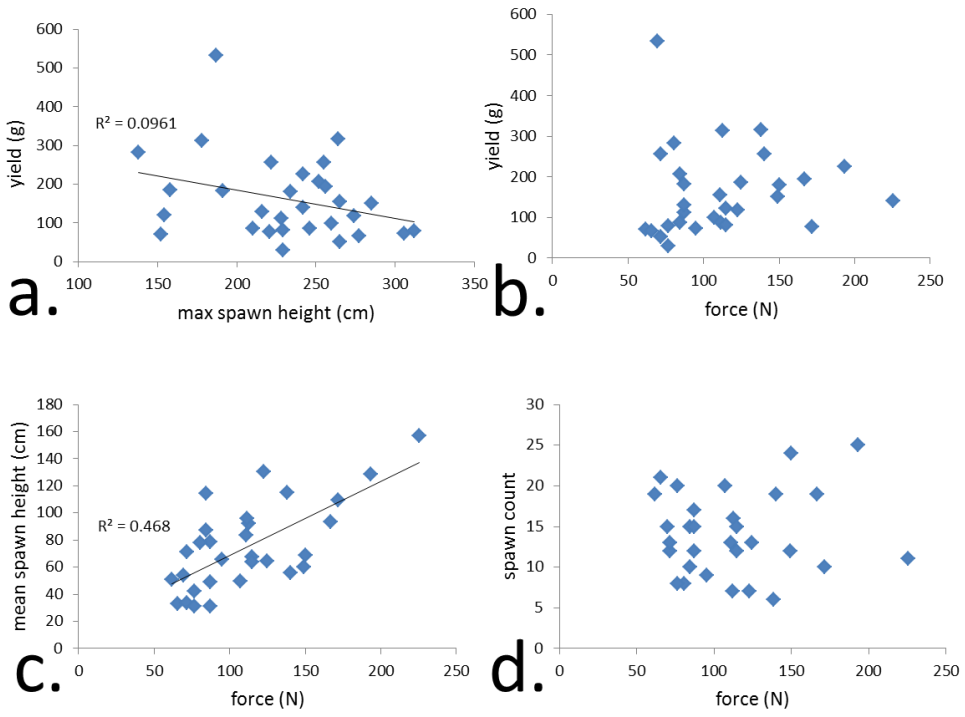


Figure 65: Raspberry yield was inversely correlated with maximum spawn height (a), but was not correlated with root re-enforcement (b). Spawn height (c) was positively correlated with root re-enforcement, but spawn counts (d) were not.

The lack of significant effect of either biofumigant treatment on any of the growth parameters of the raspberry plants was reflected in the lack of effect on the total number of nematodes in the soil (Fig. 66A), or on the number of *Pratylenchus* (Fig. 66B) and *Longidorus* nematodes (Fig. 66C) in the soil.

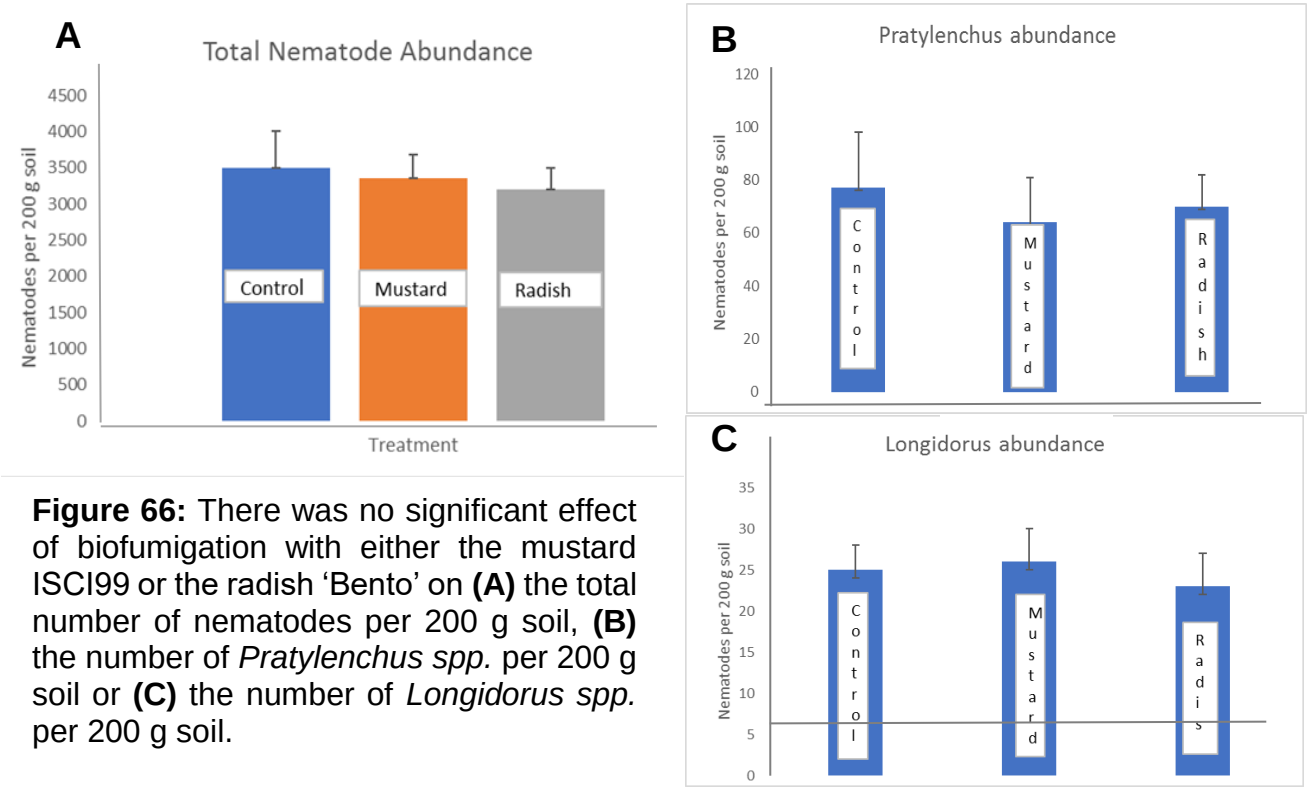


Figure 66: There was no significant effect of biofumigation with either the mustard ISCI99 or the radish 'Bento' on **(A)** the total number of nematodes per 200 g soil, **(B)** the number of *Pratylenchus spp.* per 200 g soil or **(C)** the number of *Longidorus spp.* per 200 g soil.

Conclusions

- The biofumigant cover crops mustard 'ISCI99' and radish 'Bento' had no significant effect on raspberry growth or productivity.
- Taller raspberry plants tended to have stronger root-reinforcement, although increases in height often meant less fruit production.
- No significant differences resulting from incorporation of biofumigant crops were found in either the total nematode abundance or the abundance of *Pratylenchus* and *Longidorus* nematodes (the latter are vectors of viruses that infect raspberries).

Carrot trials

Two carrot trials were set up in 2016/17 at different locations known to harbour FLN (Fig. 67). The aim was to grow a biofumigant crop and then subsequently plant carrots in the following season and assess nematode damage, whilst also sampling for numbers of FLN.

Elsoms site (52° 48' 50.4" *N*; 0° 07' 44.4" *W*) with sandy loam soil**:** The trial was set up as replicated microplots with five replicates of three plant varieties (*R. sativus* Bento; *B. juncea* ISCI99; wheat) plus fallow to give a total of 20 plots.

Howell Fen site (53° 00' 10.8" *N*; 0° 16' 51.6" *W*) with sandy loam soil**:** The second trial used replicated mini strips in a commercial carrot crop. There were four reps x three plant varieties as for Trial 1 = 16 mini-strips in total.

Both trials were supplemented with N and S using granular fertiliser to a rate of 100 kg/ha N and 20 kg/ha S for growth of the biofumigants. Fallow plots were treated in the same manner as the other plots at incorporation of the biofumigant/control plants.

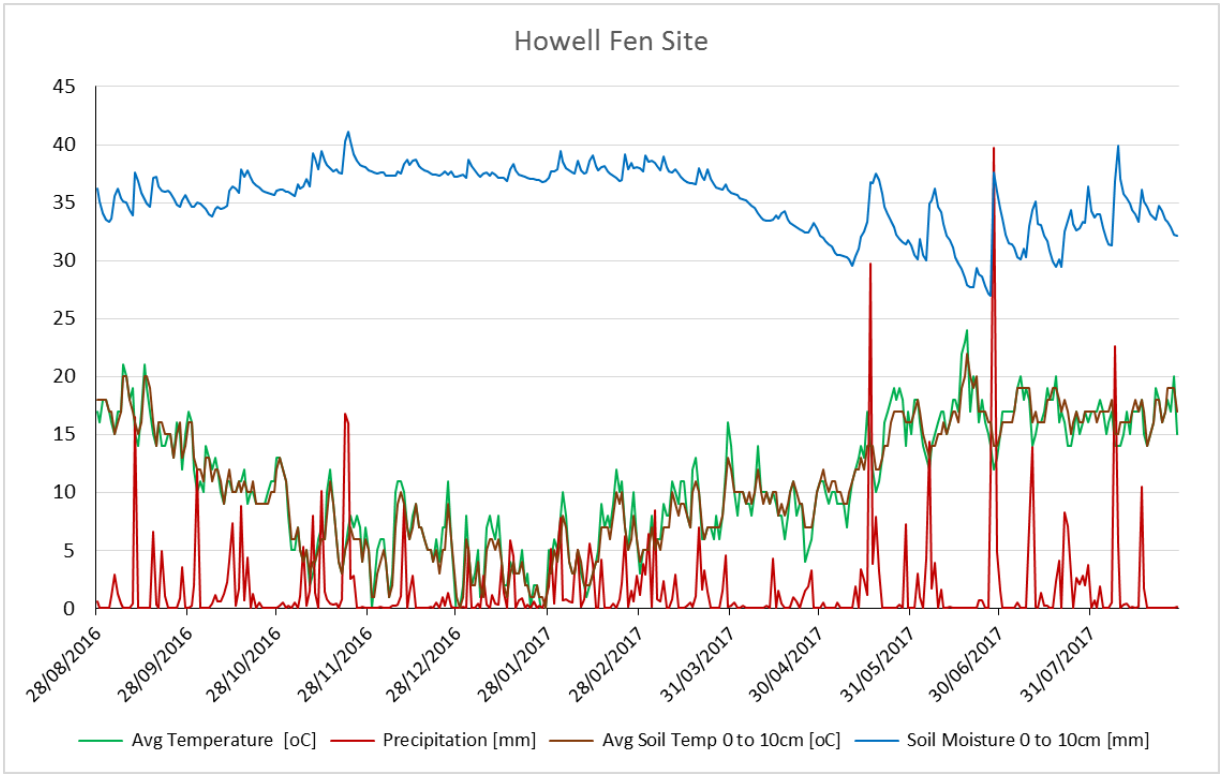


Figure 68: Average air temperature, daily precipitation, average soil temperature to 10 cm depth, and soil moisture to 10 cm depth for the Howell Fen site over the time period from biofumigant drilling to carrot harvest.

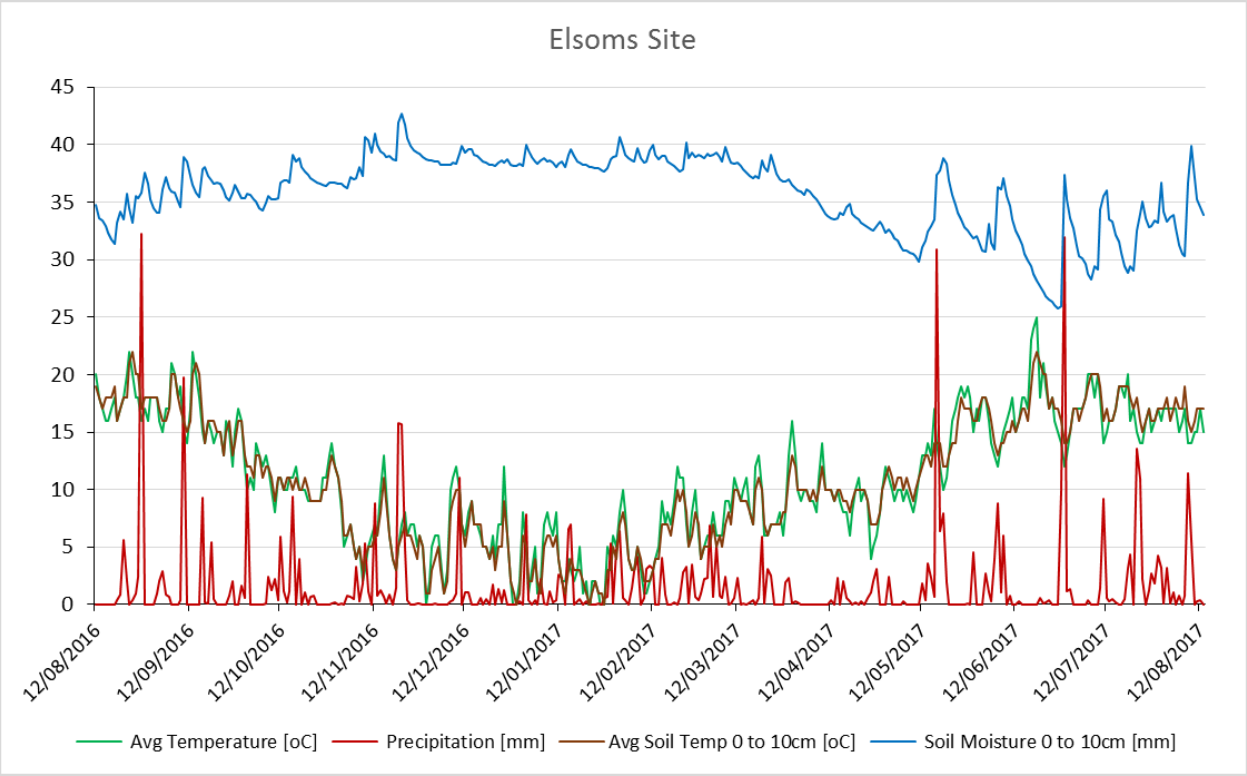


Figure 69: Average air temperature, daily precipitation, average soil temperature to 10 cm depth, and soil moisture to 10 cm depth for the Elsoms site over the time period from biofumigant drilling to carrot harvest.

For each trial, soil was sampled at both pre-drilling of biofumigant (14/08/16 for Elsoms; 07/09/16 for Howell Fen) and pre-incorporation (October 2016) and sent to the James Hutton Institute for analysis of the FLN numbers and species. Key FLN associated with carrot were assessed from these samples. Further soil samples were analysed pre- (Feb/March 2017) and post- (Sept/Oct 2017) carrot cropping. The biofumigant crops were sampled for biomass (Fig. 70) and samples were also taken and frozen for glucosinolate analysis at incorporation. Carrots were drilled in 2017 and the crops managed and harvested to assess yield and nematode damage.

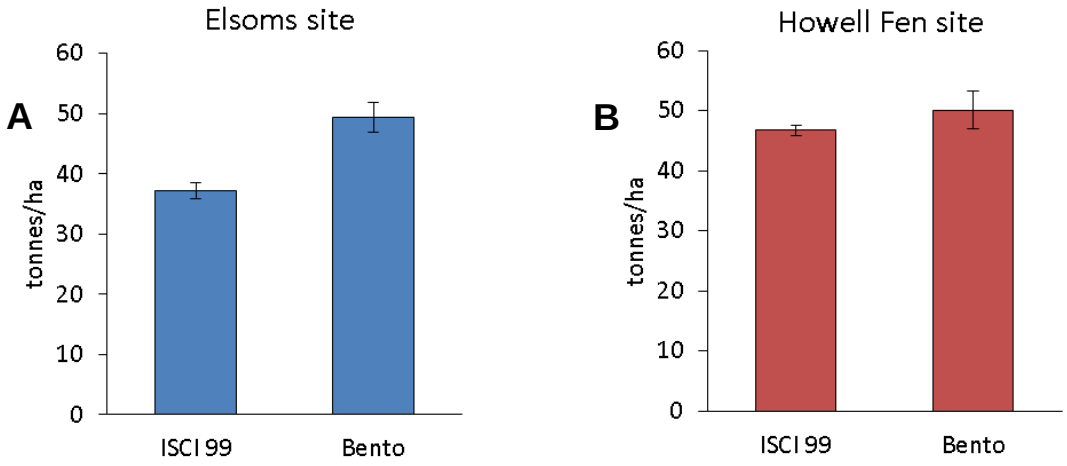


Figure 70: Biomass (tonnes/hectare) at incorporation of the mustard 'ISCI99' or the radish 'Bento' at **(A)** the Elsoms site and **(B)** the Howell Fen site. The biomass of 'ISCI99' was significantly (P=0.0009) higher at the Howell Fen site than at the Elsoms site. There was no difference in 'Bento' biomass between the two sites.

At the Elsoms site there were no differences between the control plots or any of the treatments in either mean carrot yield or proportion of carrots displaying visible FLN damage (Fig. 71). A low proportion of carrots displayed signs of FLN damage in all plots. Although all treatments that involved incorporation of mulched plant material resulted in a decrease in mean proportion of damaged carrots, the high variability meant that this was not significantly different from the fallow control.

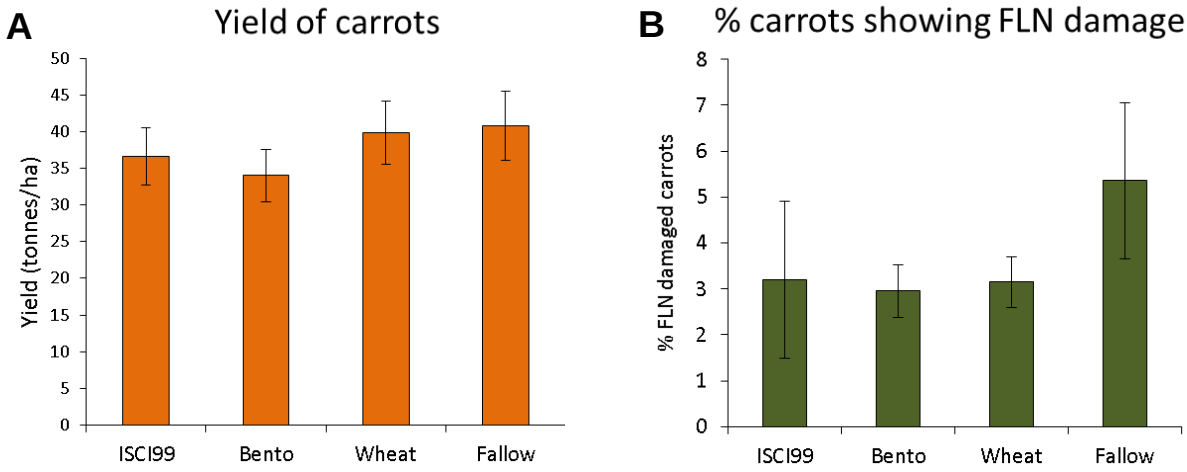


Figure 71: There was no significant effect of biofumigation at the Elsoms site with either the mustard ISCI99, the radish Bento or incorporation of wheat on **(A)** the mean yield of carrots or **(B)** the % of carrots showing FLN damage.

At the Howell Fen site, as the trial took place within a commercial crop the yield was graded into commercial categories and the total yield was slightly higher than for the Elsoms site. Although there was no significant effect of the treatments on total carrot yield, the biofumigation with 'ISCI99' resulted in a significant increase in pre-pack carrot yield (Fig.

72A). This represents the most valuable yield category. There was also a significant reduction in the % carrots displaying FLN damage in the plots treated with 'ISCI99' when compared with the control, fallow plots (Fig. 72B). Possibly the effect at Howell Fen is related to the higher biomass of ISCI99 at this site, although the mean content of the major glucosinolate (sinigrin) in leaf tissue of those plants was significantly lower (15.7 mg/g dry tissue) than for plants at the Elsoms site (22.8 mg/g dry tissue; n=4, p=0.025, T-test).

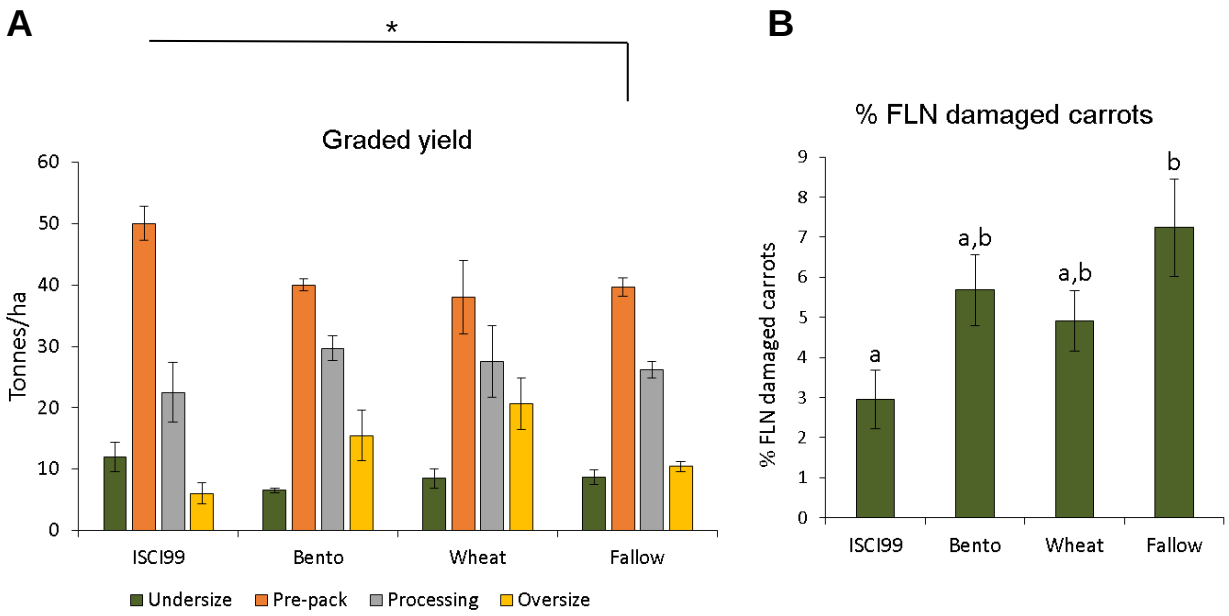


Figure 72: At the Howell Fen site biofumigation with the mustard 'ISCI99' **(A)** significantly increased the yield of pre-pack grade carrots and **(B)** significantly reduced the % of FLN damaged carrots compared with the fallow control.

2.10. Objective 5: Determine the potential of biofumigation to control soil-borne pathogens.

The soil-borne fungus *Rhizoctonia solani* causes stem and stolon canker and black scurf of potatoes. Whilst fungicide treatment of seed tubers is an important control strategy, soil-borne inoculum is less amenable to chemical control. *Verticillium dahliae* causes symptoms of Verticillium wilt. Many economically important plants are susceptible including tomatoes, potatoes and soft fruit such as strawberry. *Verticillium* can persist in soil for many years in the absence of a susceptible crop. Infection is through the roots, and management of the disease is difficult. Fungicides are generally not economical for control of Verticillium wilt. Soil fumigation with high concentrations of metam-sodium is effective, but fumigation is an expensive and hazardous control tactic. In this Objective we built on our preliminary data that showed susceptibility of *R. solani* to a range of ITCs to investigate the efficacy of biofumigation for the control of these two soil-borne fungal pathogens.

We had originally planned to collaborate with Dr. James Woodhall of FERA for this part of the project, however Dr Woodhall's move to the University of Idaho necessitated a change of strategy. All of the fungal work therefore took place at Leeds. Fungal isolates and advice on the experimental design were obtained from Dr Woodhall prior to his move. We first extracted fungal DNA and used general and specific PCR primers to confirm the identity of the *R. solani* and *V. dahliae* cultures that we had grown from the stocks provided by FERA.

Rhizoctonia solani

R. solani has 13 anastomosis groups (AGs) that are genetically isolated as they are unable to form hyphal connections between each other. The pathogen is found worldwide, with different AG groups having different host ranges. At least 5 AGs are pathogenic on potato: AG2-1, AG3, AG4, AG5 & AG8. More than 90% of UK potato isolates are AG3, the remainder are AG2-1. Therefore, we have focused on these two groups for our work with *R. solani*, with emphasis for the glasshouse trials on AG3.

Initial experiments used agar plate assays to assess the effects of different ITCs on growth of the AG2-1 and AG3 anastomosis groups of *R. solani*. Each ITC was tested at eight concentrations across a 2-fold dilution series with six replicate plates for each concentration. Agar plugs were taken from the growing edge of the mycelial mass on a stock culture plate and a single plug was inverted and transferred to the centre of each ITC-containing or control plate. Plates were sealed and incubated at 20 ^{o}C in the dark. Two perpendicular diameter measurements of the mycelial growth were made over a time-course of growth and the mean diameter for each plate recorded. Measurements were stopped when growth of the control cultures approached the edge of the Petri dish (Fig. 73). At this point, plugs were removed from any plates where no visible growth had occurred and transferred to fresh plates lacking ITC.

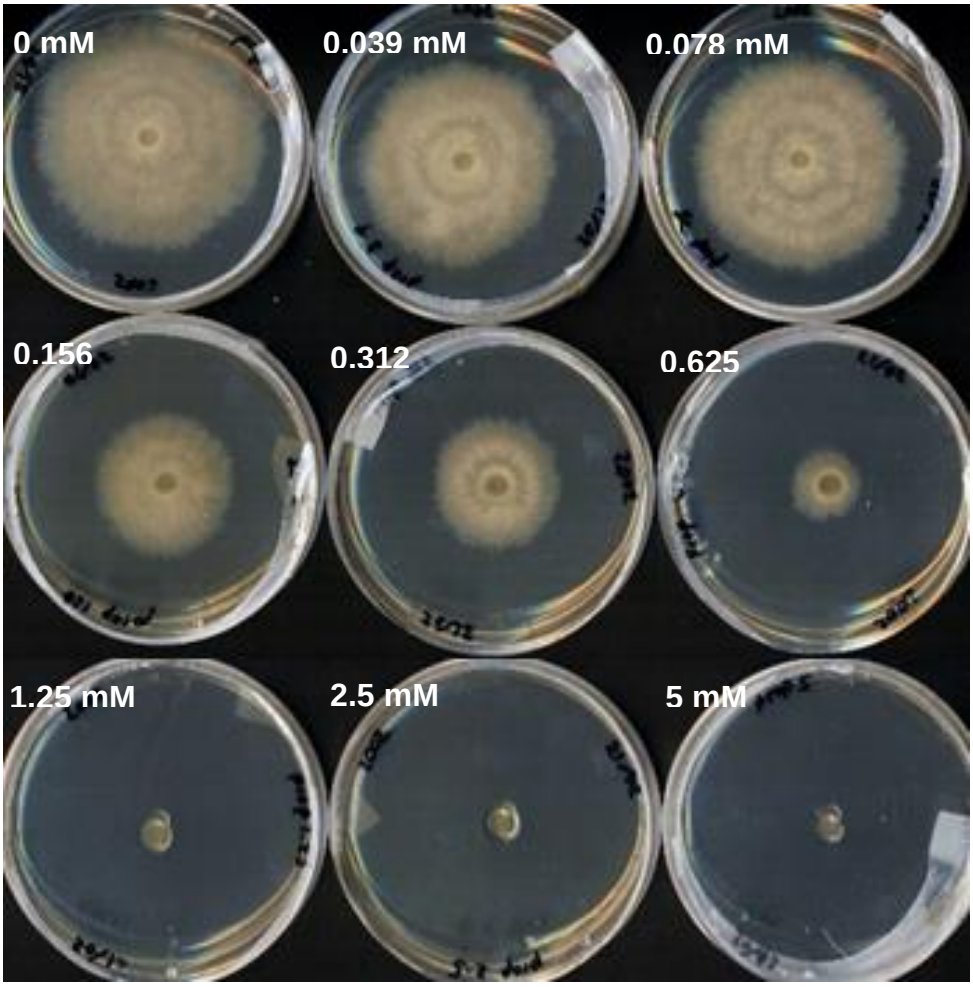


Figure 73: Growth of *Rhizoctonia solani* AG2-1 on potato dextrose agar containing a dilution series of propyl ITC. Representative plates are shown at the conclusion of the experiment when the control culture had grown to almost fill the Petri dish. For this ITC, which was the least potent against both anastomosis groups, no growth occurred at 1.25 mM or above.

The AG3 isolate of *Rhizoctonia* was more sensitive to all tested ITCs than AG2-1. The % growth inhibition relative to the control was calculated for each strain exposed to each ITC concentration. The AG3 isolate was most sensitive to 3-(methylthio)propyl isothiocyanate with 88.2% inhibition achieved at only 0.039 mM ITC (Fig. 74). 2-phenylethyl ITC at the same concentration was also highly effective at controlling mycelial growth with a 70% reduction. Both ITCs at the next highest concentration of 0.078 mM completely stopped *R. solani* growth. Benzyl and 2-propenyl isothiocyanate were the next most toxic compounds in relation to AG3, with both having an ED≥50 of 0.078 mM (Table 2), although they did vary in their abilities to halt growth entirely. A concentration of 0.156 mM for benzyl ITC was sufficient to completely prevent growth, whilst a higher concentration of 0.312 mM was necessary for 2-propenyl ITC to produce the same outcome. Finally the weakest ITC against *R. solani* AG3 was propyl isothiocyanate requiring 0.625 mM for complete inhibition, though it was noted that growth was more than halved in relation to the control when plugs were grown on 0.156 mM agar.

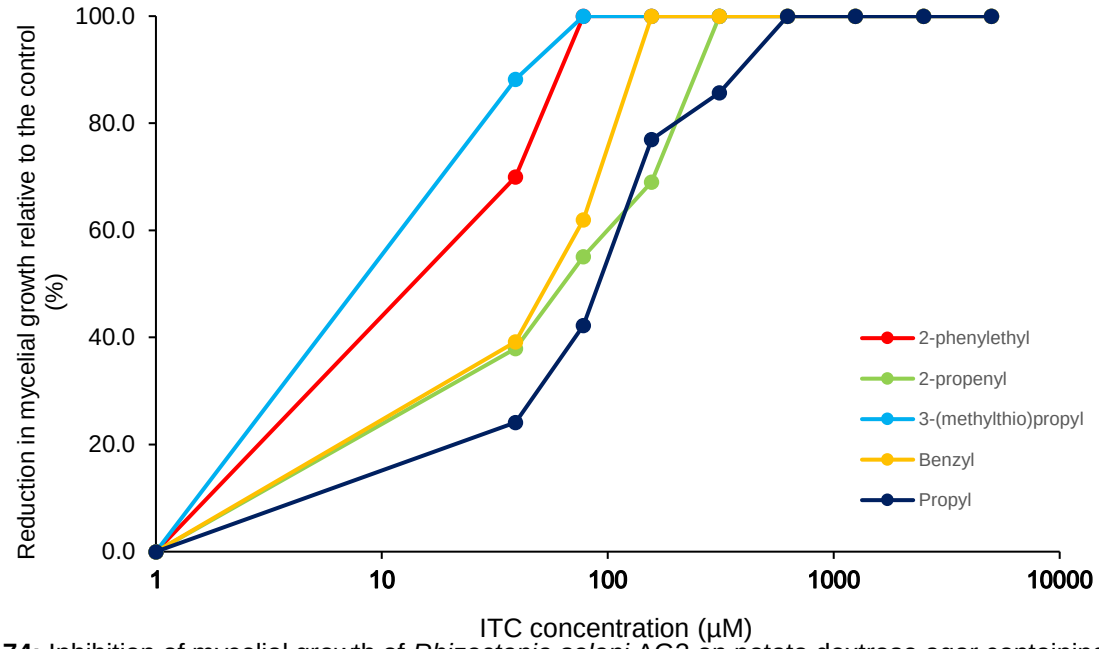


Figure 74: Inhibition of mycelial growth of *Rhizoctonia solani* AG3 on potato dextrose agar containing dilution series of a range of isothiocyanates. Inhibition is presented as % reduction in mycelial growth relative to control plates containing no ITC. n = 6 for each ITC/concentration.

AG2-1 *R. solani* was markedly less susceptible to growth inhibition by all tested ITCs (Fig. 75). 2-phenylethyl ITC for example failed to stop growth entirely at any concentration; this was in stark contrast to its performance against AG3 where it possessed one of the highest efficacies. 2-propenyl and 3-(methylthio)propyl ITCs appeared to be the most effective when considering the concentrations at which 100% reduction in growth was attained. Although these two compounds halted fungal growth at the lowest concentrations, 2-phenylethyl and benzyl were more potent in reducing growth by ≥50% with 0.312 mM being sufficient. As for AG3, propyl ITC had the weakest effect with dilutions of 0.625 mM and 2.5 mM being required for ≥50% and 100% inhibition, respectively.

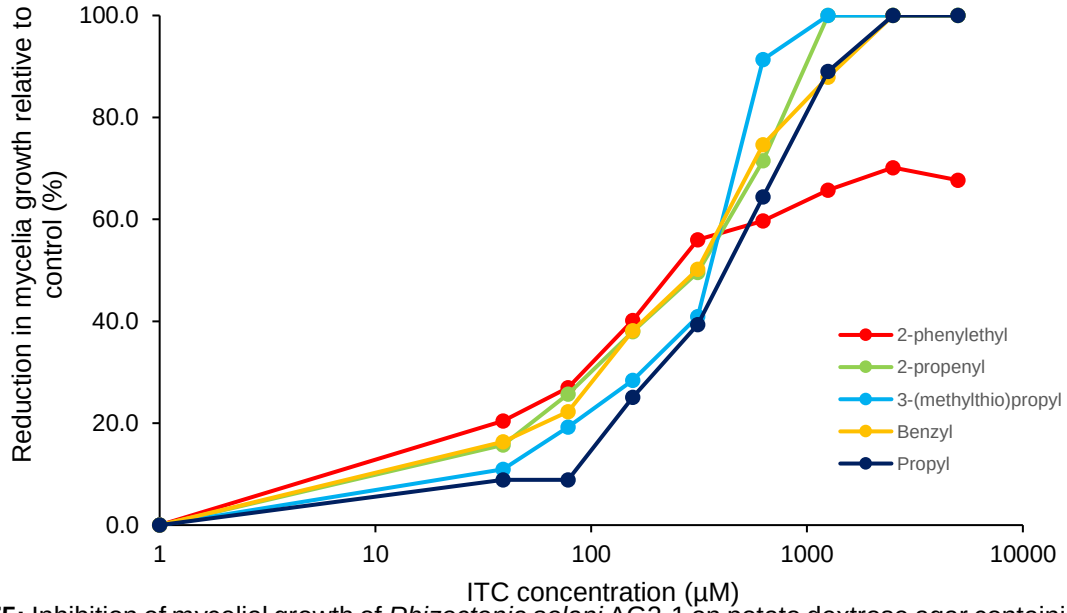


Figure 75: Inhibition of mycelial growth of *Rhizoctonia solani* AG2-1 on potato dextrose agar containing dilution series of a range of isothiocyanates. Inhibition is presented as % reduction in mycelial growth relative to control plates containing no ITC. n = 6 for each ITC/concentration.

Species	ITC	mM	
		$ED_{\geq 50}$	ED_{100}
***R. solani* AG3**	2-phenylethyl	0.039	0.078
	2-propenyl	0.078	0.312
	3-(methylthio)propyl	0.039	0.078
	Benzyl	0.078	0.156
	Propyl	0.156	0.625
***R. solani* AG2-1**	2-phenylethyl	0.312	-
	2-propenyl	0.625	1.250
	3-(methylthio)propyl	0.625	1.250
	Benzyl	0.312	2.500
	Propyl	0.625	2.500

Table 2: Effective doses of ITCs resulting in ≥50% and 100% reductions ($ED_{\geq 50}$ and ED_{100}, respectively) in fungal mycelia growth of *R. solani* AG3 and *R. solani* AG2-1.

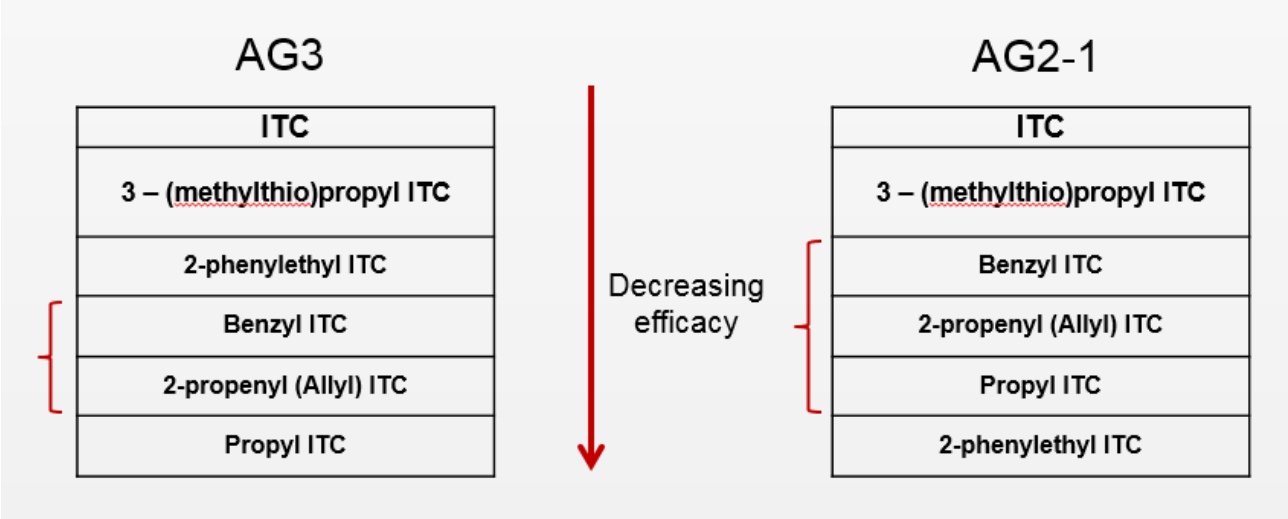


Table 3: The rank order of efficacy for ITCs was similar, but not identical for *R. solani* AG3 and *R. solani* AG2-1. Bracketed ITCs have similar efficacy against the particular *R. solani* isolate.

The re-growth potential of those fungal plugs that had shown no apparent growth whilst on plates containing ITCs was assessed following transfer to non-supplemented plates. In most cases the effect of the ITCs on *R. solani* appeared to be fungicidal rather than

fungistatic as in general those plugs that exhibited no growth on ITC plates did not begin to re-grow following their transfer. However, there were a handful of occasions where growth was seen to re-occur when plugs were no longer exposed to the ITCs. Some AG3 plugs grown on 0.078 mM 3-(methylthio)propyl ITC and 0.625 mM propyl ITC (Fig. 76) that had shown no growth initially, presented signs of fungal growth following transfer to ITC-free plates. Benzyl ITC concentrations of 2.5 mM and 5 mM had provided complete control of AG2-1 based upon growth measurements; although upon assessing them for re-growth it was observed that all of the fungal plugs at both concentrations were able to resume growth.

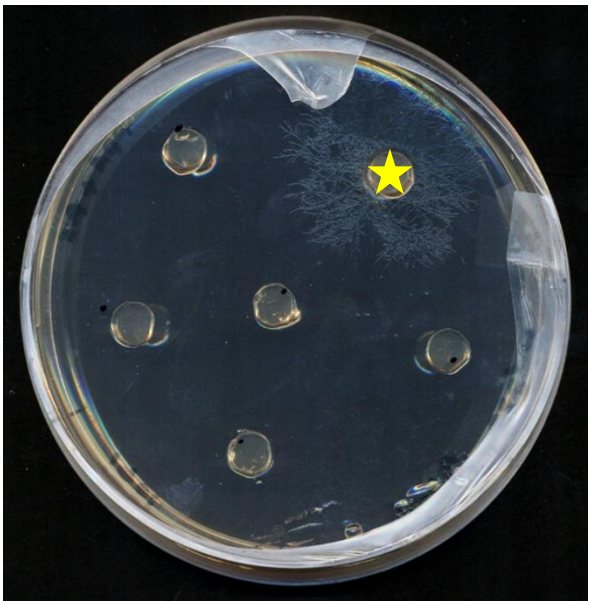


Figure 76: Regrowth of mycelium when plugs of *Rhizoctonia solani* AG3 were transferred from potato dextrose agar containing 625 µM propyl ITC to an unamended control plate. Regrowth was observed for only one of the 6 plugs – highlighted.

The previous assays showed the concentrations of ITCs required to inhibit mycelial growth, but the minimum length of time these compounds needed to be in contact with the fungi to illicit such responses was unclear. As our previous work in the field had shown a rapid decline in ITC in the soil following biofumigation, time limited ITC exposure assays were carried out to determine the minimum length of direct contact required for complete fungicidal activity. The two lowest concentrations of each ITC that caused complete inhibition of fungal mycelia growth of *R. solani* AG3 were used. As before, ITC was incorporated into PDA plates, and plugs were placed on them. However plugs were only left on the plates for 2, 4, 8, 16 and 24 hours at which point they were transferred to unamended PDA and growth was observed. Those fungal plugs grown initially at 0 mM exhibited normal fungal mycelia following their transfer to unamended PDA, suggesting transfer between plates did not negatively affect fungal mycelia growth. The lowest concentration of each ITC that had previously caused 100% inhibition of AG3 growth under prolonged exposure was unable to replicate that effect under any of the time limited exposures tested, although they did reduce growth in comparison to their respective controls. Despite the lowest ITC dilutions failing to control *R. solani* AG3, the higher concentrations of 2-propenyl (Fig. 77) and 2-phenylethyl did completely suppress growth after both 16 and 24 hours of exposure. Contrastingly, neither benzyl nor 3-(methylthio)propyl produced complete control at either 16 or 24 hours. Despite none of the exposure times shorter than 16 hours halting growth entirely, it was evident that it was reduced to various extents, with increased exposure length corresponding to greater reductions in subsequent mycelial expansion.

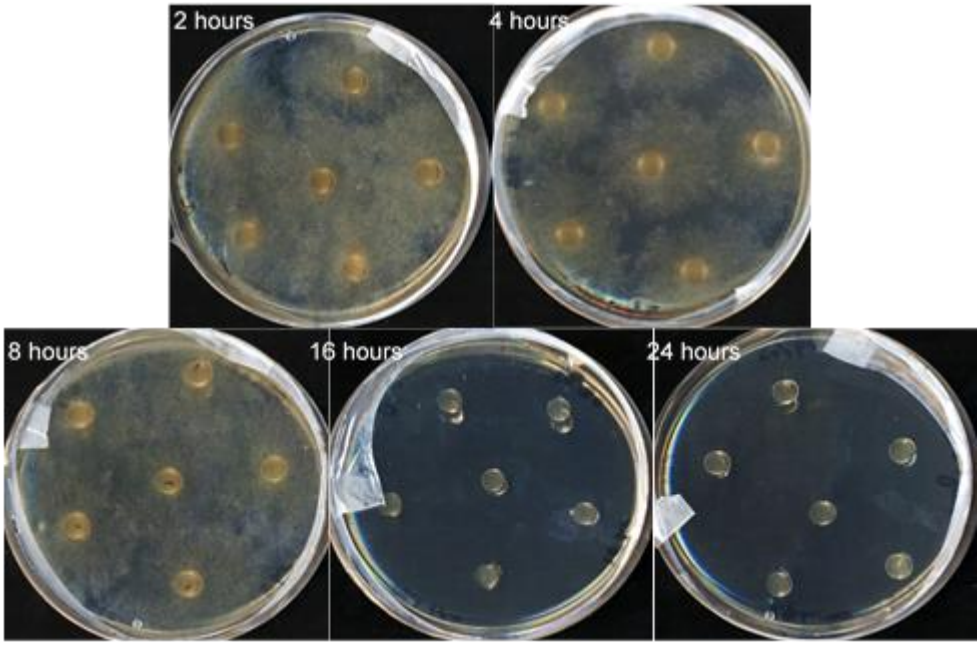


Figure 77: Timed ITC exposure assay. *R. solani* AG3 after 4 days on unamended PDA following exposure to 0.625 mM 2-propenyl ITC for 2, 4, 8, 16 and 24 hours.

The timed exposure assays clearly revealed the requirement for a period of prolonged contact with effective concentrations of isothiocyanates in order to achieve adequate fungal suppression. These results suggest that the compounds must be present within the soil, and be maintained at effective concentrations, for a minimum of approximately 16 hours. In addition to our own work, one study estimated that the half-life of 2-phenylethyl ITC was 16 hours, though this was seen to be reduced in highly water saturated soil and elevated temperatures. Based on the minimum effective exposure times seen here, if ITCs decline so rapidly their ability to control *R. solani* AG3 could be placed in some doubt, as effective ITC concentrations may not persist in the local soil environment for an adequate length of time.

Rhizoctonia solani glasshouse trial

A glasshouse trial was set up to test the effects of *B. juncea* ISCI99 on *R. solani* in soil. *Brassica juncea* 'ISCI99' and maize control plants were grown from seed in 7" pots filled with Petersfield Potting Supreme No 2 compost, under day lengths of 16 hours at 20 ^{o}C. 'ISCI99' was grown until the mid-flowering stage at which point it was harvested, along with the maize. 900 g of leaf and stem material from each plant species, equating to approximately ten ISCI99 plants and six maize plants, were collected and then macerated using a food processor.

Fungal inoculum was initially prepared by seeding bags of sterilised sand/oatmeal mix with mycelial plugs of *R. solani* AG3 from agar plates. These were incubated for 50 days with regular inspection and intermittent kneading but the majority of the bags became

contaminated and the remaining did not provide sufficient inoculum for the trial. As an alternative, 2.25 g (dry weight) of *R. solani* AG3 sclerotia was collected from approximately 6 week old plates and divided into three portions of 0.75 g. Each portion was ground using a pestle and mortar until powder-like, at which point it was mixed thoroughly into 15 kg of John Innes no.3 compost. For the inoculum only treatment this mixture was used to fill 7" pots with no further additions. For the maize and ISCI99 treatments their respective chopped 900 g of plant material was incorporated and mixed thoroughly into the inoculated compost, which was then used to fill 7" inch pots. Additionally 7" pots were also filled with unamended John Innes no.3 compost as a no inoculum control treatment. The moisture content of each pot was checked and the soil was watered until a moisture level of 25-30% was reached. The pots were then left under 16 hour days at 20 ^{o}C in the glasshouse for one week, whilst being watered occasionally to prevent the soil from drying out. After a week, chitted cv. Désirée potato tubers of a similar size were selected and a single tuber was planted into each pot. There were 6-8 replicates for each treatment.

Figure 78: Set up of glasshouse trial to determine the potential for biofumigation to suppress disease symptoms of *R. solani* on potato. Maize and brown mustard ISCI99 plants immediately prior to maceration (left); macerated mustard tissue being incorporated into fungal inoculated compost (right).

The number of aerial stems and plant height, which was measured from the base of the plant to the highest leaf, were recorded after ten weeks just prior to potato harvest. Both the weight and number of tubers produced by each plant were also quantified. After washing, plants and tubers were assessed for signs of *R. solani* disease, namely black scurf on tubers and dark lesions on stems and stolons. The severity of black scurf on all tubers was scored using the following scale: 0 = no sclerotia present, 1 = extremely low levels of sclerotia, 2 = minor amounts of sclerotia, 3 = moderate levels of sclerotia and 4 = severe amounts of sclerotia.

There were no significant differences between the four treatments for any of the plant growth traits measured (Fig. 79).

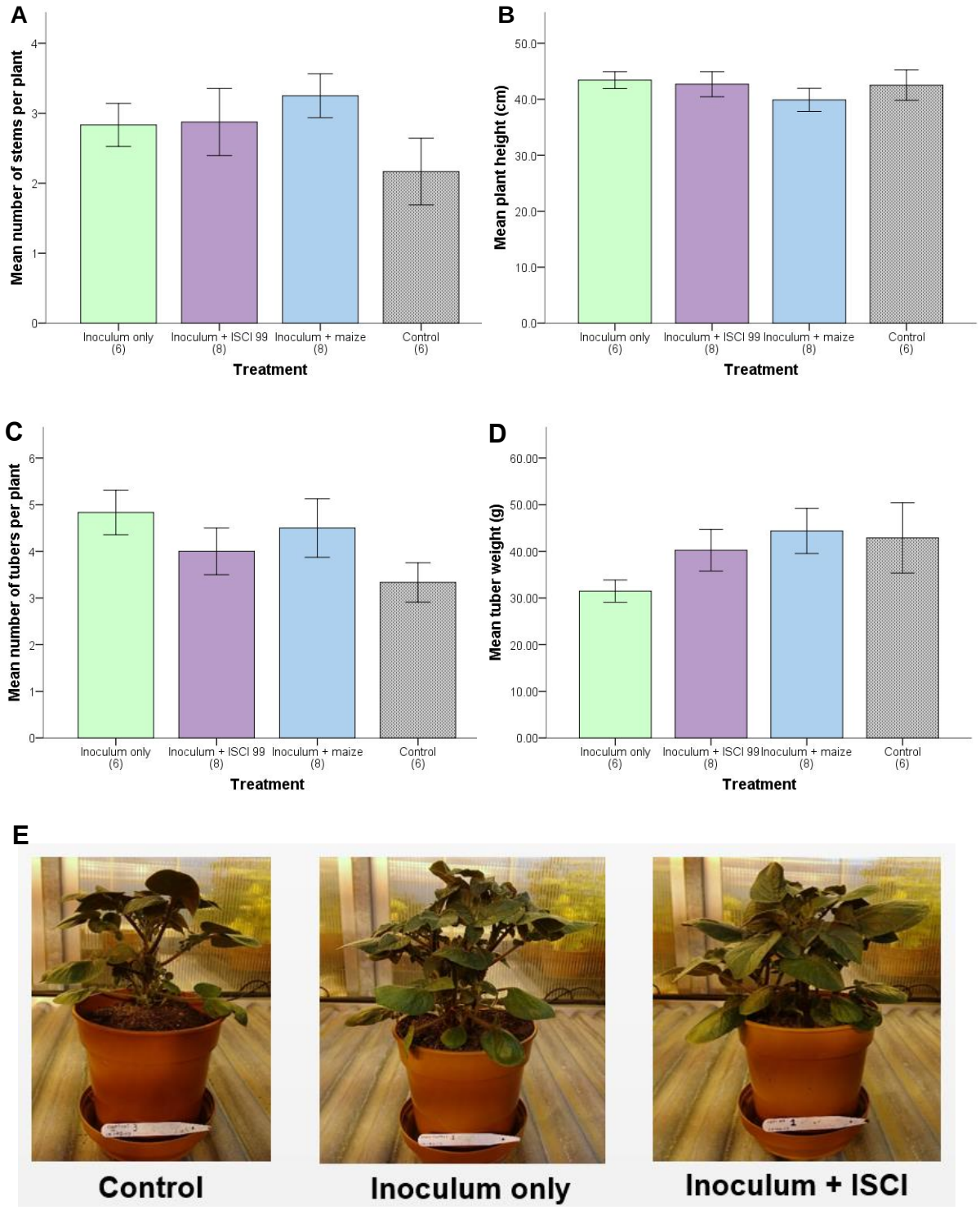


Figure 79: Soil treatment effects on growth parameters of potato plants at 10 weeks post planting. **(A)** Mean number of stems per plant. **(B)** Mean plant height. **(C)** Mean number of tubers per plant. **(D)** Mean tuber weight. Numbers in brackets give number of plants per treatment. **(E)** Representative potato plants for three of the treatments. Error bars indicate standard error.

Disease symptoms of *R. solani* were observed on both stolons and tubers of plants grown in all three treatments containing inoculum, although there was no evidence of stem canker.

None of the control plants showed any symptoms. The incidence of stolon canker and black scurf, as well as black scurf severity is shown in Fig. 80. There was no significant difference between inoculated treatments in the average incidence of stolon canker. Average black scurf incidence and severity did not differ significantly between the inoculated treatments. Despite not being significant, those tubers from the pots with incorporated maize tissue appeared to be most affected by black scurf, with an average incidence of over 90% and an average black scurf severity score of 2.3. In contrast, tubers from pots where the mustard ISCI99 had been incorporated into inoculated soil showed a lower average incidence of canker development, whilst black scurf was also seen to be less severe with an average score of 1.6.

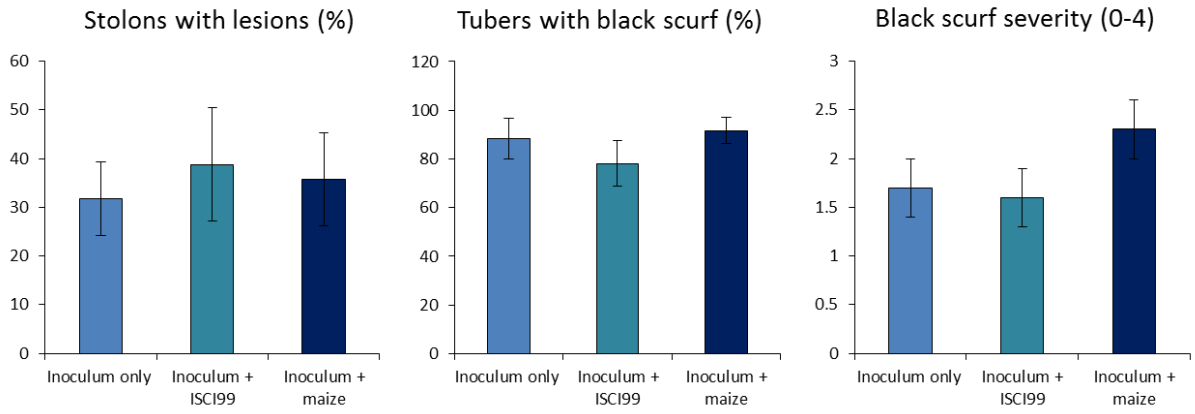


Figure 80: Soil treatment effects on *R. solani* disease symptoms. Treatment of the soil with neither macerated maize tissue nor macerated tissue of the mustard ISCI99 resulted in any significant effect on incidence of stolon canker or tuber black scurf. Error bars indicate standard error of the mean.

Verticillium dahliae

The ITCs were also tested for their efficacy in controlling growth of *Verticillium dahliae* in agar plate assays. The sensitivity of this fungus to the ITCs was intermediate between the two *Rhizoctonia* anastomosis groups. Similarly to both *R. solani* isolates, *Verticillium* was most tolerant to propyl ITC with only concentrations of 1.25 mM and above causing substantial reductions in fungal growth (Fig. 81). The most effective ITCs against *V. dahliae* were 2-phenylethyl and benzyl which both gave complete control at 0.156 mM. A 0.312 mM dose of 3-(methylthio)propyl ITC provided 100% reduction in mycelia growth, whilst 2-propenyl was less effective with 1.25 mM being necessary to achieve the same result. In contrast to *R. solani* AG2-1 and AG3 there was less of a gradual effect of increasing ITC concentrations on fungal growth, with none of the chemicals apart from propyl ITC having levels that offered inhibition of between 50 - <100%. Instead inhibition jumped from relatively low effect straight to lethal doses between the ITC concentrations used here.

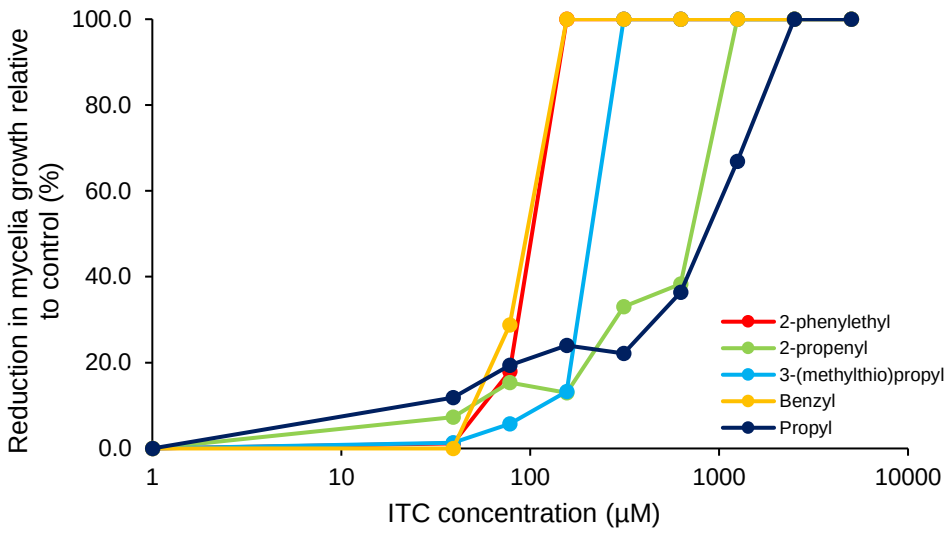


Figure 81: Inhibition of mycelial growth of *Verticillium dahlia* on potato dextrose agar containing dilution series of a range of isothiocyanates. Inhibition is presented as % reduction in mycelial growth relative to control plates containing no ITC. n = 6 for each ITC/concentration.

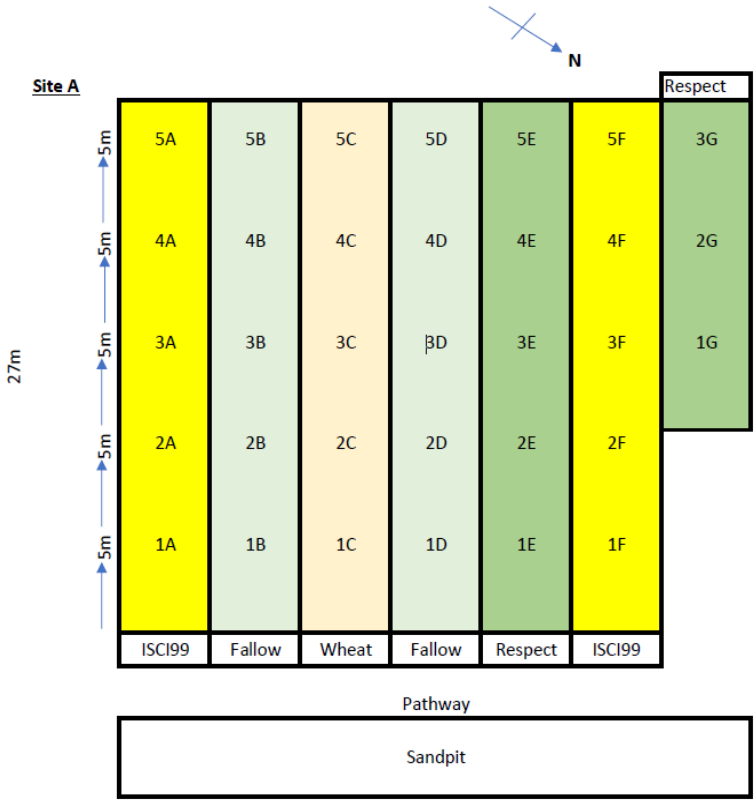
Objective 6: Analyse the impact of biofumigant crops on biotic aspects of the below ground food web.

Managing pests and pathogens in an agricultural context is a balance between efficacy of control allied with minimising the collateral impact on the environment. Beneficial nematodes occupy key nodes in soil food webs and have a significant role in soil nutrient cycling. Thus it is crucially important that biofumigation has a minimal deleterious impact on such beneficial taxa that can act as indicators of the below-ground food web status.

The impact of biofumigation on beneficial nematodes was assessed in a strip plot field trial at the Fenside Site (52° 59' 45.6" *N*; 0° 15' 3" *W*) carried out over 2018-2019 with the layout as shown in Fig. 82. The soil at the trial site is a loamy sand, with characteristics as detailed below. Two biofumigants were used: the brown mustard 'ISCI99' and the fodder radish 'Respect'. Strips were drilled on 23/08/2018. Supplementation of N and S was provided as granular fertiliser (100 kg/ha N and 20 kg/ha S). Fallow plots were treated in the same way as biofumigant plots at incorporation. Approximately 1.5 kg of soil was sampled from 5 points per strip, 5m apart, at pre-incorporation (early November 2018) and post-incorporation (11/02/2019). Soil samples were well mixed, any stones removed and then 150 g subsamples were taken for nematode extraction using the Whitehead tray method. All samples were of similar soil moisture prior to weighing.

	P		K		Mg		pH
Field Name	mg/l	index	mg/l	index	mg/l	index	
Fenside	59.2	4	218	2+	44	1	7.5

Figure 82: Layout of the strip trial for evaluation of biofumigant effects on non-target nematodes. The total length of the strips was 27 m and sampling points were 5m apart along each strip. 'ISCI99' is a brown mustard and 'Respect' is a fodder radish – both biofumigants.



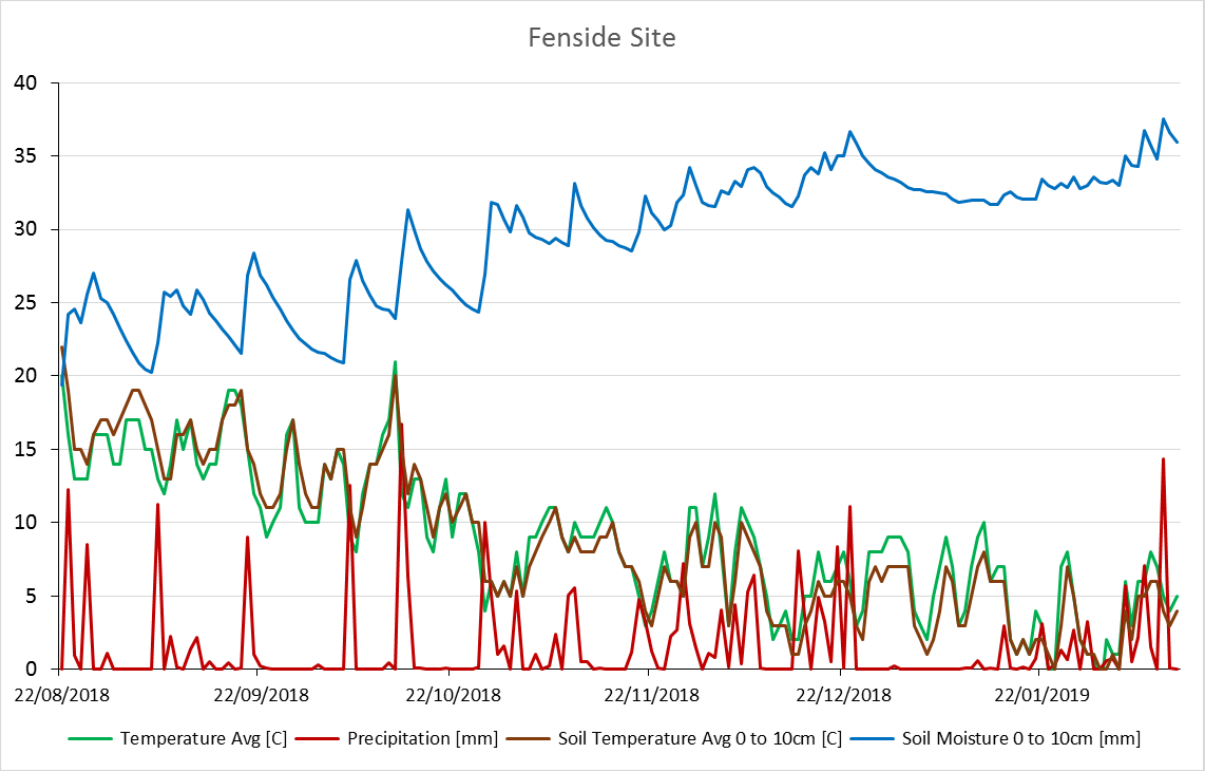


Figure 83: Average air temperature, daily precipitation, average soil temperature to 10 cm depth, and soil moisture to 10 cm depth for the Fenside site over the time period from biofumigant drilling to final soil sample collection.

The tray extraction was allowed to proceed for 3 days before nematodes were recovered in approx. 250 ml of water, settled and finally resuspended in 50 ml of water per sample. Total numbers of nematodes were determined from 1 ml aliquot counts.

For most treatments significantly more nematodes were recovered from the soil at the post-incorporation sampling, than at pre-incorporation (paired t-tests between sampling times, indicated with asterisks in Fig. 84). For one of the fallow strips there was no significant difference, whilst for the other, the difference was marginal (P=0.049).

When comparing between treatments there were no significant differences at the pre-incorporation sampling (One-way ANOVA with Tukey's post hoc test). For the samples taken approximately 14 weeks post-incorporation, the only significant difference was between the ISCI99 treatment in Strip A and the wheat in Strip C. Thus the biofumigation was clearly having no detrimental effect on the abundance of soil nematodes, either during growth of the crop, or at 3 months post-incorporation. It is possible that there was a transient effect immediately after incorporation, and that the populations had recovered by the time of sampling.

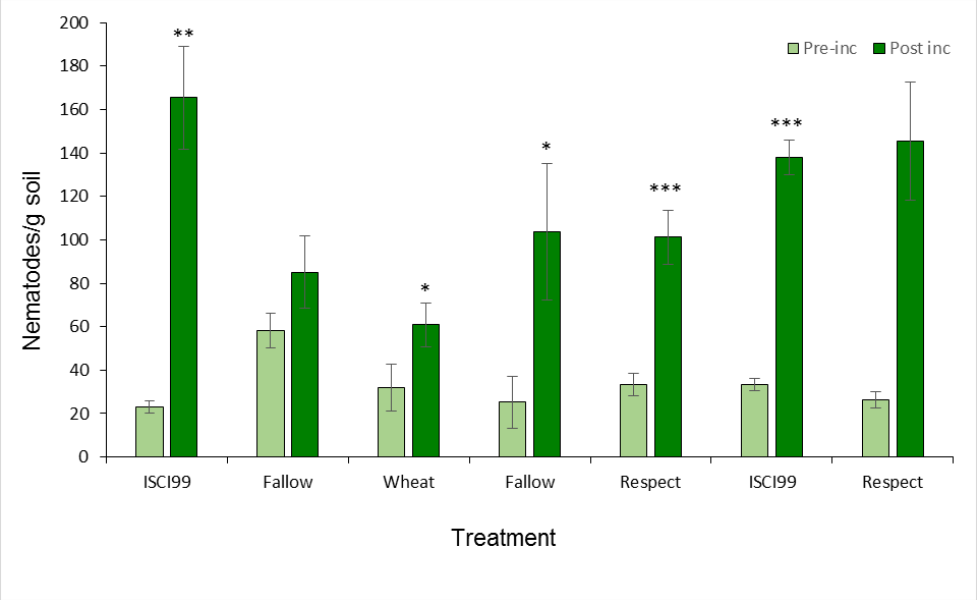


Figure 84: Number of nematodes per gram of soil for samples taken at pre-incorporation and post-incorporation of crops. The order of the treatments left to right is the same as the left to right order of the strips in the trial lay-out. Significant differences between sampling times for the same strips are shown by asterisks (* P<0.05, ** P<0.01, ***P<0.001; n=5, Paired t-tests). There were no significant differences between treatments at pre-incorporation (One-way ANOVA with Tukey's post hoc test). Only the first ISCI99 strip and the wheat strip were significantly different at the post-incorporation sampling (P=0.01).

3. Conclusions

Widespread grower uptake of biofumigation is currently limited by several factors, including a lack of data on the factors affecting its consistent efficacy. The combined findings from this project will provide agronomists with the information required to offer evidence-based advice to growers on the suitability of biofumigation as a nematode management tool in different scenarios. If chosen as a management practice they will be able to devise optimal deployment strategies to ensure that biofumigation delivers maximum efficacy.

We have defined the brown mustard (ISCI99) and radish (Bento) biofumigant cultivars of choice based on their biofumigation potential and clarified the optimum growth stage for incorporation.

Importantly, we found that biofumigant seeding rates have no significant impact on biofumigation potential. This new knowledge suggests that grower costs can be minimised by reducing seeding rate without negatively affecting biofumigation potential.

We found that the main biofumigation compound is lost from soil within 24 hours after incorporation. This is important knowledge both for informing the most effective incorporation techniques and when designing realistic *in vitro* exposure assays with pests and pathogens.

Our finding that biofumigation was effective in clay loam but not sandy loam could affect the management advice given to growers, based on their soil type. However, we also find that biofumigation effects on potato cyst nematode are highly variable, even within a single trial, making it difficult to confidently endorse it as an effective tool for management of PCN.

The impact of biofumigation on beneficial nematodes was assessed in a field trial. There was no evidence of biofumigation having a detrimental effect on the abundance of soil nematodes, either during growth of the crop, or at 3 months post-incorporation. It is possible that there was a transient effect immediately after incorporation, and that the populations had recovered by the time of sampling.

References

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Appendix I: Improved methodology for Glucosinolate analysis

In the course of analysing the biofumigant samples by HPLC, the standard extraction techniques were improved to provide a new method that gives cheaper, quicker and more accurate results. The ISO standard method for glucosinolate extraction involves boiling freeze-dried, milled material in 70% methanol at 75°C followed by a low concentration (0.05-0.3U/ml) desulfatase treatment. The current alternative method involves boiling the milled plant material in water which avoids the hazards associated with boiling methanol, however this is not useful for all glucosinolates since indole glucosinolates, such as glucobrassicin are degraded by this process (Fig. 14).

It was observed that the freeze-drier used for the initial step of the extraction process can have a large impact on the apparent glucosinolate concentration. This may be one source of the large variation in reported glucosinolate concentrations that are found in the scientific literature. Therefore, a method which does not involve a freeze-drying step could increase reliability of the data and allow more accurate comparisons between experiments conducted by different groups.

Extraction of fresh tissue directly into 80% methanol at room temperature was found to provide equally efficient glucosinolate extraction as the current method with hot methanol. The myrosinase enzyme which degrades glucosinolates in disrupted plant tissues was found to be inactive in 80% methanol (Fig. 15) so the lower temperature can be used. In addition, a higher concentration of desulfatase (0.5-1.0 U/ml) was found to improve glucosinolate quantification.

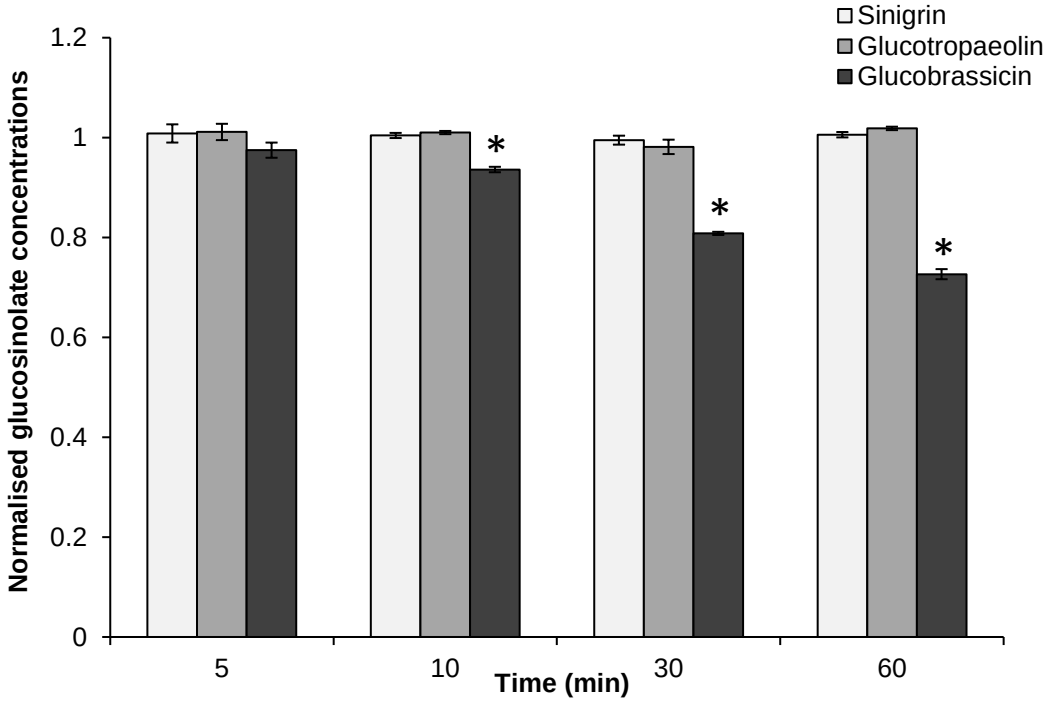

Figure 14: Concentrations of representative aliphatic (sinigrin) and aromatic (glucotropaeolin) glucosinolates were not reduced over the course of an hour when incubated in water at 100°C. The representative indole glucosinolate (glucobrassicin) is thermally degraded at 100°C. Asterisks represent significant difference from concentration at T=0 (paired t-test, $p<0.05$).

The improved method of wet tissue extraction directly into cold methanol was found to be comparable to the ISO standard method of freeze-dried tissue extraction directly into boiling methanol. 80% cold methanol can be used instead of boiling methanol to extract glucosinolates across a broad spectrum of brassicacae species and tissue types (Fig. 16). With the exception of glucoraphasatin in *R. sativus* shoots, replacing hot 70% methanol with cold 80% methanol did not significantly reduce glucosinolate concentrations, yet marginally increased recovery of sinalbin in *S. alba* and sinigrin in *B. juncea*.

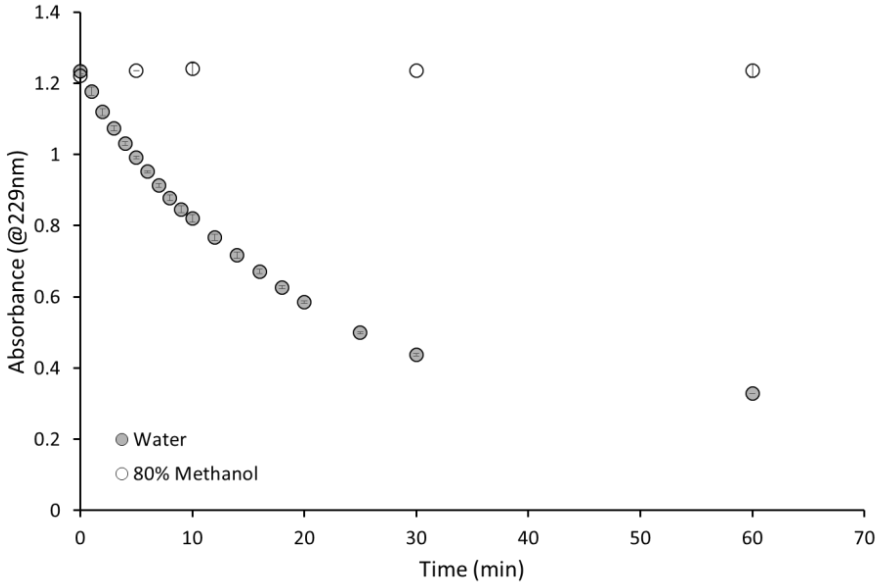

Figure 15: Spectrophotometric analysis of sinigrin hydrolysis kinetics in water and 80% methanol (n=3) by purified myrosinase (0.05 mg/ml) at room temperature (25°C).

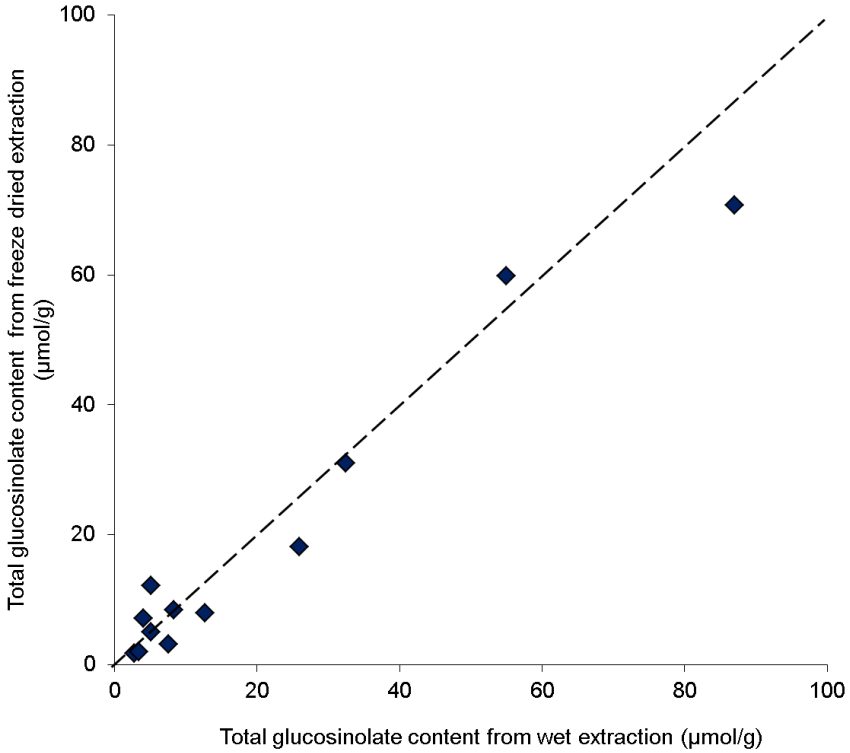

Figure 16: Comparison of glucosinolate yields between wet tissue extraction directly into cold methanol and freeze dried tissue extraction directly into boiling methanol.