Understanding the Inhibitory Effects of Plant-Derived Isothiocyanates and Biofumigation on Potato Cyst Nematodes

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Abstract

Management of potato cyst nematodes (PCN) has become more challenging due to nematicide restrictions. This has led to research into alternative control methods such as biofumigation. Biofumigation exploits the release of toxic isothiocyanates (ITCs) from glucosinolate (GSL) hydrolysis during the breakdown of Brassicaceae plant tissue. The type and concentration of ITCs released varies between plant species. Biofumigation and ITCs have been shown to control soil-borne pathogens however no single ITC achieves broad-range control.

The main aim of this study was to determine if biofumigation can be used to control PCN, namely *Globodera pallida*. *In vitro* and glasshouse studies identified a key ITC which inhibited *G. pallida*, with ITC effectiveness depending upon ITC type, concentration and exposure period. The GSL profiles of different Brassicaceae spp. were analysed and results indicated that GSL content varies between species and throughout plant development. The biofumigation process was investigated in glasshouse and field trials and analysis of GSL profiles allowed a comparison of cultivars with respect to potential ITC release. Cultivars which released the identified key ITC were able to suppress encysted *G. pallida* under controlled conditions. External factors impacted on the effectiveness of biofumigation in field trials. In order to determine if biofumigation adversely affects soil microorganisms, shifts in soil microbial communities were investigated. ITC application under controlled conditions and biofumigation under field conditions had little effect on soil microorganisms. Transient shifts in communities occurred in response to biofumigation under controlled conditions. The greatest response of soil communities was to factors independent of biofumigation.

Results from this study will feed back into the development of integrated PCN management strategies involving biofumigation as well as into biofumigant breeding programmes.

Dedication

I dedicate this thesis to my little sister, Amy, and my partner-in-crime, Gordon.

Amy, you continuously make me proud and push me to think differently. You can always come to me as your big sister and hopefully this encourages you to achieve your dreams.

Gordon, you have been my rock throughout. You should take your contribution as a sounding board, nutrition supplier and comfort giver, as evidence of your awesomeness.

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Chapter 1. Introduction

1.1. Overview

In the UK around six million tonnes of potatoes are produced annually. Potato cyst nematodes (PCN) are major potato pests and have been identified in 48% of potato fields in England and Wales with 89% of these fields infested with *Globodera pallida*, 5% infested with *G. rostochiensis* and 6% infested with both (Dybal *et al.*, 2016). In Scotland 23% of ware potato have been found to be infested with PCN (47.8% G. *pallida* and 52.2% *G. rostochiensis*) (Evans, 1999). Control of these species traditionally relies on crop rotation, host resistance and nematicide use. Due to the European Council regulation (EC) No 1107/2009 under Directive 91/414/EEC, which changed risk-based assessment of plant protection products to hazard-based criteria, increasing emphasis is being placed on the reduction of nematicide use as there are concerns regarding the possible negative impact on the environment. In response, a considerable amount of interest is being shown in the development of environmentally-friendly, economically-viable and effective pest management strategies (Matthiessen and Kirkegaard, 2006).

One such strategy is biofumigation. Biofumigation is the suppression of soil pests and diseases by volatile hydrolysis products released into the soil after the incorporation of glucosinolate-containing plant tissue, from Brassicaceae spp. (Angus *et al.*, 1994). This hydrolysis has the potential to release breakdown products such as isothiocyanates (ITCs), nitriles and thiocyanates (Cole, 1976; Fenwick, Heaney and Mullin, 1983; Borek *et al.*, 1994) of which ITCs are considered the toxic product required to suppress pathogens (Lazzeri, Tacconi and Palmieri, 1993). Biofumigation and ITCs appear to have nematicidal activity against a range of nematode species and previous *in vitro* research has indicated that certain glucosinolate (GSL) hydrolysis products can cause *G. rostochiensis* and *G. pallida* mortality. More recent research on the effects of Brassicaceae material and ITCs on encysted second-stage juveniles (J2) have shown that different cultivars have varying effects on the viability and hatch of *G. pallida*. Most studies are focussed on the effect of a select few Brassicaceae species and GSL breakdown products, namely *Brassica juncea* and its major GSL, sinigrin. As different species contain a number of different GSLs able to release various concentrations of ITCs, there is a need to screen a wide range of ITCs and Brassicaceae against *G. pallida* in order to determine which cultivars will be most effective as a PCN biofumigant.

1.2. Potatoes

In terms of human consumption, potatoes (*Solanum tuberosum)* are the fourth most important food crop grown globally, after the three major grains – wheat, rice and maize (Oerke, 2005;

Fiers *et al.*, 2012). In the UK, five to six million tonnes of potatoes are produced annually (AHDB Potatoes, 2017). Around half of potatoes produced are used in the fresh market whereas the rest are distributed to a range of food processing markets including crisps and chips. Potatoes are one of the most important vegetables produced in Britain; fresh potatoes account for 22% of consumer expenditure on fresh vegetables, with annual retail sales of around one billion pounds (Vasileiou and Morris, 2006).

1.2.1. Potato pathogens

Potato crops are susceptible to over forty pests and diseases which can be both air- and soil-borne including insects, nematodes, viruses, bacteria and fungi (Fiers *et al.*, 2012). It has been estimated that 40% of global potato loss is due to weeds, pests and pathogens with animal pests, including nematodes, accounting for 11% of crop loss (Oerke, 2005).

Soil-borne diseases can be split into two groups based on potato symptoms; those damaging tubers and those damaging other plant parts (Gudmestad, Taylor and Pasche, 2007).

Tuber symptoms are divided into galls, blemishes and rots (Fiers *et al.*, 2012). Galls consist of outgrowth and tuber deformation and are commonly formed by powdery scab, common scab, wart and root-knot nematodes (Vovlas *et al.*, 2005; Bouchek-Mechiche *et al.*, 2006; Merz and Falloon, 2008; Fiers *et al.*, 2012). Blemishes occur on the tuber skin and are economically important due to consumer preferences. Blemishes can appear on the tuber surface as spots (black dot, black scurf, skin spot or powdery scab; Aqeel, Pasche and Gudmestad, 2008; Merz and Falloon, 2008; Woodhall *et al.*, 2008; Fiers *et al.*, 2012), areas of uneven colour presenting as a scab (common or netted scab; Bouchek-Mechiche *et al.*, 2006) or as a silver surface colouration (silver scurf; Cunha and Rizzo, 2004). Rots include dry and soft rots (charcoal rot, leak, bacterial soft rot, black leg and stem rot; Garibaldi, Gilardi and Gullino, 2006; Fiers *et al.*, 2012), flesh discoloration (pink rot) or vascular ring discoloration (ring rot, brown rot, *Verticillium* wilt and *Fusarium* dry rots; Fiers *et al.*, 2012; Ochiai *et al.*, 2007; Peters *et al.*, 2008).

Soil-borne diseases which damage other plant tissues include diseases such as blackleg, stem canker and stem rot which can form stem lesions (Garibaldi, Gilardi and Gullino, 2006; Woodhall *et al.*, 2008; Fiers *et al.*, 2012). Alternatively, leaf symptoms such as cell death (necroses) and loss of colouration (chloroses) can be caused by *Phoma* leaf spot or *Verticillium* wilt (Ochiai *et al.*, 2007; Fiers *et al.*, 2012). Root lesions caused by nematodes (root-knot and potato cyst nematodes) can lead to either necroses or rot and are potential sites for other soil microorganisms to enter the potato plant (Vovlas *et al.*, 2005; Fiers *et al.*, 2012).

Favourable conditions for potato disease development are frequently the same as the conditions needed for potato growth: a temperature between 10°C and 25°C, high moisture content and neutral pH therefore the incidence of disease may be high if precautions are not taken (Fiers *et al.*, 2012).

1.2.2. Potato pathogen control methods

Until recently, pesticides were the most common pest control method utilised. They can be applied in a number of ways including as a fumigant, powder, spray or granule (Tsror *et al.*, 2000; Errampalli *et al.*, 2006; Hide *et al.*, 2009). Since pesticide use is becoming more restricted, many chemicals are no longer permitted in Europe and their use is declining. Methyl bromide was previously used as a broad-spectrum soil fumigant to control soil-borne pests and diseases in high value crop lines, including potato (Ciancio and Mukerji, 2008). Concerns about its negative impacts on the environment and human health, such as contamination of drinking water and ozone depletion, led to the fumigant being banned in many countries and necessitating a search for alternative soil-borne disease control measures.

Other control methods include: crop rotations, making use of resistant varieties, altering fertilization and water management, adding a delay between haulm killing and harvest, and biological control (Ciancio and Mukerji, 2008; Fiers *et al.*, 2012), all of which are most effective when incorporated into an integrated pest management system.

Crop rotations of three to four years have proven effective at controlling soil-borne potato pathogens in several studies (Fiers *et al.*, 2012), but this effect is very much dependent on the host range of the pathogen and its ability to survive in the absence of a host. Certain pests and pathogens able to persist for long periods are not as easily controlled using crop rotation – examples of this include *G. pallida, Synchytrium endobioticum,* and *Spongospora subterranea* (Hampson, 1985; Christ, 1989; Minnis, Haydock and Eva, 2004; Fiers *et al.*, 2012); therefore additional practices will be required to effectively control these pathogens.

One area in potato disease control is cultivar resistance. Resistant cultivars are able to defend against pathogens by triggering the production of antimicrobial agents, activating defence genes and initiating cell death (Levine *et al.*, 1994). Wild *Solanum* species containing disease resistant genes are potential candidates for crossing into the *S. tuberosum* genome to reduce infection of the commercial potato crop (Jansky and Rouse, 2003). In addition, varieties containing anthocyanin colour pigments (red, purple and blue) are being more commonly grown as they are thought to provide greater resistance to diseases, such as soft rot, compared to white and yellow non-anthocyanin containing cultivars (Wegener and Jansen, 2007); this is not necessarily the best way to combat potato diseases economically as consumer choice

dictates the need for potatoes with light skin and flesh to be available on the market. Although potato resistance to disease does occur (Merz and Falloon, 2008), broad-range resistance to pathogens is difficult to achieve and alternative control methods are still required.

Applying a mixture of organic and inorganic fertilisers supplies potatoes with the required micronutrients needed to become stronger and less susceptible to disease (Davis *et al.*, 1994; Panique *et al.*, 1997). Fertilisers can contribute to the suppression of soil-borne pathogens by altering soil properties such as soil pH, nutrient availability and microbial activity (Liu *et al.*, 2015) which changes the soil environment so that it is unfavourable. Alternatively, the addition of fertilizer, especially those containing high nitrogen, can increase potato foliar development and humidity providing better conditions for pathogens such as *Pectobacterium* spp. to flourish (Fiers *et al.*, 2012).

Factors involved in planting, dehaulming, lifting and harvesting can have an effect on potato disease development. Planting at low densities increases plant yield due to increased growth space and lower disease spread compared to planting at high densities. Adjusting planting, dehaulming and harvesting dates can also reduce the incidence of disease due to unfavourable temperatures and reduced time for disease development (Fiers *et al.*, 2012). After harvest, attention needs to be paid when handling tubers during storage as the introduction of wounds to the tuber skin can increase the incidence of diseases such as silver scurf, gangrene, pink rot, black leg, soft rot and *Fusarium* dry rot (Hide, 1994; van Vuurde and de Vries, 1994; Salas *et al.*, 2000; Marcinkowska, Roze-Kałużny and Kałużny, 2005; J. C. Peters *et al.*, 2008; R. D. Peters *et al.*, 2008). Methods to reduce disease development in storage include limiting mechanical handling, curing harmed areas, avoiding exposure to light and ensuring plenty of ventilation (Fiers *et al.*, 2012).

A major area of research when considering potato disease is the application of non-chemical natural control agents (Fiers *et al.*, 2012). Fumigation by natural oils has been studied as a biological control agent for various tuber diseases such as dry rot, gangrene, black scurf and stem canker (Bång, 2007); *in vitro* garlic, thyme and sage essential oil volatiles displayed antifungal properties against *Helminthosporium solani*, *Fusarium solani*, *Phoma foveata* and *Rhizoctonia solani* where growth was inhibited. Fish emulsion incorporated into soil reduced scab incidence by *Streptomyces* spp. and increased tuber yield by up to 170% (Abbasi, Conn and Lazarovits, 2006). Adding a foliar spray containing potato defence gene inducers such as salicylic acid, di-potassium hydrogen phosphate, and tri-potassium phosphate can enhance potato plant defence against pathogens (Mahmoud, 2007). Incorporating bioagents such as *Pseudomonas putida* into soil was shown to reduce the incidence of potato brown rot disease

due to direct *Rastonia solanacearum* pathogen suppression (Mahmoud, 2007). The incorporation of Brassicaceae crops as a green manure, in the process known as biofumigation, has also been associated with the reduction of soil-borne potato pathogens due to the release of volatile toxic compounds (Angus *et al.*, 1994; Kirkegaard *et al.*, 1998; Sarwar *et al.*, 1998); this will be discussed in detail in 1.6.

1.3. Potato Cyst Nematodes

PCN are major potato pests that can remain dormant in the soil for over ten years in the form of cysts containing up to 600 eggs (Jones and Jones, 1974; Antoniou, 1989). PCN is thought to be present in almost all potato-growing areas (Brown, 1969).PCN is estimated to cause an economic loss of £50 million per year (DEFRA, 2004). There are two main species of PCN; *G. pallida* (Stone) Behrens and *G. rostochiensis* (Wollenweber) Behrens. *Heterodera* (*Globodera*) *pallida* was only described in 1973 (Stone, 1973) so any earlier publications referring to *H. rostochiensis* could refer to either species. *Globodera rostochiensis* was originally the predominant species in the UK until the introduction of resistant potato cultivars decreased populations allowing *G. pallida* to replace it as the dominant species with over 90% of infested UK fields containing this species (Minnis *et al.*, 2002; Trudgill *et al.*, 2003). *Globodera pallida* can be classified into three pathotypes, Pa1-Pa3, and *G. rostochiensis* can be classified into five pathotypes, Ro1-Ro5, based on the nematodes ability to multiply on a potato host (Kort *et al.*, 1977). PCN have a narrow host range and can only survive on a limited range of solanaceous crops including potato, tomato, aubergine and certain weeds (Fiers *et al.*, 2012).

1.3.1. Potato cyst nematode life cycle

In the absence of potatoes, PCN J2 remain dormant within their eggs inside the cyst which is formed from the hardened body of the female after death (Figure 1.1) (Ellenby, 1946; Clarke, 1968; Clark, Shepherd and Dart, 1972). PCN are able to survive in the absence of a host for many years due to their ability to enter dormancy (diapause or quiescence) in order to synchronize their life cycle to the potato host (Hominick, Forrest and Evans, 1985; Perry, 1989). Whilst in the state of diapause, PCN are more resistant to nematicides and changing environmental conditions (Spears, 1968; Elling *et al.*, 2007; Palomares-Rius *et al.*, 2013). Diapause is normally broken after a period of cold, such as winter, as potato growth begins when temperatures start to increase and soil moisture content becomes optimal (Muhammad, 1994). The J2 move from diapause to quiescence, in the absence of a host or when hatching conditions are unfavourable, where metabolism is lowered but development and hatch can be readily resumed as soon as conditions become more favourable (Perry and Moens, 2011).

PCN J2 are stimulated to hatch from cysts by host-specific hatching factors secreted from potato roots where they then infect potato roots (Forrest and Perry, 1980; Forrest and Farrer, 1983; Devine *et al.*, 1996; Jones *et al.*, 1997; Byrne, Maher and Jones, 2001). It is believed that this chemical release leads to a series of events within the PCN cyst beginning with a change in the permeability of the eggshells, the release of egg solutes, such as trehalose, and the uptake of water and oxygen which stimulates J2 movement and initiates hatching (Johnson, Dropkin and Martin, 1958; Ellenby and Perry, 1976; Atkinson and Ballantyne, 1977a; Clarke, Perry and Hennessy, 1978; Perry, 1989).

Once hatch has been initiated, J2 use their stomatostylet to cut a hole in the eggshell where they proceed to exit through one of the natural cyst wall openings (Doncaster and Shepherd, 1967). *Globodera pallida* J2 have a prolonged hatching pattern and can continue emerging for up to ten weeks (Evans, 1983; Whitehead, 1992). Hatching is higher on exposure to younger potato root diffusate compared to older (Byrne, Maher and Jones, 2001) therefore they are likely to infect potato roots early in the plant's growth. In comparison, *G. rostochiensis* J2 have a shorter hatching pattern of around six weeks (Evans, 1983; Whitehead, 1992). A greater hatch of *G. rostochiensis* occurs in response to older potato plants (Byrne, Maher and Jones, 2001) suggesting infectivity later in the potatoes life cycle.

Once hatched, the J2 need to infect a host rapidly to survive as they are only infective for six to eleven days and are vulnerable to environmental conditions due to the lack of cyst wall protection (Robinson, Atkinson and Perry, 1987). Infection of potatoes by PCN occurs when the hatched J2 enters the root by puncturing through the cell walls with its stylet. Interactions between the J2 and the host plant are mediated by a number of effector proteins secreted from the J2 which can alter plant cellular functions, suppress defence responses and modify host cell walls in order to assist in the infection of the roots by the nematodes (Cotton *et al.*, 2014; Thorpe *et al.*, 2014; Ali *et al.*, 2015). The J2 then remain attached to the potato root at the established feeding site until development through J3-J4 stages to mature male or immature female is complete. External factors can influence the ratio of male to female formation during development as a mechanism to increase reproduction and progeny populations. A limited nutrient supply (potato root system) leads to a larger proportion of male PCN whereas an abundant supply of potato roots and feeding sites leads to the majority of PCN developing into females.

Prior to death, the males detach and fertilise the still attached females; females remains during egg development. At this point differences between the species can be seen in the colour development of females; *G. pallida* remains white until fully mature and *G. rostochiensis* females passes through a golden stage for 4-6 weeks (OEPP/EPPO, 2013). When mature, the

females die and their skin hardens to form the protective cyst around the eggs inside. Once formed, the cyst detaches from the potato root where it remains in the soil until a further host potato crop is planted.

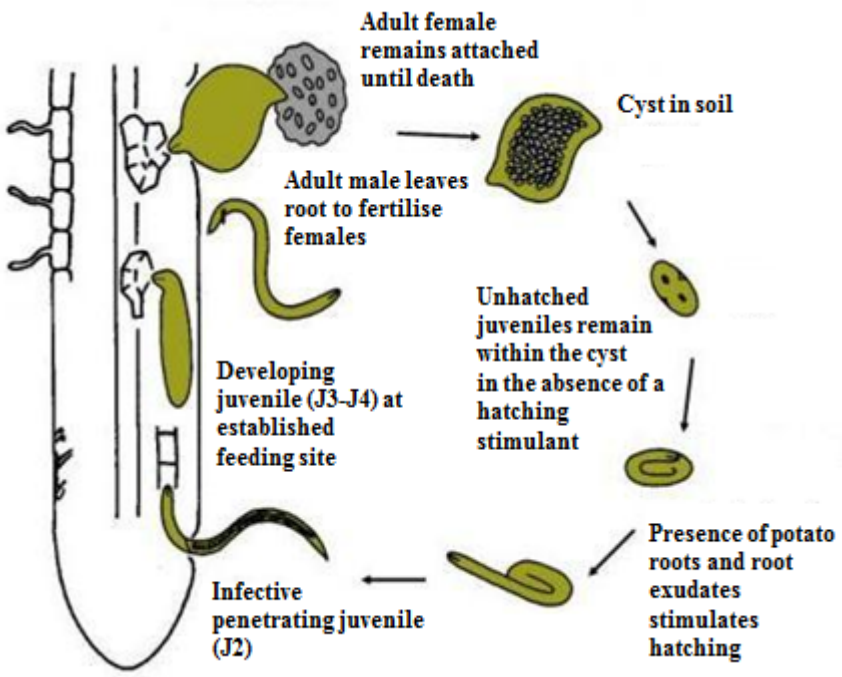


Figure 1.1. Potato cyst nematode lifecycle

1.3.2. Potato cyst nematode symptoms

The symptoms of PCN infection can vary greatly on potatoes (Figure 1.2) (Pylypenko, Phillips and Blok, 2008). PCN infection can cause patches of poor growth in crops with the occasional yellowing, wilting or death of foliage. Tuber size can be reduced as a result of foliage damage but it can also be reduced in the absence of foliar damage and when few visible symptoms are present. With respect to tuber damage, blemishes may appear. Large PCN populations can cause senescence, stunting or proliferation of lateral roots (Cronin *et al.*, 1997); this reduced root system can lead to decreased water uptake and plant death. The absence or presence of symptoms in different crops can often lead to the misdiagnosis of the pathogen or the lack of PCN detection entirely.

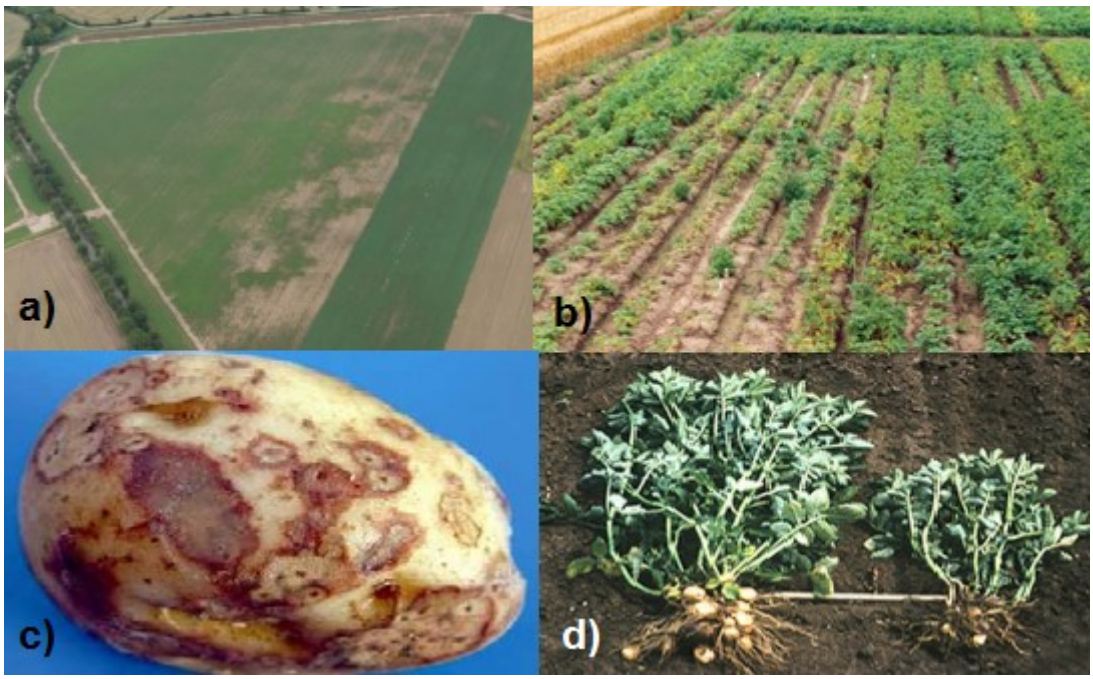


Figure 1.2. PCN symptoms on potatoes. A) Patches of poor potato growth (Barworth Agriculture, UK) B) Foliage yellowing, wilting and death (Scottish Crop Research Institute (SCRI), UK) C) Tuber blemishes (SASA, UK) D) Comparison of a healthy and PCN infected potato plant with reduced: root systems, tuber size and foliage growth (University of Hamburg, Germany, Bugwood.org).

1.3.3. Control of potato cyst nematodes

The ability of PCN to survive within the cyst in the absence of a host crop provides challenges for their eradication. Furthermore, the withdrawal of widely used nematicides due to the introduction of EU legislation (European Council regulation (EC) No 1107/2009 under Directive 91/414/EEC), which changed risk-based assessment of plant protection products to hazard-based criteria, has increased the urgency for implementing an alternative PCN control strategy.

Nematicides have been a particular focus for pesticide withdrawal due to their toxicity to humans and the environment. Since they are applied to the soil directly they can be easily absorbed into plants and contaminate groundwater (Oka, 2010). The broad-spectrum nematicide methyl bromide was a commonly applied chemical for PCN control, as previously mentioned in 1.2.2, and its phase out in 2005 had a large impact on nematode control strategies (Oka, 2010). Granular oximecarbamate nematicides, such as those containing oxamyl, are being more commonly used and can be effective against *G. rostochiensis* however they may be inadequate for *G. pallida* control. This is due to the short persistence of oxamyl in soil and the extended period of emergence of *G. pallida* J2 allowing invasion of potato roots after the nematicide has broken down (Whitehead, 1992; Ryan *et al.*, 2000). In

contrast, a nematicide containing fluensulfone, a new chemical which has lower mammalian toxicity than previous chemicals, is being studied for its ability to reduce *G. pallida* root infection and multiplication (Norshie, Grove and Back, 2016). Fluensulfone acts by reducing J2 mobility, impairing metabolic processes, reducing the J2's ability to access lipid stores and eventually causing J2 death (Kearn *et al.*, 2017).

Crop rotation is a traditional control method which is still widely used today. It is a broad-spectrum control method and where specialisation is required, as is the case for PCN, it may not be effective on its own (Cronin *et al.*, 1997). Potato rotations for PCN control are typically every five to seven years. A study by Trudgill *et al.* (2003) suggested that this is too short a period to prevent *G. pallida* populations increasing, even when in conjunction with nematicide application; this was attributed to dormant PCN being able to survive for years in combination with dormancy contributing to encysted J2 being less affected by chemical application and environmental conditions (Antoniou, 1989). *Globodera pallida* populations naturally decline by 10-30% per annum whereas *G. rostochiensis* populations reduce by 20-40% per year (Whitehead, 1995) with some PCN populations being found to have a natural decline rate of up to 69% per annum in the absence of a host (Haley, 2004). Therefore, longer rotations would be advantageous if used as the sole control method. A model from a later study suggested that rotations of at least eight years are required for *G. pallida* control, but including other control approaches could reduce this time (Trudgill, Phillips and Elliott, 2014).

Certain commercial UK potato cultivars, such as Maris Piper and Cara, have full resistance to *G. rostochiensis* (Ro1) but there is a lack of commercially-sold fully-resistant cultivars to *G. pallida* in the UK so crops are still vulnerable to damage (Danquah *et al.*, 2010). There are a few cultivars partially resistant to *G. pallida* yet their resistance can vary dramatically with a 10-90% reduction in infection depending on the *G. pallida* pathotype (Trudgill *et al.*, 2003); no single potato resistance gene provides resistance to all three *G. pallida* pathotypes (Strachan *et al.*, 2016). Although extremely variable, it has been suggested that partially resistant cultivars in combination with an effective nematicide may be able to reduce *G. pallida* populations; unfortunately, only around 8% of potato fields are planted with one of these partially resistant cultivars and a large proportion of these are not infested with *G. pallida* (Trudgill *et al.*, 2003) so the cultivars are not being utilised to their full potential.

An alternative PCN control method is trap cropping. In general, non-tuber forming Solanaceae species are used as PCN trap crops as they produce the required PCN hatching factors and many have a high degree of resistance PCN. A study which screened ninety

species found that only those from the *Solanum* genus induced J2 hatch. Varieties of two species (*Solanum sisymbrilfolium* and *Solanum nigrum*) also showed full or high resistance to *G. pallida* and *G. rostochiensis* suggesting their potential as PCN trap crops for both species (Scholte, 2000). *Solanum sisymbrilfolium* has been found to reduce the soil infestation of *G. pallida* eggs on potato by 99% in a glasshouse trial (Dandurand and Knudsen, 2016) and by 77% in a field trial (Scholte and Vos, 2000). Although trap cropping has proved to be effective, its success is dependent on the environmental conditions where warm climates and low soil organic matter are required for adequate growth. In addition it is a costly process when considering that its sole purpose is to hatch PCN prior to commercial potato crop planting.

There are many plant products capable of producing secondary metabolites to control pests and diseases, although there is a lack of studies into the effect of these on PCN. *In vitro* studies with Marigold (*Tagetes* spp.) have suggested that leaf and root extracts are able to reduce hatch from *G. rostochiensis* eggs but suppression is dependent upon cultivar and environmental factors (Chitwood, 2002). The effectiveness of Marigold at suppressing soil pests, including nematodes, is attributed to its production and release of the natural toxin, α-terthienyl (Marles *et al.*, 1992; Hooks *et al.*, 2010). Another example of plant extracts being exploited for PCN control is the application of aqueous garlic extracts to *G. pallida* J2 which, during *in vitro* studies, was able to increase J2 mortality (Danquah *et al.*, 2011). Garlic releases the sulphuric compound, allicin, whose breakdown products are the compounds responsible for nematicidal suppression (Auger *et al.*, 2004). Interestingly, Danquah *et al.* (2011) found that when *G. pallida* cysts were exposed to low concentrations of garlic extracts for eight weeks subsequent hatch was stimulated. They suggested that the reason for this was that the garlic extract contained a stimulant not present in potato root diffusate or water which has the ability to induce the hatch of J2 which would otherwise have remained dormant until the next potato crop. The natural plant based product Dazitol has been shown to significantly reduce *G. pallida* multiplication with the added benefit of increasing potato tuber size (Martin, Turner and Fleming, 2007); the active ingredients in Dazitol are allyl ITC (from mustard oil) and capsaicin (from chilli) which are both understood to act by inducing nematode mortality.

PCN suppression by bacteria has been investigated in several studies. *Rhizobium etli* and other rhizobacteria strains can induce systemic resistance in potato roots to *G. pallida* infection hence reducing the ability of PCN to penetrate the root (Racke and Sikora, 1992; Hasky-günther, Hoffmann-hergarten and Sikora, 1998; Reitz *et al.*, 2000). One novel control

method involves using chitinase-producing bacteria to reduce J2 hatch from *G. rostochiensis* by interfering with the chitin contained within the eggshell surrounding the J2 (Cronin *et al.*, 1997). During a series of *in vitro* studies, it was found that purified chitinase inhibited J2 hatch by 70% compared to an untreated control after two weeks. In addition, 100% of chitinase-producing bacteria tested against PCN cysts significantly inhibited egg hatch but to different extents.

Herbicides have also been suggested as an alternate control method of PCN. Active compounds from thiocarbamate herbicides were tested against *G. rostochiensis in vitro* where cycloate, pebulate, vernolate, tri-allate and lenacil significantly inhibited hatch (Perry and Beane, 1989). As these compounds are known to lead to membrane disintegration and altered permeability of plant cells, this hatch suppression was attributed to the ability of the herbicides active compounds to alter the permeability of the lipid layer of the PCN eggshell. Further to this, *in vitro* hatching assays with chloridazon and tri-allate in pelleted form led to a reduction in hatch from *G. rostochiensis* cysts by up to 70%. When transferred to pot trials no reduction in hatch was observed (Beane and Perry, 1990). One explanation for this is that herbicide action is reduced in the soil due to dissipation into the environment leading to a lower concentration of compounds coming into direct contact with the cysts.

1.4. Biofumigation and Glucosinolate Hydrolysis

Biofumigation is the suppression of soil pests resulting from volatile hydrolysis products released into the soil after the incorporation of GSL-containing plant tissues (Kirkegaard *et al.*, 1993). The presence of GSLs and the enzyme myrosinase in Brassicaceae spp. is well-documented (Cole, 1976; Fenwick and Heaney, 1983; Sang *et al.*, 1984; Brown and Morra, 1995). Both compounds remain separate whilst the plant tissue is intact, and upon disruption, GSL hydrolysis occurs (Lazzeri, Tacconi and Palmieri, 1993; Donkin, Eiteman and Williams, 1995; Buskov *et al.*, 2002). This hydrolysis has the potential to release breakdown products such as ITCs, nitriles and thiocyanates (Cole, 1976; Fenwick and Heaney, 1983). It is understood that the ITCs are the active hydrolysis product able to suppress soil pests.

1.4.1. Glucosinolates

GSLs are found exclusively within the dicotyledon group of flowering plants, containing around 200,000 species, occurring predominantly within the order Brassicales (Fenwick and Heaney, 1983). At least 130 glucosinolates have been identified within this order which is made up of multiples families (Fenwick, Heaney and Mullin, 1983; Daxenbichler *et al.*, 1991; Fahey, Zalcmann and Talalay, 2001) of which Brassicaceae is the most studied as it contains the genus *Brassica* which is of interest with respect to food, human health and pest control

(Hayes, Kelleher and Eggleston, 2008; Hopkins, van Dam and van Loon, 2009; Clarke, 2010).

The biosynthesis of GSLs has been well studied using *Arabidopsis thaliana* as a model (Mithen, 2001; Sønderby, Geu-Flores and Halkier, 2010; Wang *et al.*, 2011). During biosynthesis there are three independent stages that the initial aldoxime chemical compound undergoes to form the parent GSL (Mithen and Campos-de Quiroz, 1998). Firstly, side chain elongation occurs to produce a series of methionine homologues. Secondly, the glucone core structure is formed and sulphur is incorporated. The last stage of GSL formation involves variable secondary modifications on the side chains.

GSLs are sulphur containing beta-thioglucosides which consist of a common structure of a sulphonated oxime moiety and a variable side-chain derived from one of the eight natural amino acids (Brown *et al.*, 1991; Mithen, 2001). Due to the nature of the amino acid precursors, GSLs can be split into aliphatic (alanine, leucine, methionine or valine precursors), aromatic (phenylalanine or tyrosine precursors) and indolic (tryptophane precursor) classifications (Sønderby, Geu-Flores and Halkier, 2010). The side chains of these GSLs, once formed, can then undergo further chemical modifications such as elongation, hydroxylation, methylation, glycosylation, oxidation and acylation (Fahey, Zalcmann and Talalay, 2001; Sønderby, Geu-Flores and Halkier, 2010; Avato *et al.*, 2013).

GSLs are localised to plant cells containing high levels of sulphur, referred to as S-cells (Koroleva *et al.*, 2000). S-cells cluster between the endodermis and the phloem cells of vascular bundles and are found close to or in direct contact with myrosin cells (Bones and Rossiter, 1996). Within these S-cells, GSLs are thought to be sub-localised to the vacuole (Grob and Matile, 1980; Yiu *et al.*, 1984; Kelly, Bones and Rossiter, 1998).

1.4.2. Myrosinase

GSLs coexist within plant tissues with myrosinase, a glycosylated thioglucosidase responsible for the hydrolysis of GSLs which are expressed within the vacuole of myrosin cells (Bones and Rossiter, 1996; Mithen, 2001; Kissen, Rossiter and Bones, 2009). Several forms of myrosinase have been identified in Brassicaceae spp. (Lönnerdal and Janson, 1973; Lenman *et al.*, 1990) and expression of the different forms have been found in *Brassica napus* tissue (Lenman *et al.*, 1993). All forms appear to hydrolyse GSLs so the myrosinase type has little relevance to biofumigation efficiency.

In addition to the myrosinase found within plant cells, the enzyme can also be produced by soil microorganisms and fungi (Borek, Morra and McCaffrey, 1996; Sakorn *et al.*, 2002; Gimsing, Kirkegaard and Bruun Hansen, 2005; Gimsing *et al.*, 2007). Gimsing *et al.* (2007)

found that benzyl ITC could be formed in soil from benzyl GSL in the absence of added myrosinase or Brassicaceae plant material demonstrating the presence of GSL-hydrolysing myrosinase in the soil. Although myrosinase can be present in the soil in low concentrations in the absence of GSL-containing plant tissue, the presence of Brassicaceae spp. may stimulate production by microorganisms which can enhance GSL hydrolysis from the plant material for higher levels of breakdown product (Gimsing and Kirkegaard, 2009).

1.4.3. Glucosinolate hydrolysis mechanism

The glucosinolate-myrosinase enzyme complex, also called the "mustard oil bomb" is an intricate system characterised by the compartmentalisation of myrosinase and the substrate GSL within the vacuole of cells (Kissen, Rossiter and Bones, 2009). When the tissue cells are damaged, GSL and myrosinase come into contact and GSL hydrolysis occurs. There are two steps in the hydrolysis of GSL. In the first step, myrosinase breaks down the thioglucoside linkage in the GSL releasing glucose and an unstable aglycone in the presence of water. During the second step, the unstable aglycone undergoes a series of rearrangements to produce breakdown products such as ITCs, nitriles, thiocyanates, epithionitriles and oxazolidines (Cole, 1976; Fenwick and Heaney, 1983; Sønderby, Geu-Flores and Halkier, 2010).

The most common volatile products of hydrolysis are ITCs. Nitriles and epithionitriles are alternate hydrolysis products to ITCs. The least common of the hydrolysis products are thiocyanates and oxazolidines. The nature of the products released are dependent upon multiple factors; the GSL side chain structure, hydrolysis conditions and the presence of co-factors (Cole, 1976; Tookey, VanEtten and Daxenbichler, 1980; Burow *et al.*, 2006). Borek *et al.* (1994) determined that during 2-propenyl GSL (sinigrin) hydrolysis, allyl nitrile formation was more common when pH was below 4.0 whilst at higher pH values allyl ITC dominated. In addition to the influence of pH on product formation, they also showed that the addition of iron (II) promoted nitrile formation whereas the addition of iron (III) inhibited the hydrolysis reaction. Similar effects of iron on nitrile formation have been noted in a more recent study (Hanschen *et al.*, 2015).

1.4.4. Additional protein interactions

The determination of type and quantity of breakdown products released from GSL hydrolysis can sometimes involve genetic control by cofactors such as epithiospecifier proteins (ESP) or nitrile specifier proteins (NSP) although not all Brassicaceae spp. contain these proteins (Lambrix *et al.*, 2001). The synthesis of nitriles and epithionitriles from alkenyl GSLs, although influenced by pH, is regulated by the activity of ESP (Kaoulla, MacLeod and Gil,

1980; MacLeod and Rossiter, 1985; Williams *et al.*, 2008). Genetic variation at the ESP locus specifically affects the amount of nitrile produced altering the ratio of ITC: nitrile production (Zhang, Ober and Kliebenstein, 2006). The mode of this effect is not currently understood (Kissen, Rossiter and Bones, 2009).

Myrosinase-binding proteins (MBP) have also been identified in Brassicaceae spp. where they co-localise with myrosinase in plant cells (Lenman *et al.*, 1990; Geshi and Brandt, 1998; Geshi *et al.*, 1998; Rask *et al.*, 2000). Although MBP form complexes with myrosinases, their role has not currently been fully established. It has been hypothesised that MBPs can negatively affect the ITC: nitrile ratio in GSL degradation in association with ESPs (Zhang, Ober and Kliebenstein, 2006).

1.4.5. Isothiocyanates

ITCs are secondary metabolites produced during GSL hydrolysis and are responsible for the bitter peppery taste of cruciferous vegetables. ITCs are reactive electrophiles whose central carbon can undergo rapid addition reactions with biological nucleophiles, such as amines and thiols, and covalently modify proteins (Drobnica, Kristián and Augustín, 1977; Borek *et al.*, 1994; Brown and Hampton, 2011). ITCs react up to a thousand times faster with thiol groups than with amino groups so proteins containing cysteine residues are particularly sensitive to modification due to the amino acids thiol side chain (Drobnica, Kristián and Augustín, 1977). The ability of ITCs to react with proteins is the foundation for their general toxicity to various organisms. ITCs are volatile; differences in volatility depend on the length and structure of the ITC side chains (Brown and Morra, 1997) and their disappearance is generally rapid. ITCs hydrolyse through two steps, first by breaking down into a thiocarbamic acid and then by decomposing rapidly into a protonated amine (Joseph *et al.*, 1992).

1.4.6. The role of glucosinolate hydrolysis

GSL hydrolysis is activated in response to plant damage (Brown and Morra, 1996); therefore it is sensible to assume that one function of GSL breakdown is defence, where breakdown products can act against plant pathogens, insects and generalist herbivores as either a poison or deterrent (Rask *et al.*, 2000; Bednarek *et al.*, 2009; Clay *et al.*, 2009). Both allyl ITC and Brassicaceae plant extracts suppressed the growth and decreased the survival of the specialist herbivore, *Pieris rapae* (Agrawal and Kurashige, 2003) which supports the notion that GSL hydrolysis is an effective defence strategy employed by Brassicaceae spp.

Some pests have developed strategies to counteract the defensive effect of GSLs. GSLs can be detoxified by sulfatases or NSPs in the diamond back moth *Plutella xylostella* or cabbage white butterfly *P. rapae,* respectively (Ratzka *et al.*, 2002; Wittstock *et al.*, 2003). Other

insects, such as the turnip sawfly *Athalia rosae* or the cabbage aphid *Brevicoryne brassicae*, act by tolerating GSLs through sequestration; the insects accumulate the GSLs as a secondary metabolite and use it in their own defence systems (Müller *et al.*, 2001; Bridges *et al.*, 2002; Kazana *et al.*, 2007). Although effective against some soil-borne pests, GSL hydrolysis will not have broad-range activity and it is likely that more pests will develop strategies to defend against its toxicity.

GSLs and ITCs are also believed to play a role in the prevention and treatment of human diseases (Dinkova-Kostova and Kostov, 2012). The consumption of cruciferous vegetables rich in ITCs, specifically sulforaphane and erucin, has been linked to decreased cancer risk (Higdon *et al.*, 2007; Herr and Büchler, 2010; Clarke *et al.*, 2011; Azarenko, Jordan and Wilson, 2014) and studies have shown that ITCs have anti-cancer properties (Khor *et al.*, 2008; Munday *et al.*, 2008). Protein modification is likely to be a central aspect to the anti-cancer properties of ITCs as they can: influence carcinogen metabolism, impair tumour development, modify inflammatory responses and induce cell death (Brown and Hampton, 2011; Cavell *et al.*, 2011). In addition to their potential in cancer treatment, ITCs are being considered as central system disease drugs (Martelli *et al.*, 2012, 2014; Citi *et al.*, 2014). ITCs release hydrogen sulphide on breakdown. Hydrogen sulphide is a gasotransmitter involved in the regulation of respiratory, cardiovascular and nervous systems and drugs containing this compound might counteract diseases of these vital systems.

1.5. Brassicaceae Glucosinolate Profiles and Isothiocyanate Release

A large area of research with respect to biofumigation has been focussed on the GSLs present in different Brassicaceae species and their potential to release ITCs. Different GSLs release different ITCs in varying concentrations; in addition, Brassicaceae species contain different GSLs in variable concentrations depending on plant tissue and growth stage. These key factors mean that cultivars need to be selected carefully for use against soil-borne pests in order to be effective biofumigants. Further to this, the fate of the ITCs once released into the soil is important in determining how efficiently the GSLs convert to ITCs and how long the period of toxicity will last.

1.5.1. Brassicaceae glucosinolate content

The GSL profiles of multiple Brassicaceae cultivars have been reported previously (Kirkegaard *et al.*, 1998; Fahey, Zalcmann and Talalay, 2001; Bellostas, Sørensen and Sørensen, 2007). Kirkegaard and Sarwar (1998) studied the GSL concentration of thirteen *Brassica* spp. root and shoot samples and found a large variation in total production. Antonious *et al.* (2009) screened ten different Brassicaceae cultivars for GSL content and

showed that each accession had varying GSL content with cultivars of *B. juncea* generally containing a higher GSL concentration compared to *B. napus, Brassica campestris* and *Eruca sativa*. Bellostas *et al.* (2007) identified that sinigrin, the precursor for allyl ITC, was the predominant GSL in three *Brassica* species; *Brassica carinata, Brassica nigra* and *B. juncea* accounting for over 90% of the GSL content in seeds and 50% in green tissues. In contrast, *Brassica rapa* was composed of multiple GSLs (but-3-enyl, 2-hydroxybut-3-enyl and 2-hydroxypent-4-enyl GSLs) at lower concentrations. This demonstrates the extreme variability of GSL levels among species.

There is also significant variation in GSL profiles among different tissues of single plants, influenced by environmental conditions and the developmental stage of the plant itself. In one study, the overall GSL concentration in *B. carinata, B. nigra* and *B. juncea* tissue varied depending on growth stage with older plants containing higher concentrations. GSL concentration was also variable among different tissues: lower in roots and leaves but higher in reproductive organs (Bellostas, Sørensen and Sørensen, 2007). When exploring the effect of age on leaf GSL content, *B. napus* GSL concentration increased rapidly in developing leaves until they reached forty days after planting after which time concentration decreased until sixty days after planting (Porter *et al.*, 1991). These studies, and others like them, have shown that the type and concentration of GSL in each part of the plant depends on the tissue, developmental stage of the plant and the effect of environmental factors (Brown *et al.*, 2003; Velasco *et al.*, 2007; Avato *et al.*, 2013). These studies highlight the need to investigate the effect of plant growth and development on individual GSL accumulation.

It is thought that there is a higher concentration and greater diversity of GSLs in the roots of Brassicaceae spp. compared to the shoots (Gardiner *et al.*, 1999; Dam, Tytgat and Kirkegaard, 2009). Gardiner *et al.* (1999) showed that the highest concentration of 2-phenylethyl GSL in *B. napus* was in the roots with very little in the shoots. In a separate study, 2-phenylethyl GSL was identified in *B. napus* with a higher concentration in root tissue compared to shoot tissue (Morra and Kirkegaard, 2002). Studies focussing on above-ground tissues from Brassicaceae cultivars may underrepresent the true concentrations of ITC-releasing GSLs, but it should be noted that although GSL concentration is often higher in roots, their contribution to total GSL concentration is limited by their low biomass (Kirkegaard and Sarwar, 1998) so roots may not influence soil-borne pathogens as strongly as above-ground material.

1.5.2. Potential isothiocyanate release

The potential ITC release of different species is of great interest in biofumigation research. Allyl ITC, produced from sinigrin, is thought to be the most abundant breakdown product

from a range of *Brassica* spp. (Cole, 1976; Gardiner *et al.*, 1999; Harvey, Hannahan and Sams, 2002). Cole *et al.* (1976) analysed the breakdown products released from common eight week old Brassicaceae where they identified a variety of ITCs and nitriles. In *B. juncea* the most abundant product was allyl ITC, 2-phenylethyl and isopropyl ITC were also detected. The highest concentration of allyl ITC was released from *B. nigra* and the highest 2-phenylethyl ITC concentration from *B. juncea*. In contrast, *B. napus* released a variety of ITCs consisting of isopropyl, 3-butenyl, 4-pentenyl and 2-phenylethyl in relatively low ITC concentrations compared to the other species. Although allyl ITC is described as the most abundantly produced in *Brassica* spp., its release is not universal further highlighting the high variation associated with GSL content and ITC release among species.

It has been described on multiple occasions that measuring GSL content does not accurately predict concentrations of the toxic breakdown products, ITCs, when released into soil (Kirkegaard and Sarwar, 1998; Morra and Kirkegaard, 2002; Gimsing and Kirkegaard, 2006). For this, additional factors need to be considered including: temperature, pH, soil texture, water content and the microbial community.

In one study, the concentration of GSLs in the tissue was greater than the concentration of ITCs released in 450 *Brassica* samples (Warton, Matthiessen and Shackleton, 2001). The authors suggested several reasons for this including: incomplete GSL hydrolysis, fast volatisation of the ITCs into the atmosphere before measurement, or the reaction of the strong electrophilic ITCs with nucleophilic compounds released from the tissue which would have led to the rapid breakdown of ITCs before detection. In addition there were differences between GSLs with respect to ITC concentration released indicating that not all GSLs hydrolyse the same way. The authors hypothesised that this was most likely due to either differences in myrosinase activity between species or in the time required to arrange the structure of the breakdown products formed.

In vitro experiments indicate that only 5% of the potential ITC release from GSL hydrolysis is achieved when Brassicaceae tissue is disrupted as it would be under field conditions (Gardiner *et al.*, 1999). Similarly, Morra and Kirkegaard (2002) presented that only 1% of the GSLs identified were released as ITCs in soil 24hrs after incorporation of *B. juncea* and *B. napus*; increasing GSL concentrations did not lead to a proportional increase in ITC release.

As a result of ITC release studies, increasing the efficiency of GSL to ITC conversion during hydrolysis has been recognised as being just as important as the GSL profiles of the various Brassica cultivars themselves (Morra and Kirkegaard, 2002; Matthiessen and Kirkegaard, 2006). One study addressed this by including a variety of methods to increase ITC formation:

tissue pulverisation, rapid incorporation, increasing soil moisture and irrigation. The maximum ITC release efficiency achieved from *B. napus* and *B. juncea* was 26% and 56%, respectively (Gimsing and Kirkegaard, 2006). Ensuring optimum conditions for ITC conversion is extremely important and by achieving this, released ITC concentrations can be greatly increased.

1.5.3. Formation and breakdown of isothiocyanates in soil

In addition to identifying which ITCs are produced and in what quantity, the persistence of ITCs in soil is important. Warton *et al.* (2001) found that several ITCs, including 2-phenylethyl, formed within the first 6hrs of hydrolysis with very little production after this time; allyl ITC also formed quickly (within 5-17hrs in different species), and there was some further release after 24hrs post-hydrolysis. A study by Gimsing and Kirkegaard (2006) showed that ITCs can be identified in soil for up to twelve days after incorporation of *B. napus* and *B. juncea*. ITC formation reached its highest concentration in soil after 30 mins. ITC production from *B. napus* reached concentrations of 300nmol g^{-1} 2hrs after incorporation but this decreased by 90% after 24hrs (Brown *et al.*, 1991). Similarly, the highest concentration of ITCs was identified immediately after *B. napus* and *B. juncea* tissue incorporation with little production after 4 days (Morra and Kirkegaard, 2002). Gardiner *et al.* (1999) identified maximum ITC concentrations at 30hrs after incorporation of *B. napus*. Bending and Lincoln (1999) identified the highest concentrations 96hrs after *B. juncea* incorporation (measurements were made only 48hrs after incorporation so any earlier ITC release may have been missed). These studies show that ITC formation and breakdown in soil is rapid, and that there are variations between ITCs in rates of production and also between *Brassica* spp. in ITC release.

When considering ITC breakdown, an *in vitro* study estimated the half-life of allyl ITC in soil to be 16hrs with disappearance becoming more rapid as soil water content and temperature increased (Petersen *et al.*, 2001). Similarly, a second study determined that the half-life of allyl ITC in six different soil compositions was between 20-60hrs depending on the soil type with shorter half-lives in soils containing higher organic carbon (Borek *et al.*, 1995), most likely due to the ability of ITCs to react with nucleophilic chemicals in organic matter (Drobnica, Kristián and Augustín, 1977; Brown and Hampton, 2011). These values are consistent with studies completed by Gardiner *et al.* (1999), who recorded a 75% decrease in ITC concentration within 42hrs of the maximum concentration being released, and Brown *et al.* (1991) who detected a 90% decrease in ITC concentration within 22hrs of the maximum soil concentration being recorded. The results from these studies suggest that any pest control

due to the release of ITCs is most likely to occur within the first few days after plant incorporation and GSL hydrolysis.

Microbial degradation of ITCs can contribute to their rapid disappearance in soil. A study comparing fresh and autoclaved soil showed that the half-lives of allyl, benzyl and 2-phenylethyl ITC increased from a few hours to a few days when autoclaved (Warton, 2003). In addition, allyl ITC concentrations were found to be three times higher in autoclaved soil compared to non-autoclaved suggesting an additional role for soil microorganisms in ITC hydrolysis (Price *et al.*, 2005). 2-phenylethyl ITC was almost non-existent after 44hrs in non-sterile soil compared to a slow decrease in concentration over 91hrs in sterile soil (Rumberger and Marschner, 2003). In contrast, Borek *et al.* (1995) did not detect differences between sterile and non-sterile soil on the breakdown of allyl ITC. They only monitored over 48hrs so differences may have occurred out with this period. Enhanced biodegradation would have a negative impact on biofumigation as the exposure period of pests and pathogens to ITCs would be reduced.

1.6. Effect of Biofumigation on Soil-borne Pests

1.6.1. Effect of biofumigation on fungal pathogens

Several biofumigant products have been shown to control various fungal diseases. One of the first biofumigation studies looked at the effects of *B. napus* and *B. juncea* root tissue on the *Gaeumannomyces graminis* which causes take-all of wheat (Angus *et al.*, 1994). They established that both species were able to inhibit fungal growth *in vitro. Brassica juncea* was more effective than *B. napus* and the major ITC released was different in each, 2-phenylethyl and methyl respectively. A later study also determined that *B. napus* suppressed the take-all fungal pathogen in pot and field experiments where the predominant GSL in the root tissue was 2-phenylethyl (Kirkegaard *et al.*, 1998). Both *B. juncea* and allyl ITC were able to inhibit mycelial growth from *Sclerotium rolfsii*, a common fungus which causes southern blight, *in vitro*; the damaged *Brassica* tissue was more effective than the ITC on its own (Harvey, Hannahan and Sams, 2002). This suggests that either allyl ITC works in combination with other released chemicals to inhibit fungal growth or ITC concentrations were higher in the tissue than tested *in vitro* where 528.80µmol L^{-1} allyl ITC inhibited 90% of mycelial growth. There are differences between these studies in the major ITC released from *B. juncea,* likely due to the different tissues used.

In an *in vitro* study by Walker *et al.* (1937), published before biofumigation was established, allyl, phenyl and methyl ITCs were shown to inhibit the growth of *Colletotrichum circinans* with allyl ITC being the most effective. Allyl ITC was also screened against a number of

other fungal pathogens: *Botrytis allii, Aspergillus niger, Aspergillus alliaceus* and *Gibberella saubinetii* and shown to effectively reduce growth of all (Walker, Morell and Foster, 1937). A later *in vitro* study noted that the effect of ITCs on fungal pathogens varied between pathogen and ITC concentration, with the greatest reduction in *Colletotrichum coccodes* and *Helminthosporium solani* growth after 2-phenylethyl ITC exposure and *R. solani* growth after exposure to benzyl or methyl ITC (Taylor, Kenyon and Rosser, 2014).

1.6.2. Effect of biofumigation on soil invertebrates

Although not well investigated, a few studies have explored the effect of biofumigation on soil invertebrates and insects. One study considered the effect of soil incorporated *B. napus* on wireworm mortality and behaviour and found that the worms were repelled by the treatment (Brown *et al.*, 1991). The toxicity of methyl, allyl, benzyl and 2-phenylethyl ITC were screened against weevil larvae both *in vitro* and in the presence of soil (Matthiessen and Shackleton, 2005); all ITCs were effective *in vitro* with methyl ITC the most active and least impacted by the presence of soil or low temperatures. Allyl ITC was affected by different soils and temperatures but was still able to increase larvae mortality. Benzyl and 2-phenylethyl ITC decreased in effectiveness as the temperature was lowered and were ineffective in the presence of peat soil.

More recently, *Brassica oleracea* (wild cabbage) containing high levels of sinigrin and 3-butenyl GSL reduced the survival and reproduction of the beneficial soil invertebrates, springtails (*Folsomia candida*) and earthworms (*Eisenia andrei*) (Zuluaga *et al.*, 2015; Fouché, Maboeta and Claassens, 2016). This crop is not a commonly used biofumigant species but these results do show that the effects of common biofumigant *Brassicas* on non-target beneficial soil organisms need to be addressed.

1.6.3. Effect of biofumigation on nematodes (excluding potato cyst nematodes)

The mortality rate of the free living nematode, *Caenorhabditis elegans*, increased after exposure to allyl ITC and the parent GSL, sinigrin; AITC was more toxic to the nematodes than sinigrin (Donkin, Eiteman and Williams, 1995) and low ITC product formation from the GSL was suggested as the cause for this discrepancy. A similar study exploring the effect of allyl ITC on a number of nematode species showed that the ITC was the most toxic to *C. elegans* (Yu *et al.*, 2005). Benzyl ITC has also been identified as toxic to *C. elegans* where mortality after exposure *in vitro* was rapid and occurred within the first 3hrs (Nagesh *et al.*, 2002). Although *C. elegans* is free-living, it is known to be a potential mushroom pest (Grewal and Richardson, 1991) and is a good test species due to its easy culturability making it useful for initial efficiency studies.

In contrast to the *C. elegans* studies, a field trial found no effect of ITC exposure or *B. juncea* biofumigation on a range of free-living nematodes (Vervoort *et al.*, 2014). Changes which did occur were attributed to other factors involved in biofumigation such as mechanical disturbance, green manure addition and an absence of host plants to infect.

The sugar beet cyst nematode *Heterodera schachtii* is another major pest whose control has been impacted by the change in EU pesticide legislation. An *in vitro* study conducted with GSLs purified from Brassicaceae exhibited an increase in juvenile mortality rate dependent on the concentration and exposure period of: sinigrin, 3-butenyl GSL, benzyl GSL and 4-methylthio-3-butenyl GSL (Lazzeri, Tacconi and Palmieri, 1993). Sinigrin was the most effective and led to 100% juvenile mortality after 24hrs at a concentration of 0.5% w/v. A later study examining the effects of allyl ITC on *H. schachtii* mortality and hatch demonstrated that the nematode was fairly resistant to the ITC with high concentrations required for hatch inhibition (Yu *et al.*, 2005).

Allyl ITC has also been established to cause mortality of the soybean cyst nematode *Heterodera glycines* and the lesion nematodes *Pratylenchus penetrans* and *P. neglectus*. The soybean cyst nematode was more affected than the lesion nematodes (Yu *et al.*, 2005). Further research on the control of *Pratylenchus* species has indicated that several biofumigant crops have the ability to suppress the pest including *B. oleraceae* (Kago *et al.*, 2013), *B. juncea* (Yu *et al.*, 2007; Mazzola *et al.*, 2009)*, B. napus* and *Sinapis alba* (Mazzola *et al.*, 2009).

The majority of studies investigating biofumigation have been focussed on the control of root-knot nematodes from the genus *Meloidogyne* and *Tylenchus*. Yu *et al.* (2005) determined that allyl ITC could increase both *Meloidogyne incognita* and *Meloidogyne hapla* mortality whilst also inhibiting hatch. Benzyl and 2-phenylethyl ITC have also been established to induce mortality in *Meloidogyne javanica* and *Tylenchus semipenetrans* juveniles (Zasada and Ferris, 2003). Allyl ITC was similarly effective although a higher concentration was required. In contrast to this, a later study proposed that allyl ITC was the most toxic pure ITC when screened against *M. javanica* (Wu *et al.*, 2011). Although there is a disagreement between which ITC is most effective between these studies, they all agree with work by Lazzeri *et al.* (2004) within which several GSLs including sinigrin, 2-phenylethyl and benzyl GSL released ITCs which reduced *M. incognita* activity. These results suggest that these three GSLs and resulting ITCs are desirable for use in root-knot nematode control.

In addition to understanding the effect of ITCs on mortality, the effect of sub-lethal doses of ITCs on root-knot nematodes has been considered. Low concentrations of benzyl ITC have been shown to both reduce the infectivity of *M. incognita* juveniles on soybean and inhibit egg production (Zasada *et al.*, 2009) providing evidence that low ITC concentrations which

are not nematicidal, may still have a suppressive effect on infectivity and reproduction. This is consistent with a later study, where hatch from the second generation of *M. incognita* eggs was also reduced after initial treatment with benzyl ITC (Halbrendt *et al.*, 2010). This implies long-term consequences for progeny not directly exposed to the ITC.

When studying the control of root-knot nematodes, *Brassica hirta*, containing high levels of benzyl GSL, led to 100% *M. javanica* mortality in soil whereas *B. juncea,* containing sinigrin, reduced *M. javanica* survival by 65% (Zasada and Ferris, 2004). Both cultivars were also able to significantly reduce *T. semipenetrans* levels. Similar effects of different *Brassica* cultivars on *M. javanica* have been demonstrated previously (McLeod and Steel, 1999).

Although research with biofumigation and root-knot nematodes is promising, many *Brassica* spp. are hosts to these pests so there is the possible disadvantage of an increase in population if not researched correctly (McSorley and Frederick, 1995; McLeod and Steel, 1999; McLeod, Kirkegaard and Steel, 2001). This opens up the possibility of using *Brassica* cultivars as a trap crop instead of as a biofumigant as explored successfully for *M. hapla, M. chitwoodi* and *M. incognita* (Ploeg, 2008).

In addition to suppressing plant-parasitic nematodes, several studies have indicated that biofumigation can adversely affect non-target beneficial nematodes (Fourie *et al.*, 2016). Green manures of *B. juncea* and *S. alba*, applied as a potato pest biofumigant, were found to reduce levels of a number of entomopathogenic nematodes (EPN) (Ramirez *et al.*, 2009). Furthermore, the entomopathogenic nematodes, *Steinernema feltiae* and *Steinernema riobrave*, used to control *M. chitwoodi* were not as effective in the presence of biofumigation (Henderson *et al.*, 2009). These studies suggest that due to interference, the two methods of pest control may not be effective if combined into integrated pest management programs.

1.6.4. Effect of biofumigation on potato cyst nematodes

It is well documented that biofumigation and ITCs have nematicidal activity against a range of different nematode species but as these toxic effects vary among pest it is important to research the effects on the species of interest. Before biofumigation became an established methodology to control soil pests, *in vitro* assays found that mustard root diffusate had the ability to inhibit *G. rostochiensis* J2 hatch from cysts previously stimulated by potato root diffusate (Ellenby, 1945; Forrest and Farrer, 1983; Forrest, 1989). Aside from the exudate causing encysted J2 mortality, several alternate theories were suggested as stimulation with potato root diffusate after mustard root diffusate exposure was able to initiate further hatching. One hypothesis was that the mustard root exudate contained a substance which was able to change the permeability of the cyst or eggshell, whereas a second was that the

diffusate could act directly on the J2 by reducing movement and their ability to pierce through the egg (Forrest and Farrer, 1983). Forrest (1989) identified that the decreased hatch was not due to increased mortality but instead due to inhibition of hatch, potentially due to lower concentrations of ITCs having a nematostatic effect instead of nematotoxic effect.

Since then several studies have demonstrated that exudates from a range of Brassicaceae spp. can be used to control PCN. In a toxicity assay with *G. rostochiensis* J2, 2-phenylethyl GSL breakdown products had the greatest effect on mortality (Buskov *et al.*, 2002). A similar effect was noted with benzyl GSL and sinigrin breakdown products at longer exposure periods. Sinigrin hydrolysis products were also revealed to cause 100% *G. rostochiensis* J2 mortality *in vitro* at 24hrs exposure (Pinto, Rosa and Santos, 1998). More recently, a study into the effect of Brassicaceae extracts on *G. rostochiensis* populations found that all extracts reduced the number of newly formed cysts on potato roots *in vitro* (Aires *et al.*, 2009). This was dependent on the type and concentration of GSLs, with tissues containing high levels of sinigrin and 2-phenylethyl GSL leading to the lowest cyst count. Similarly, a significant effect of *S. alba*, *B. napus* and *Raphanus sativus* green manures on *G. rostochiensis* viability and multiplication in a pot trial has been published; the GSL content of tissue was not recorded so it is unknown which breakdown products led to this effect (Fatemy and Sepideh, 2016). These studies investigated the effect of GSL breakdown products on *G. rostochiensis* mortality and multiplication, although there is a distinct lack of information on the effect of pure ITCs on *G. pallida* and *G. rostochiensis* J2 mortality.

It has been demonstrated that Brassicaceae green manures added to *G. pallida* cysts in soil can lead to over 95% mortality of encysted J2 (Lord *et al.*, 2011); although several cultivars had an effect *in vitro* and in soil microcosms, the most effective green manures contained high levels of sinigrin, the precursor for allyl ITC. In a later study, *B. juncea* and *R. sativus* were able to reduce hatch and increase mortality *in vitro* at relatively low applications (Ngala, Woods and Back, 2015a). *Brassica juncea* contained high levels of sinigrin whereas *R. sativus* contained high concentrations of 2-phenylethyl GSL in the root and 4-methylsulfinylbutyl GSL in the leaf suggesting that the breakdown ITCs from these three GSLs would be able to effectively control PCN. Pure allyl ITC was found to reduce *G. pallida* hatch from encysted eggs by 50% *in vitro* within 2hrs exposure. When exposed to *B. juncea,* containing sinigrin, hatch was unaffected (Brolsma *et al.*, 2014). The authors suggested that the lack of effect *in vivo* was due to lower concentrations of allyl ITC compared to *in vitro* as the ITC release efficiency from plant tissue is both low and highly variable in soil. In an alternate study, *R. sativus* suppressed encysted *G. pallida* in soil during

plant growth by increasing mortality (Ngala, Woods and Back, 2015b). When taken into the field, both *B. juncea* and *R. sativus* had a biofumigant effect on encysted *G. pallida* and were able to reduce post-potato harvest PCN populations (Ngala *et al.*, 2014).

In comparison, a study found no effect of *S. alba* on *G. rostochiensis* hatch under field conditions; the lack of control in this case is most likely due to the release of an unstable ITC (4-hydroxybutyl ITC) from this species(Valdes, Viaene and Moens, 2012). Plant extracts from *S. alba, B. napus* and *R. sativus* were not found to be nematicidal towards *G. rostochiensis* with all treatments leading to an increase in hatch *in vitro* (Valdes *et al.*, 2011). The authors suggested that the addition of green manures increased hatch due to either eggshell permeability changes or direct J2 stimulation priming J2 for rapid hatch. It is not evident why this study differs so drastically to other studies and the lack of GSL analysis means that a comparison cannot easily be made.

The effects of biofumigation on PCN have been shown to vary dramatically in many ways. *In vitro* studies are promising and different ITCs and Brassicaceae tissues have been shown to increase J2 mortality and reduce hatch from encysted eggs. Differences between studies become evident when research moves into *in vivo* and field trials: several cultivars were found to reduce populations in some studies but not others. The most likely reasoning behind this is the wide variation in the GSL content of different cultivars and the difficulty of consistently growing plants in order to optimise ITC release under variable external conditions.

1.7. Soil Microbial Diversity

Soil microbial diversity is a way of assessing the "health" of soils. Soil health is defined as the ability of soil to function as a vital living system to sustain plant and animal productivity, maintain water and air quality, and promote plant and animal health (Doran and Zeiss, 2000). This makes it of importance in agriculture as it can be an important indicator of changes in environmental quality, food security and economic viability (Herrick, 2000). Soil microbial diversity and communities, including bacterial and fungal, can affect this through its impact on key soil functions (Kirk *et al.*, 2004).

Soil microorganisms play a role in many important soil nutrient cycles and pathways including the cycling of organic compounds such as nitrogen, by mineralizing and decomposing organic matter, and they can influence above-ground ecosystems and help plants deal with various stresses through their roles in plant nutrition, plant health, and soil structure (Kirk *et al.*, 2004; Boyle *et al.*, 2008; Rincon-Florez, Carvalhais and Schenk, 2013). Soil communities can influence soil nutrient availability through various oxidation, reduction and solubilisation reactions and affect nutrient uptake and plant growth through the release of

growth stimulating or inhibiting substances (Adesemoye and Kloepper, 2009; Yang, Kloepper and Ryu, 2009; Compant, Clément and Sessitsch, 2010). This means that changes in the soil communities can affect above-ground ecosystems through altered soil processes and nutrient production which in turn can influence plant growth.

Recent studies have shown that plants are able to create desirable soil microbial communities by demonstrating host specificity and attracting beneficial microorganisms to suppress soil pathogens, enhance growth, increase yield and reduce stress, with soil properties also playing a role in these plant-microorganism interactions (Berendsen, Pieterse and Bakker, 2012; Rincon-Florez, Carvalhais and Schenk, 2013). An example of pathogen suppression through soil communities was demonstrated by Mendes *et al.* (2011) who determined that *R. solani* was suppressed by soil containing the disease-suppressive taxa: Proteobacteria, Firmicutes and Actinobacteria.

Soil diversity can be easily affected by stresses from outside sources including: degradation of plant material, loss of organic matter, erosion, pollution, acidification or climate change (Chapman, Campbell and Artz, 2007). Any loss in the ability of the microbial biomass to maintain its original functions can be interpreted as likely to impact on key soil processes, such as nitrogen cycling.

Our inability to identify and categorise soil species limits our knowledge about soil microbial diversity and creates an issue when attempting to understand and study how changes in these species affect different processes. A lack of bacterial culture practices means that approximately only 1% of the soil bacterial population can be characterised (Hugenholtz, Goebel and Pace, 1998; Leckie, 2005). As 1% is such a small percentage of the total estimated number of species, it is improbable that the culturable bacteria are representative of the total number of bacteria so our knowledge of microorganisms is likely to be biased towards those that can be grown successfully (Torsvik *et al.*, 1998; Kirk *et al.*, 2004). In order to better characterise the structure and function of the soil microbial population, molecular methods have been adopted such as DNA hybridisation, terminal restriction fragment polymorphism and denaturing gradient gel electrophoresis. There are not many culture-independent studies published, although positive results have been collected with respect to identifying and characterising different microbial communities (Leckie, 2005; Ramsey *et al.*, 2006; Malik *et al.*, 2008; Rincon-Florez, Carvalhais and Schenk, 2013; Stefanis *et al.*, 2013).

1.7.1. Effect of agricultural practices on soil microbial communities

Intensive agricultural practices can lead to physical, chemical and biological changes in the soil which can compromise soil nutrient cycles and plant health. The effects of agricultural

approaches such as pesticide, nematicide and herbicide use on the soil microflora are important because the structure and function of soil microbial communities may be impacted which will have an effect on key soil processes (Pankhurst *et al.*, 1996; Singh, Walker and Wright, 2002).

Several studies have been completed exploring the effect of different agricultural practices on microbial community diversity and function. Farming practices such as organic, low-input and conventional systems can give structurally different microbial communities (Bossio *et al.*, 1998). In addition, specific agricultural practices have been shown to cause shifts in the soil microbial communities including; tillage (Ibekwe *et al.*, 2002), compost amendments (Bernard *et al.*, 2012) and crop rotation (Orr *et al.*, 2011). Although it is unknown if these shifts in the soil communities are negative or positive for soil processes, any alteration to the original soils community profile will impact on soil nutrient cycling and plant growth.

One of the major soil processes of interest is nitrogen cycling, due to this a number of studies have been completed looking at the effect of common agricultural practices on bacterial groups involved. The long-term application of mineral fertilisers to agricultural soils can enhance the abundance of ammonia-oxidising bacteria (AOB), involved in the nitrogen cycle, suggesting a positive influence of fertiliser application with respect to this specific nutrient cycle (Okano *et al.*, 2004; Ai *et al.*, 2013); this is to be expected as fertiliser added to agricultural soil normally consists of compounds involved in the nitrogen cycle such as nitrogen and ammonia. Crop rotation can also increase the activity of nitrogen-fixing bacteria with would benefit the soil process (Orr *et al.*, 2011). A later study indicated that nitrogen-fixing bacteria can be impacted by pesticides, in this case the chemical application negatively affected the bacterial group providing evidence that chemical use would adversely affect the nutrient cycle, in turn negatively affecting above-soil ecosystems (Orr *et al.*, 2012).

It is thought that only 0.1% of pesticides reach their target organism when incorporated into the soil so this would suggest that 99.9% is being released into the environment (Pimentel, 1995). The large amount of pesticide entering the soil can have adverse effects on the microbial system and there is a need to understand the response of the microbial communities to these compounds with regards to changes in community structure caused by selective pressure after pesticides are applied. The application of the chemical nematicide, metham sodium, has been shown to inhibit microbial activity and lead to a long-term shift in microbial communities both *in vitro* and in field studies (Spyrou, Karpouzas and Menkissoglu-Spiroudi, 2009; Omirou *et al.*, 2011). Carbofuran can stimulate microbial growth in soil and butachlor can reduce the growth of the same soil microorganisms (Lo, 2010). In the same review other

pesticides, such as propanil and chlorpyrifos, had no impact on soil microorganisms. In general, pesticides are found to negatively affect the soil community (Lo, 2010). There are exceptions to this demonstrating that the study of new chemical products is required to accurately determine if their application would impact soil processes.

The widespread and repeated use of herbicides can also negatively affect soil microbial communities. For example the degradation of a selective, post-emergence herbicide, bromoxynil, was shown to lead to significant changes in bacterial diversity and composition (Baxter and Cummings, 2008). This in turn led to the decreased decomposition and increased persistence of the chemical due to reduced bacterial activity. In an alternate study, long-term application of phenylurea herbicides decreased soil diversity and altered the functional abilities of the soil microorganisms (el Fantroussi *et al.*, 1999).

One concern with using alternative methods of pest control is the effect that these practices may have on non-target microflora. Now that pesticides and nematicides are being phased out due to their negative effects on human health and the environment, alternate methods are being introduced whose effects on soil diversity are not well understood.

1.7.2. Effect of biofumigation on soil microbial communities

Although many studies have investigated the toxic effects of biofumigation against soil-borne pests, the effects on soil microbial diversity have not been as widely researched. In spite of this, the studies which have been published have noted that biofumigation has the potential to alter the soil community.

The addition of pure ITCs to soil has varying effect on soil microorganisms. A soil bioassay with a range of ITCs showed that ITCs reduced nitrifying bacteria populations and inhibited their growth (Bending and Lincoln, 2000); of these, phenyl ITC was the most toxic and 2-phenylethyl ITC had a high fumigant effect. Similarly, 2-phenylethyl ITC inhibited microbial growth *in vitro*, depending on the bacterial or fungal species (Smith and Kirkegaard, 2002), and 2-(4-hydroxyphenyl)ethyl ITC has been shown to be strongly antimicrobial and inhibited the growth of a number of bacterial strains (Tajima *et al.*, 1998). Sinigrin and sinigrin plus myrosinase have also been shown to affect the soil bacterial community composition within seven days (Hanschen *et al.*, 2015). In contrast, a soil microcosm study utilising higher ITC concentrations found that bacterial populations were not impacted by allyl, butyl, phenyl or benzyl ITCs, with the exception of a transient increase in one population when exposed to allyl ITC (Hu *et al.*, 2015); fungal populations decreased in response to allyl ITC addition. Differences between studies are likely due to different ITC structures, differences in headspace environments leading to varying ITC volatisation speeds and diverse microbial

populations investigated in each study. As different microbial populations respond differently to various ITCs it is important to determine the effect of the ITCs in use on the microbial groups of interest.

Brassica oleracea residues incorporated into the soil have been shown to stimulate microbial activity and biomass in soil, with the AOB community structure unaffected by the treatment (Omirou *et al.*, 2011). In contrast, a later study observed changes in microbial communities, after *B. oleraceae* exposure, on a temporal basis with the largest change occurring one week after incorporation (Zuluaga *et al.*, 2015). Bernard *et al.* (2012) investigated the effect that *B. napus* green manure had on soil microbial communities and determined that incorporation increased bacterial populations and induced changes in the community structure. Similar results were suggested for the incorporation of *R. sativus* and *B. juncea* on soil microbial activity where total activity increased during growth and after incorporation of the cultivars (Ngala, Woods and Back, 2015b). *Brassica juncea* and *R. sativus* green manures have also been shown to increase the carbon substrate utilization of communities under controlled conditions with the highest effect occurring within fourteen days of incorporation (Fouché, Maboeta and Claassens, 2016). In contrast, *B. juncea* green manures had no effect on soil microbial communities one month after incorporation in a pot trial (Rokunuzzaman *et al.*, 2016). *Brassica napus* rapeseed meal incorporation has also been shown to lead to a shift in soil microbial communities as well as an increase in bacterial diversity (Wang *et al.*, 2014). *Brassica carinata* seedmeal altered both fungal and bacterial communities in a pot trial with the effect on fungal communities persisting longer than the effect on bacterial communities (Mocali *et al.*, 2015). In contrast, *B. carinata* seedmeal in a field trial had no effect on soil microbial communities (Wei, Passey and Xu, 2016) suggesting a reduced impact on non-target soil microorganisms when environmental conditions are included as factors.

There are several gaps in the research when studying biofumigation and soil microbial diversity. Firstly, a small number of studies have researched the effect of ITCs and biofumigants on microbial communities. Secondly, several of these studies provide contradicting results between the effect of ITCs and biofumigation, potentially due to the addition of green manure benefits when using biofumigation over pure chemical ITC addition. In addition, results between similar trials differed demonstrating that effects on the soil communities are highly variable and providing evidence that further experimentation is required. Lastly, most of the studies which have demonstrated an effect of biofumigants on microbial diversity have not fully investigated the persistence of these changes.

1.8. Project Aims and Objectives

The main aim of this study is to determine if biofumigation has the potential to be used as an alternative control method for PCN, namely *G. pallida*. In order to achieve this, several key objectives will be addressed. These include to:

1. identify and assess the toxicity of ITCs to PCN *in vitro*
2. increase the efficiency of key ITCs against encysted *G. pallida in vitro*
3. determine any suppressive effects of key ITCs on encysted *G. pallida* in soil under controlled conditions
4. analyse Brassicaceae spp. GSL profiles over plant development to identify potential *G. pallida* biofumigant cultivars and optimum incorporation times
5. investigate the biofumigant effect of Brassicaceae cultivars on encysted *G. pallida* under controlled and field conditions
6. assess the effect of ITCs and biofumigation on soil microbial communities under controlled and field conditions

Although a significant amount of research has been carried out on biofumigation mediated by Brassicaceae plants, there are still large gaps in our understanding of the processes involved. As a consequence of experimentation carried out during this project, it is envisaged that detailed information on Brassicaceae spp. GSL profiles will be collected. Knowledge on the toxic and suppressive effects of GSL breakdown products on PCN will also be developed. Additionally, the sub-lethal effects of these Brassicaceae spp. on nematode reproduction will be established. Further to this, changes in soil microbial communities when exposed to ITCs and biofumigant cultivars will be explored. This work will feed back into cultivar breeding programmes and aid in the development of future biofumigation strategies as an alternative to pesticides when considering the control of soil-borne potato diseases.

Chapter 2. Materials and Methods

2.1. Chemicals

All chemicals and kits were obtained from Sigma Aldrich unless otherwise stated (Table 2.1).

Product	Supplier
Acetone	Rathburn Chemicals, UK
Meldola's Blue Dye	Avonchem Ltd., UK
PerfeCTa® qPCR ToughMix	Quantabio, MA, USA
PowerSoil DNA Isolation Kit	Cambio, UK
Purified Agar	Fisher Scientific, UK
Self-indicating Soda Lime	Fisher Scientific, UK
10% CO_2 in Nitrogen	Calgaz, UK
Vydate® 10G	DuPont (UK) Ltd., UK
Nemathorin® 10G	Syngenta, UK

Table 2.1. Chemicals and kits not obtained from Sigma Aldrich

2.2. Isothiocyanates

All isothiocyanate (ITC) liquid solutions were made up to the desired concentration using distilled water (dH_2O). ITCs used throughout this study include: allyl (AITC), benzyl (BITC), 2-phenylethyl (PEITC), methyl (MITC), propyl (PITC), isopropyl (IITC), ethyl (EITC), phenyl (PHITC), butyl (BUITC) ITC and sulforaphane (SUL) (Figure 2.1).

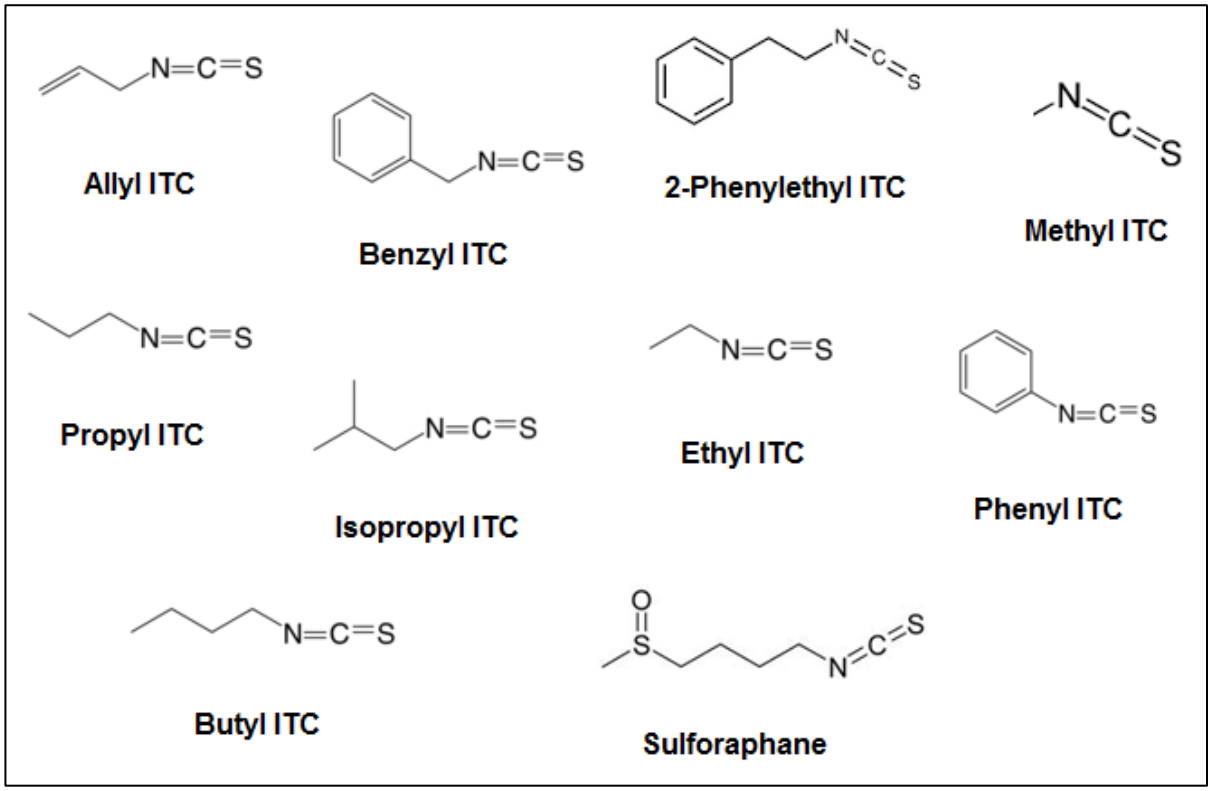


Figure 2.1. Isothiocyanate structures

2.3. Viability Studies

2.3.1. Juvenile toxicity assays

Batches of ten *G. pallida* Pa2/3 or *G. rostochiensis* Ro1 cysts (2.5.3; SASA, UK) were soaked in potato root diffusate (PRD; SASA, UK) in wells of a six-well suspension plate (Greiner Bio-One, Austria). Plates were stored in the dark at room temperature (18 ± 1°C) for a week to stimulate hatch. Hatched live juveniles (J2) were identified using a Wilovert HF microscope (25X magnification; Hund Wetzlar, Germany) and collected for experimentation by transfer to a new six-well plate using a pipette. A minimum of thirty hatched J2 were exposed to 3 mL ITC solutions at different initial concentrations. A dH_2O negative control treatment was included in each assay. Treated J2 were stored in the dark at room temperature for 72hrs and only exposed to light during mortality counting which occurred three times. Mortality was determined every 24hrs over the 72hr period with a Wilovert HF microscope. Dead J2 were counted in each well directly and then removed so as to not be counted in future assessments; the nematode was considered dead when it was immobile and did not respond to stimuli in the form of pricking by a needle. Occasionally nematode death occurred during pricking before state of living had been determined leading to exclusion of these J2 in the final count; living J2 were counted alongside dead J2 in the last 24hr assessment in order to determine accurate total percentage mortality.

2.3.2. Cyst hatching assays

Hatching assays were performed with *G. pallida* and *G. rostochiensis* cysts in wells of six-well suspension plates. Plates were stored in the dark at room temperature throughout with the exception of during counting when the cysts were exposed to light. Batches of cysts were soaked in dH_2O for three days prior to exposure to 3 mL ITC treatment or direct transfer to 2 mL PRD. If exposed to ITCs, a dH_2O negative control treatment was included in each assay; after treatment, cysts were transferred to PRD. PRD was refreshed weekly throughout each assay. Hatched J2 were counted at regular intervals, using a Wilovert HF microscope, for four weeks or until the rate of hatch had overcome its peak. Emerged J2 at each count were discarded.

2.3.3. Meldola's blue dye viability assays

Meldola's Blue Dye (MB) stain was applied to cysts either independently or after a hatching assay. When used in the absence of a hatching assay, cysts were soaked in dH_2O for a week at room temperature in the dark to hydrate cysts. When used directly after a hatching assay, pre-soaking in dH_2O was excluded from the protocol.

Cysts were exposed to 0.05% (w/v) MB (Avonchem Ltd., UK) for seven days. In order to remove excess stain, cysts were soaked in dH_2O for 24hrs before viability determination. Cysts were crushed with a micropestle and eggs were rinsed into a 15 mL tube, topped up with dH_2O (1 mL for each cyst in the sample) and gently mixed. A 1 mL aliquot was transferred into wells of a six-well suspension plate and both unstained (viable) and stained (nonviable) unhatched J2 within eggs were counted using a Wilovert HF microscope.

2.4. Glasshouse Protocols

2.4.1. General glasshouse conditions

Pot trials were set up under glasshouse conditions and incubated at day/night temperatures of 20 ± 2°C/18 ± 2°C under a 16hr photoperiod. Pots were watered twice daily with the exception of during the time period when pots were sealed. No pesticides or fertilisers were added for the duration of the trials.

2.4.2. Potato root diffusate collection

PRD for the hatching of J2 was collected from potato cyst nematode (PCN) susceptible cv Desiree tubers (SASA, UK) planted and grown under glasshouse conditions at Science & Advice for Scottish Agriculture. Tubers were planted in 2 L pots which were three-quarter filled with John Innes No. 2 soil (57.91% sterilised loam, 24.82% peat, 16.55% coarse sand, 0.62% fertiliser mix [40% hoof and horn meal, 40% superphosphate, 20% sulphate of potash] and 0.11% ground limestone) and maintained under glasshouse conditions. Plants were left for four to six weeks to allow the development of roots before diffusate collection began. Diffusate was collected following a previously published protocol (Widdowson, 1958). Briefly, dH_2O was added to each pot until fully saturated before passing 200 mL dH_2O through each pot allowing diffusate collection. This was passed though the pots twice more and diffusate from each plant was combined and filtered through a 320mm diameter filter paper (MACHEREY-NAGEL, Germany), to remove large particles and dirt. Collected diffusate was stored at -20ºC. Collection occurred weekly for ten weeks after which time all collected PRD was pooled, aliquoted into 200 mL volumes and stored at -20ºC until use. When required, PRD was defrosted and stored at 4ºC in the dark until use.

2.4.3. Potato cyst nematode production

Globodera pallida Pa2/3 and *G. rostochiensis* Ro1 cysts were produced by multiplication on susceptible cv Desiree tubers planted and grown in 5 L pots filled with John Innes No. 2 soil under glasshouse conditions. Batches of cysts were enclosed within muslin bags and placed at

4-5cm depths in soil. Plants were grown for sixteen weeks after which time the plants were cut and soil was dried in preparation for cyst collection (2.7.1).

2.4.4. Soil preparation

Soil types were made by combining John Innes No. 2 soil with horticultural sand (Keith Singleton Horticultural Products, UK) or clay soil (Barworth Agriculture Ltd., UK). The soil texture of John Innes No. 2 was determined using the Mason Jar Soil Test method. Individual soil components (sand, clay and silt) were expressed as a percentage of the total soil and compared to the soil textural triangle (Figure 2.2). The weight of sand and clay required to get the desired composition of each soil type was determined from the John Innes No. 2 soil texture and a soil texture calculator (United States Department of Agriculture, no date). Sandy silt loam soil was composed entirely of John Innes No. 2 soil. Clay loam soil was composed of a 1:4 ratio of clay to John Innes No. 2 soil. Sandy loam soil was composed of a 1:1 ratio of horticultural sand to John Innes No. 2 soil. The correct texture of different soils were confirmed with jar soil tests before use. Soil was transferred to 2 L pots in preparation for experimentation.

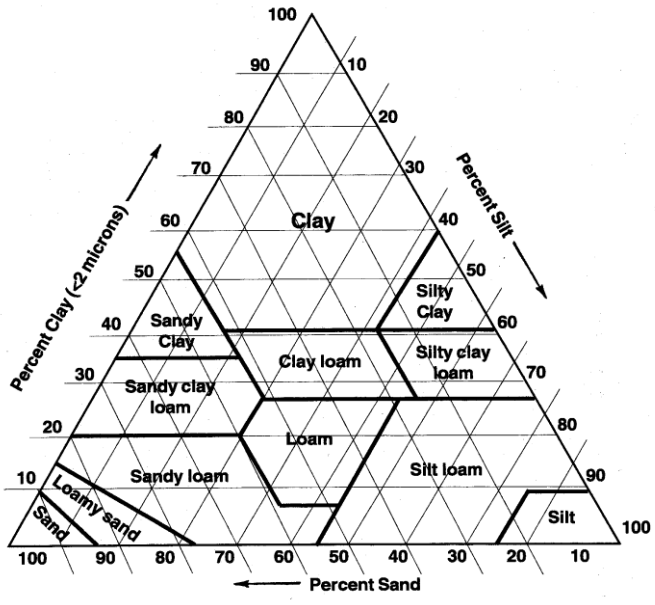


Figure 2.2. Soil textural triangle (United States Department of Agriculture, no date).

2.4.5. Plant sampling

Plant samples for GSL analysis were composed of different plant organs in relation to the overall plant composition. Upper leaves, lower leaves, stems, stalks, flowers (if present) and seed pods (if present) were cut from one plant per sample and combined prior to freeze-

drying. The ratio of each organ in the sample was determined by a visual inspection of the individual plants at the time of collection.

2.5. Biofumigation Field Trials

Two field trials were carried out at naturally *G. pallida* infested field sites in Lincolnshire, UK. All trial preparation, maintenance and sampling prior to analysis was carried out by Barworth Agriculture Ltd. PCN soil samples were composed of thirty cheese-cored soil samples $plot^{-1}$ at a soil depth of 10-20cm randomly taken and combined to give a final weight of ~500g soil $plot^{-1}$. Field sites were prepared by ploughing to 20-25cm and tilling with a disc harrow (custom Simba seed drill) to produce a seed bed. 100kg ha^{-1} nitrogen and 18kg ha^{-1} sulphur were added as fertilisers in each trial prior to sowing. Brassicaceae cultivars were sown and left to grow for eight to ten weeks. Plant material (65-80t ha^{-1}) was incorporated into plots at 12-15cm depth by flail mowing (Del Morino flail mower) then rotovating after which fields were rolled (flat roller) to seal the soil.

2.6. Soil Sample Collection

2.6.1. Potato cyst nematode collection

Cysts were collected from soil using a Fenwick can extraction method followed by acetone floating. Soil was placed in an 85µM sieve and showered with H_2O in order to separate the soil into organic material retained on the sieve, heavy sand which collected at the bottom of the Fenwick can and light debris containing cysts which floated to the top and was collected in a 25µM sieve. The collected cyst-containing debris was transferred to a filter paper and dried prior to acetone washing.

Material was transferred into a conical flask filled with acetone (Rathburn Chemicals, UK), mixed and left for several minutes to allow debris separation and cysts to float to the top. Debris and cysts which floated to the top were collected in a filter paper. This process was repeated several times until there was no more cyst-containing debris to collect. Cyst samples were rinsed with H_2O to remove excess acetone and left to dry. Cysts were separated from any remaining debris by hand under a SWF10X S-4400 microscope (Euromex, Netherlands). Cysts were stored at 4°C in the dark for a minimum of four weeks before use.

2.6.2. Soil microbial collection

At least three rhizosphere soil samples were taken at a soil depth of 10-20cm and combined to obtain a composite sample for each treatment replicate in each pot trial. Field soil samples were composed of thirty cheese-cored samples $plot^{-1}$ at a soil depth of 10-20cm randomly taken and combined. The fresh soil samples were sieved (mesh size <2mm) to remove large

particles and plant debris. Soil was stored in 50 mL Falcon tubes in the dark at 4°C for up to one month before analysis and 2g subsamples were stored in Eppendorf tubes at -80°C prior to DNA extraction.

2.7. Molecular Biology

2.7.1. Soil DNA extraction

Microbial genomic DNA was extracted from 0.25g of soil using the Mo Bio PowerSoil DNA Isolation Kit (Cambio, UK) following the manufacturer's instructions. The total DNA concentration (ng μL^{-1}) of each sample was determined using the Nucleic Acid program on a NanoDrop ND-1000 Spectrophotometer V3.8.1 (Labtech International Ltd., UK). Extracted DNA samples were stored at -20°C until use.

2.7.2. Quantitative polymerase chain reaction (qPCR) primer and probe design

In order to design qPCR primers and probes for identifying bacteria, commonly found soil bacterial genus of interest were identified in published studies. At least three sequences for the gene of interest from a minimum of three bacterial species per genus were downloaded from the GenBank NCBI database. Multiple sequences were downloaded and aligned in order to check the quality and continuity between sequences and organisms. Sequences were aligned using ClustalW alignment method in Geneious v9.1.6 and potential primers and probes were designed to the aligned sequences using the 'Design New Primers' function in Geneious v9.1.6 with the pre-set primer design conditions. Primers and probes were designed to sequences where there were less than four variable bases between sequences of interest in the probe binding site and at least one of the forward or reverse primer binding sites. All probes were dually labelled with the fluorescent dyes fluorescein (6-FAM) 5' reporter and Black Hole Quencher (BHQ-1) 3' quencher. Designed primers and probes were subsequently synthesized by Eurofins Scientific, UK.

2.7.3. qPCR DNA standards

DNA standards for each targeted bacterial set were purchased as genomic DNA (DSMZ, Germany and ATCC, UK). Standards were prepared by making serial dilutions from the stock genomic DNA. The 1:10 serial dilutions were made in Sigma H_2O with the first dilution shaken at 450 rpm at 4°C for 24hrs and the subsequent three 10-fold dilutions prepared from the previous serial dilution and shaken at 450 rpm at 4°C for 1hr. Once prepared, the DNA concentration (ng μL^{-1}) of each standard was determined using the Nucleic Acid program on a NanoDrop ND-1000 Spectrophotometer V3.8.1. Standards were stored at -20°C until use.

Total DNA concentration (ng μL^{-1}) in each standard serial dilution was used to produce a standard curve in each assay run.

2.7.4. qPCR amplification

Real-time qPCR master mixes were prepared to give a total reaction volume of 25 µL. Reactions consisted of 12.5 µL PerfeCTa® qPCR ToughMix (Quantabio, MA, USA), 1.5 µL forward primer (5ng μL^{-1}), 1.5 µL reverse primer (5ng μL^{-1}), 0.75 µL probe (5ng μL^{-1}), 7.75 µL Sigma H_2O and 1 µL of added DNA in each well of a MicroAmp optical 96-well reaction plate (Applied Biosystems, CA, USA).

Real-time qPCR assays were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA) and the associated 7900 SDS V2.4.1 software. Amplification was carried out under the following conditions; 50°C for 2 mins, 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs and 50°C for 1 min. A Sigma H_2O control and four DNA standards were included as negative and positive controls, respectively, and a standard curve was produced from the known DNA standards to quantify results. Each sample was run in triplicate.

2.8. Liquid Chromatography-Mass Spectrometry (LC-MS)

Due to a lack of access to a mass spectrometer, sample preparation, glucosinolate extraction and LC-MS analysis was completed by staff at the University of Reading, who collated and returned the results before full analysis.

2.8.1. LC-MS sample preparation

Freeze-dried plant samples were milled into fine powder using a Mini Cutting Mill (Thomas Scientific, NJ, USA) through a 2mm mesh to ensure that the samples were even and fine for extraction. Samples were stored in a cool, dry place until extractions began.

2.8.2. LC-MS sample extraction

The extraction protocol used was taken from a previous study (Bell, Oruna-Concha and Wagstaff, 2015). Briefly, two technical replicates of each biological replicate were prepared as follows: 40mg of the ground sample was heated in a dry-block at 75°C for 2 mins. 1 mL of 70% (v/v) methanol preheated to 70°C was added to each sample, which was subsequently placed in a water bath for 20 mins at 70°C. Samples were centrifuged for 5 mins at 12,000 rpm at room temperature. The supernatant was filtered using 0.22µm Arcrodisc syringe filters with Supor membranes (hydrophilic polyethersulfone; VWR, UK) into Eppendorf tubes. The volume was adjusted to 1 mL with 70% (v/v) methanol and frozen at -80°C until analysis.

2.8.3. LC-MS analysis

Immediately before LC–MS analysis, samples were diluted with 9 mL of HPLC-grade H_2O. Samples were run in a random order with QC samples. An external standard of sinigrin hydrate was prepared for quantification of GSL compounds as follows: a 12mM solution was prepared in 70% methanol then a dilution series of concentrations was prepared as an external calibration curve with HPLC-grade H_2O (896, 448, 224, 112, 56, 42, 28, 14 and 5.6ng μL^{-1}; sinigrin correlation coefficient: y = 27.371; r^2 = 0.998).

LC–MS analysis was performed in the negative ion mode on an Agilent 1260 Infinity Series LC system (Stockport, UK) equipped with a binary pump, degasser, auto-sampler, column heater and diode array detector coupled to an Agilent 6120 Series single quadrupole mass spectrometer. Separation of samples was achieved on a Gemini 3µm C_{18} 110Å (150 x 4.6mm) column (with Security Guard column, C_{18}; 4mm x 3mm; Phenomenex, Macclesfield, UK). GSLs were separated during a 40 min chromatographic run, with a 5 min post-run sequence. Mobile phases consisted of ammonium formate (0.1%; A) and acetonitrile (B) with the following gradient timetable: (i) 0 min (A-B, 95:5, v/v); (ii) 0-13 mins (A-B, 95:5, v/v); (iii) 13-18 mins (A-B, 40:60, v/v); (iv) 18-26 mins (A-B, 40:60, v/v); (v) 26-30 mins (A-B, 95:5, v/v); (vi) 30-40 mins (A-B, 95:5, v/v). The flow rate was optimised for the system at 0.4mL min^{-1}, with a column temperature of 30°C, and 25 µL sample injected into the system. Quantification was conducted at a wavelength of 229nm.

MS analysis settings were as follows: API-ES was carried out at atmospheric pressure in negative ion mode (scan range m/z 100–1500 Da). Nebulizer pressure was set at 50psi, gas-drying temperature at 350°C, and capillary voltage at 2,000V.

2.8.4. LC-MS glucosinolate identification

Compounds were identified using their primary ion mass and by comparing relative retention times with those published in the literature (Botting *et al.*, 2002; Cataldi *et al.*, 2007; Rochfort *et al.*, 2008; Lelario *et al.*, 2012). Data were analysed using Agilent OpenLAB CDS ChemStation Edition for LC-MS (Agilent, version A.02.10). The sinigrin calibration curve and relative response factors were used to calculate glucosinolate (GSL) concentrations (mg g^{-1} dry weight (DW)) where available. Where relative response factors could not be found for intact GSLs, the response factor was assumed to be 1.00. GSL concentrations in each sample were averaged over technical and biological replicates and used to determine the abundance of each GSL in the samples.

2.9. Microbial Community Level Physiological Profile (CLPP) Analysis

Analysis of basal respiration and changes in soil microbial CLPPs was performed using the MicroResp™ protocol (Campbell *et al.*, 2003) and associated components (MicroResp™, UK) as previously described. The protocol has been outlined in Appendix A.

2.10. Statistical Data Analysis

2.10.1. Analysis of variance and significance testing

Data were analysed using the statistical package Genstat v18.2. Data was tested for normality using the 'Probability Distribution Plot' function. Where required, data sets were transformed in order to provide a normal data set for accurate p-value production in the presence of high variation in the count data and large standard errors. Transformations differed depending on the optimum power-lambda determined during normality testing. Analysis of Variance (ANOVA) analysis was performed on data using the 'General ANOVA' function whose treatment structure differed depending on the factors being investigated. Significant P-values (P<0.05) were investigated with a means comparison test: Tukey's HSD, Dunnett's or a t-test.

2.10.2. Multivariate analysis

Canonical variate (CV) analysis was performed on multivariate data using the 'Canonical Variates' function in Genstat v18.2. Data were grouped by the factor being investigated in each experiment. The mean Mahalanobis distance between sets was displayed and used to measure the overall separation of groups. CV plots were produced and the first and second CV ordinates for each sample were saved and used to form scatterplots of the data sets. The significance of distances from CV analyses were investigated using the 'Multivariate Analysis of Distance' function on a distance matrix from similarities of the data. The distance matrix was formed from Euclidean tests of each included variate. The treatment structure differed depending on the factors being investigated in each experiment. The significance probabilities were determined from a random permutation test repeated 999 times and used to calculate a P-value. When a significant P-value (Pr<0.05) occurred, ANOVA analysis, followed by a means comparison test, was performed on the first and second CV ordinates.

2.10.3. Regression analysis

Regression analysis was completed using the 'Standard Curves' function in the Regression Analysis tab of Genstat v18.2. Different curve equations were tried with CO_2% as the response variate and At6n as the explanatory variate. The model, F-probability and estimates were displayed as outputs for each curve tested and the curve which fitted the most data (R^2) with the least variation (standard error) was selected (Appendix A).

Chapter 3. Assessing the Toxicity of Isothiocyanates to Potato Cyst Nematodes in Bioassays

Part of this chapter is included in a manuscript published in Nematology:

'Allyl isothiocyanate shows promise as a naturally produced suppressant of the potato cyst nematode, *Globodera pallida*, in biofumigation systems' (Wood, Kenyon and Cooper, 2017)

3.1. Introduction

The suppressive effect of biofumigation is dependent on the isothiocyanate (ITC) type, concentration and the species being targeted (Taylor, Kenyon and Rosser, 2014). Several studies have demonstrated an *in vitro* effect of glucosinolate (GSL) hydrolysis products on PCN mortality and hatch (Buskov *et al.*, 2002; Serra *et al.*, 2002; Brolsma *et al.*, 2014). Previous research studied the effect of GSL breakdown products on *G. rostochiensis* juvenile (J2) mortality with the exception of Brolsma *et al.* (2014) who studied the effect of pure allyl ITC on *G. pallida* hatch from encysted eggs. There is limited information on the effect of pure ITCs on PCN J2 mortality and their effect on encysted PCN hatch.

All studies to date have focussed on the effect of a select few *Brassica* spp. and their GSL hydrolysis products on PCN J2, namely *B. juncea* and its major GSL, sinigrin. As different cultivars contain a number of GSLs leading to the release of various ITCs (Fahey, Zalcmann and Talalay, 2001), there is a need to screen a wide range of ITCs against PCN J2 in order to determine which are effective. In addition, encysted J2 are harder to target due to the protective cyst so the effect of these ITCs on hatch is an important area to research, especially as biofumigation is applied when PCN is in this state. As ITCs are the toxic compounds, the effects of these are the focus of this study instead of the parent GSL.

The ITCs used in this study were selected according to commercial availability, use in previous studies, or those known to be produced during Brassicaceae plant tissue GSL hydrolysis (Fenwick and Heaney, 1983; Fenwick, Heaney and Mullin, 1983; Fahey, Zalcmann and Talalay, 2001). Several of these ITCs have not been previously tested for PCN toxicity; sulforaphane (SUL) was included as it has been widely investigated for its involvement in human health and cancer suppression (Higdon *et al.*, 2007; Clarke *et al.*, 2011) so it seemed pertinent to determine its biofumigation potential. ITC concentrations were chosen due to their use in a previous *in vitro* study where pure ITCs were screened against various potato fungal pathogens (Taylor, Kenyon and Rosser, 2014). As *G. pallida* is the main species of interest in this work, a greater number of ITCs were screened against this species compared to *G. rostochiensis*.

The main aims of this study were to:

- Assess the ability of ITCs to increase PCN J2 mortality
- Determine the effect of ITCs on encysted J2 hatch

3.2. Materials and Methods

3.2.1. Juvenile toxicity assays

Juvenile toxicity assays were performed as described in 2.3.1. During the study a minimum of thirty hatched *G. pallida* and *G. rostochiensis* J2 were exposed to one of several ITC solutions (BITC, PEITC, MITC, AITC, PITC and IITC against both species plus EITC, PHITC, BUITC and SUL against *G. pallida*) at three initial concentrations (12.5, 25 and 50ppm) for 72hrs. Four replicates of each treatment were completed. For each treatment J2 mortality counts were converted to percentage mortality.

3.2.2. Cyst hatching assays

Hatching assays were performed with batches of five *G. pallida* or *G. rostochiensis* cysts as described in 2.3.2. Treatments consisted of AITC, BITC, PEITC, MITC, EITC or SUL at initial concentrations of 3.125, 6.25, 12.5, 25 or 50ppm and exposure periods of 1, 4, 7, 10 or 16 days. Four replicates of each treatment were completed in each assay.

3.2.3. Data analysis

Data analysis was performed as in 2.10.1. In the juvenile toxicity assays, two-way ANOVA analysis was performed where the factors ITC x Concentration were analysed. In the hatching assays, one-way ANOVA was performed with Concentration as the factor. Significant differences ($P<0.05$) between treatments and the control were identified using Dunnett's test.

3.3. Results

3.3.1. Isothiocyanates and G. pallida *juvenile mortality*

Total *G. pallida* J2 mortality recorded over a 72hr period differed significantly depending on the ITC and concentration applied ($P<0.001$ for ITC, Concentration and ITC x Concentration; Figure 3.1). AITC was extremely effective, causing significant J2 mortality at all concentrations; 100% mortality occurred after exposure to 25ppm and 50ppm AITC. Both EITC and PEITC treatments resulted in a significant increase in mortality at all doses (12.5-50ppm). When exposed to BITC, MITC or SUL, J2 mortality increased as the dose increased with 25ppm and 50ppm concentrations significantly increasing mortality. 50ppm SUL caused 100% J2 mortality. PHITC, PITC and BUITC were less effective with only the 50ppm treatments resulting in a significant increase in J2 mortality. The least effective ITC was IITC; it had no effect on J2 mortality at any dose.

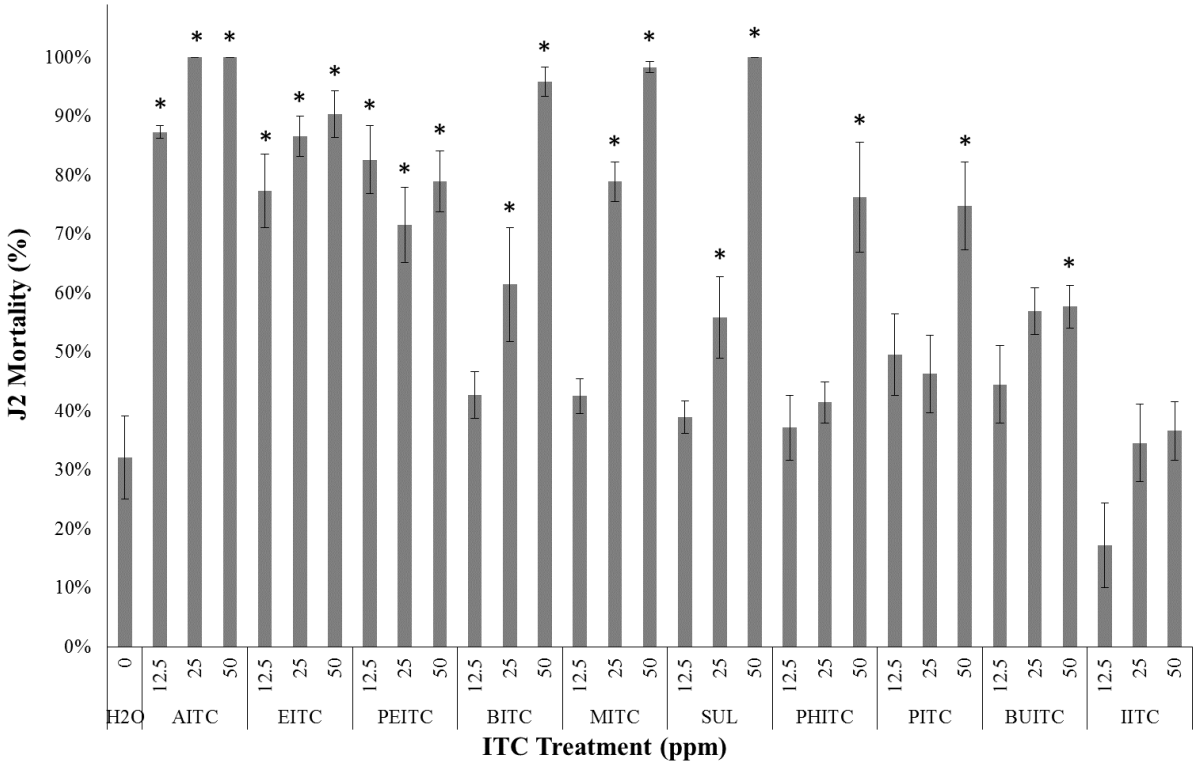


Figure 3.1. Total mortality (%) of *G. pallida* J2 after ITC exposure for 72hrs. Error bars represent the standard error. Significant differences (P<0.05) compared to the control are indicated by an asterisk.

During exposure to ITCs, the rate of *G. pallida* J2 mortality differed depending on the ITC and concentration applied (Table 3.1). Mortality occurred earlier over the 72hr period as AITC, BITC, MITC and SUL concentration increased. 100% J2 mortality occurred in response to 50ppm AITC after 24hrs exposure. Concentration had no effect on rate of mortality for EITC, PITC, PHITC, PITC, BUITC or IITC treatments, although these ITC treatments did lead to the majority of mortality occurring earlier than in the H_2O control, where the highest J2 death was noted after 48-72hrs exposure; the exceptions to this were 25ppm PHITC, PITC, IITC and 50ppm BUITC.

Treatment	Concentration (ppm)	Percentage Total Mortality (%)		
		24hrs	48hrs	72hrs
H_2O	**0**	8.06	21.49	**69.83**
AITC	**12.5**	26.95	**45.24**	27.91
	25	**60.31**	32.06	7.63
	50	**100.00**	-	-
EITC	**12.5**	**47.13**	34.28	17.99
	25	**46.87**	25.94	27.62
	50	**60.79**	27.90	10.96
PEITC	**12.5**	31.80	**49.24**	18.46
	25	18.38	**55.14**	25.19
	50	18.08	**54.25**	27.66
BITC	**12.5**	9.67	42.56	**48.36**
	25	27.82	29.21	**41.73**
	50	25.44	**58.50**	16.11
MITC	**12.5**	21.38	19.24	**57.73**
	25	**47.76**	34.12	18.52
	50	**58.48**	28.81	12.71
SUL	**12.5**	2.95	39.84	**59.76**
	25	12.70	36.68	**47.97**
	50	**87.59**	8.76	3.65
PHITC	**12.5**	30.44	**35.13**	32.79
	25	33.34	31.38	**35.30**
	50	28.26	**38.35**	34.31
PITC	**12.5**	28.84	**41.20**	28.84
	25	**33.42**	**33.42**	**33.42**
	50	35.25	**41.33**	23.09
BUITC	**12.5**	27.64	**36.85**	33.17
	25	33.15	**38.12**	28.17
	50	12.89	**42.98**	**42.98**
IITC	**12.5**	28.64	**42.96**	23.86
	25	27.00	34.36	**39.27**
	50	27.71	**39.58**	29.69

Table 3.1. *Globodera pallida* J2 mortality at each 24hr count as a percentage of total mortality. The count where the majority of mortality occurred is in bold. Data presented is the average of four replicates.

3.3.2. Isothiocyanates and G. rostochiensis *juvenile mortality*

After exposure to six ITCs, total *G. rostochiensis* J2 mortality differed depending on the ITC and concentration applied (P<0.001 for ITC, Concentration and ITC x Concentration; Figure 3.2). AITC, PEITC and BITC were extremely effective, leading to significant J2 mortality at all concentrations. 100% mortality occurred after exposure to 25ppm and 50ppm AITC. Following MITC exposure, J2 mortality increased with concentration; 25ppm and 50ppm MITC significantly increased mortality compared to the control. PITC and IITC had no effect on J2 mortality at any concentration.

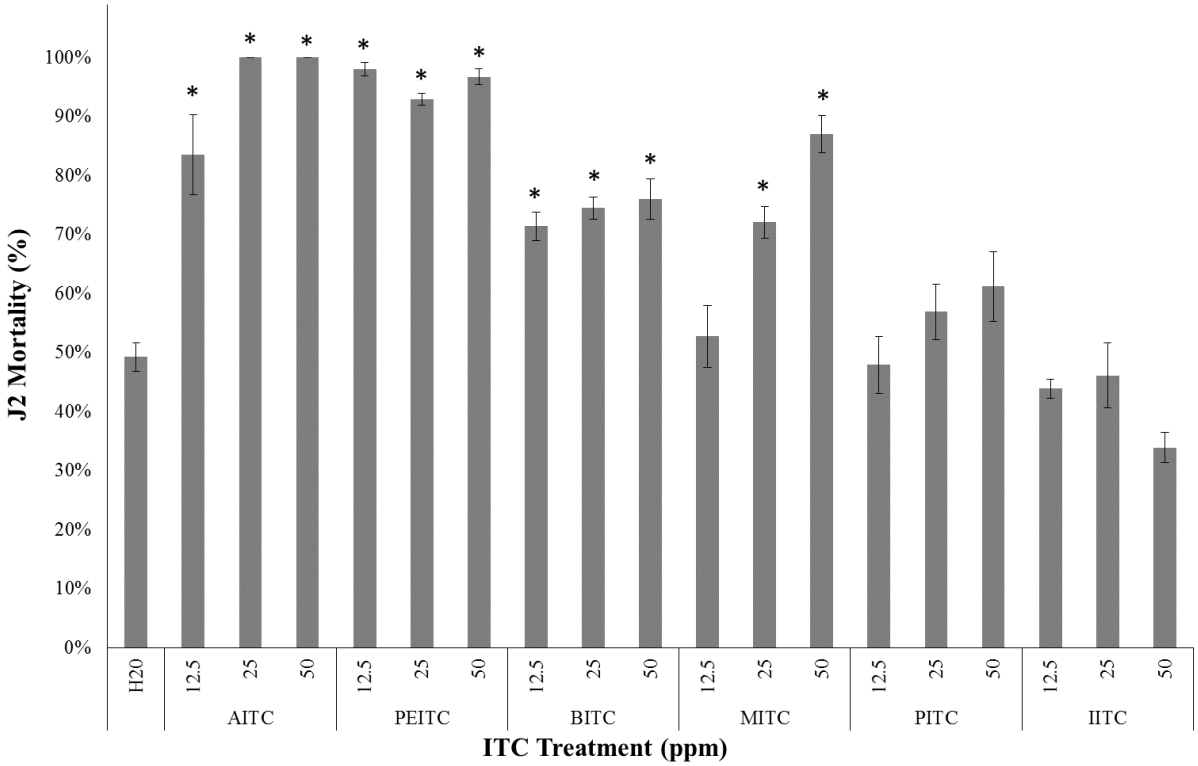


Figure 3.2. Total mortality (%) of *G. rostochiensis* J2 after ITC exposure for 72hrs. Error bars represent the standard error. Significant differences (P<0.05) compared to the control are indicated by an asterisk.

During exposure to ITCs, the rate of *G. rostochiensis* J2 mortality over the 72hr period differed slightly depending on the ITC and dose used (Table 3.2). Mortality occurred earlier over the 72hr period as AITC and PEITC concentration increased. 100% J2 mortality occurred in response to 50ppm AITC after 24hrs exposure. Concentration had no effect on rate of mortality for BITC, MITC, PITC or IITC. All PITC and IITC treatments led to a delay in J2 mortality compared to the control, where the majority of J2 mortality occurred within the 24-48hrs exposure period.

Treatment	Concentration (ppm)	Percentage Total Mortality (%)		
		24hrs	48hrs	72hrs
H_2O	**0**	24.70	**45.06**	30.24
AITC	**12.5**	29.81	**39.23**	30.95
	25	**51.90**	39.57	8.53
	50	**100.00**	-	-
PEITC	**12.5**	41.00	**47.54**	11.46
	25	**51.36**	31.94	16.71
	50	**58.83**	26.95	14.22
BITC	**12.5**	13.79	**56.28**	29.93
	25	20.97	**54.04**	24.99
	50	26.62	**43.78**	29.61
MITC	**12.5**	9.45	**54.43**	36.12
	25	16.67	**54.22**	29.11
	50	38.23	**44.10**	17.66
PITC	**12.5**	22.51	29.10	**48.39**
	25	11.04	41.06	**47.90**
	50	12.95	41.02	**46.04**
IITC	**12.5**	34.51	29.93	**35.57**
	25	26.06	35.90	**38.05**
	50	19.50	38.01	**42.49**

Table 3.2. *Globodera rostochiensis* J2 mortality at each 24hr count as a percentage of total mortality. The count where the majority of mortality occurred is in bold. Data presented is the average of four replicates.

3.3.3. Isothiocyanates and encysted G. pallida *hatch*

Globodera pallida cysts were exposed to AITC, PEITC, BITC and MITC, for 1-16 days at concentrations ranging between 3.125-50ppm. Due to a lack of positive results with these ITCs, further hatching assays with SUL and EITC were only completed for exposure periods of 1-7 days at the concentrations found to be effective against free J2 in 3.3.1. Four of the ITCs screened in the toxicity assay were excluded from the hatching assay due to a lack of activity against free J2. Although PHITC, PITC and BUITC induced mortality at the highest concentration it was thought that the addition of the cyst coating would inhibit any toxic effect these ITCs had on encysted J2.

AITC exposure did not have a consistent effect with only select concentrations after 7 and 16 days exposure significantly affecting hatch (P<0.001 at both exposure periods). After 7 days exposure, 25ppm and 50ppm AITC significantly reduced hatch and delayed hatch compared to the control (Figure 3.3). After 16 days exposure, 25ppm AITC increased hatch compared to the control (Figure 3.4); 50ppm AITC delayed hatch. None of the other treatments or exposure periods (1, 4 and 10 days) affected overall hatch. A delay in *G. pallida* hatch was evident when exposed to 25ppm and 50ppm AITC for 1 day and 50ppm for 10 days in a similar pattern to the data shown in Figure 3.3 (further data not shown).

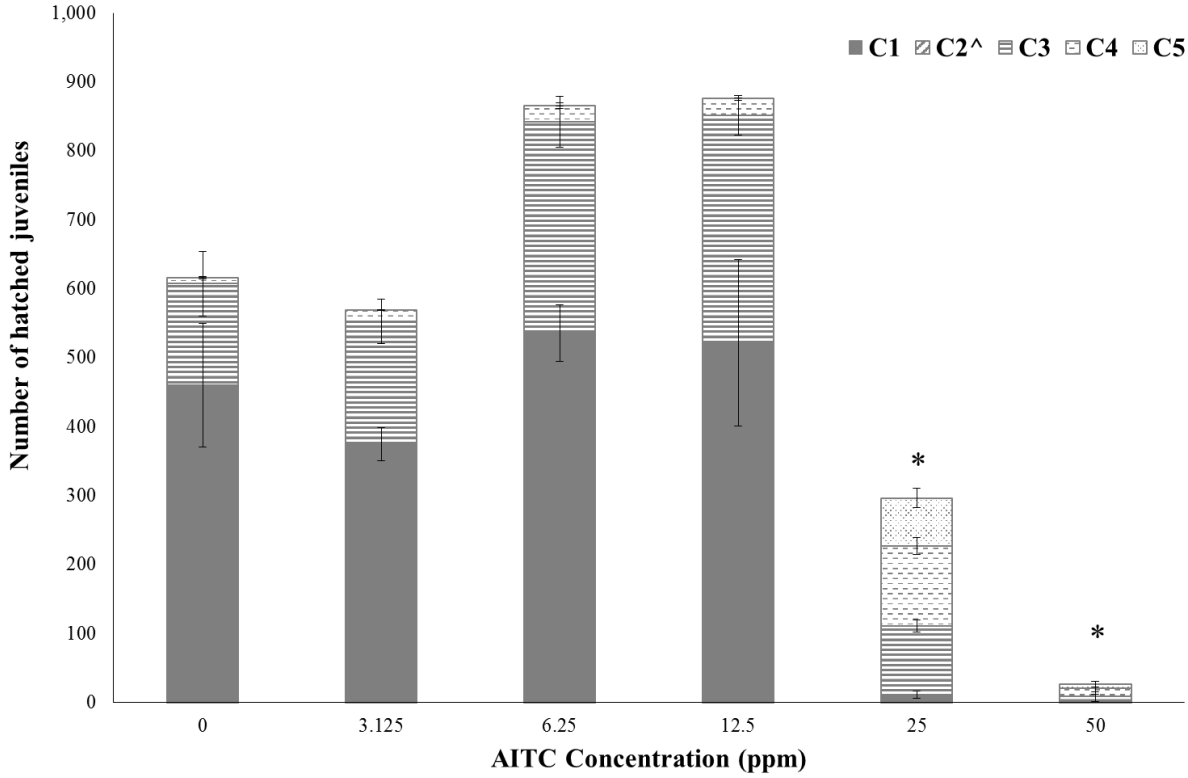


Figure 3.3. Hatch of *G. pallida* J2 when exposed to AITC for 7 days. C1-C5 indicate weekly counts and error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk. ^ C2 was not completed and C3 represents the count of both weeks.

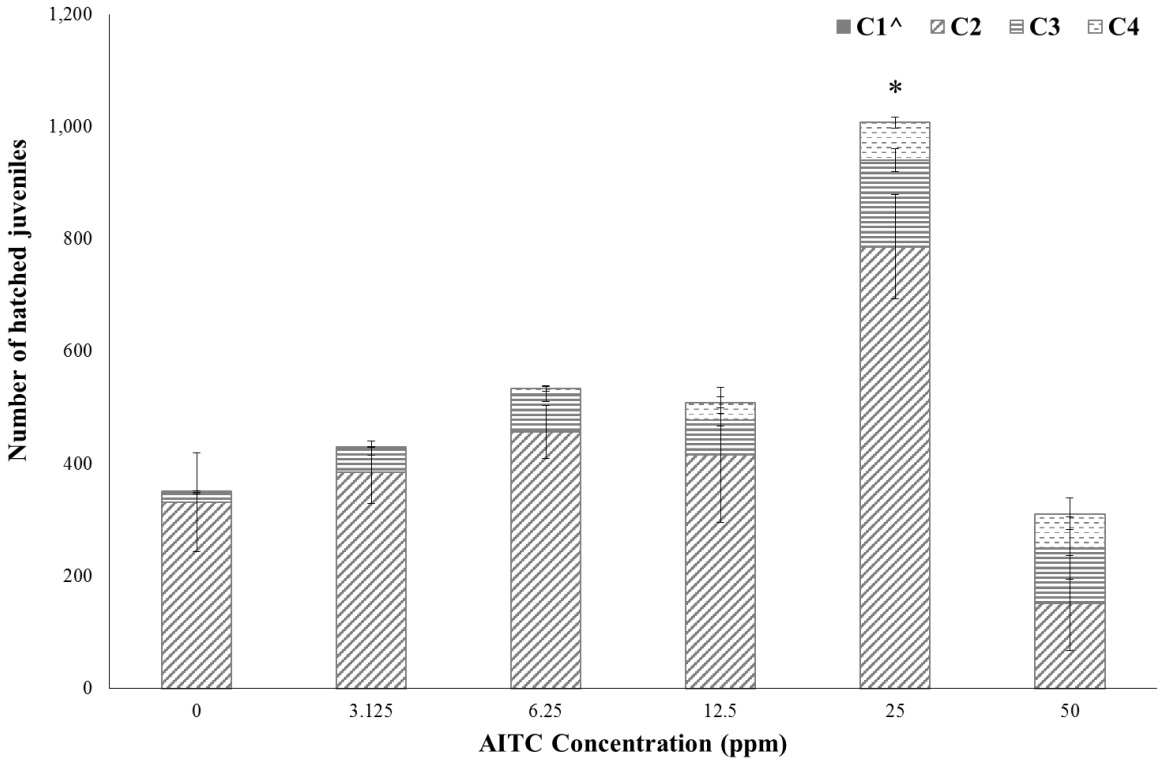


Figure 3.4. Hatch of *G. pallida* J2 when exposed to AITC for 16 days. C1-C4 indicate weekly counts and error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk. ^ C1 was not completed and C2 represents the count of both weeks.

Exposing *G. pallida* cysts to PEITC had no effect on hatch with the exception of 7 days exposure (P=0.003) where all PEITC concentrations significantly increased hatch compared to the control (Figure 3.5); there was no effect on rate of hatch. Rate of hatch was unaffected by PEITC treatment at any other exposure period (1, 4, 10 and 16 days) (data not shown).

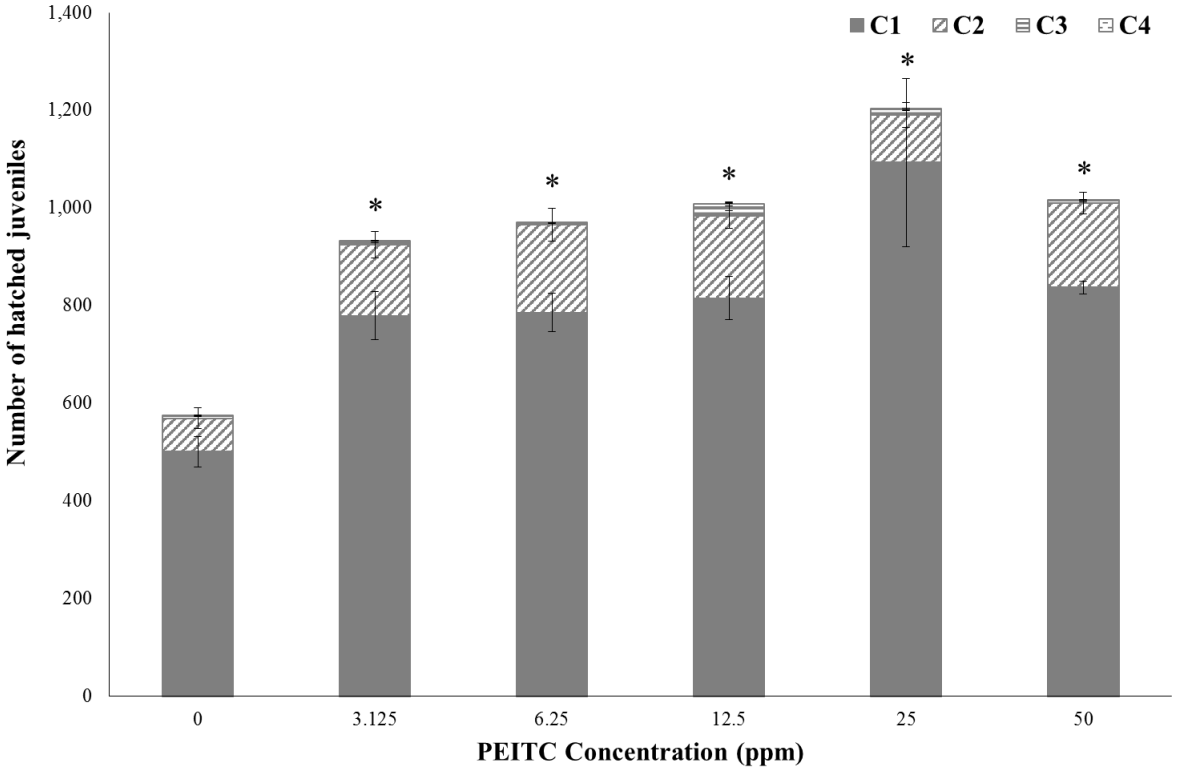


Figure 3.5. Hatch of *G. pallida* J2 when exposed to PEITC for 7 days. C1-C4 indicate weekly counts and error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

The application of BITC between 3.125-50ppm for 1-16 days exposure had no effect on total *G. pallida* hatch nor did it affect rate of hatch. Data for 7 days exposure is displayed in Figure 3.6 and is representative of the hatching results from the other exposure periods (data not shown).

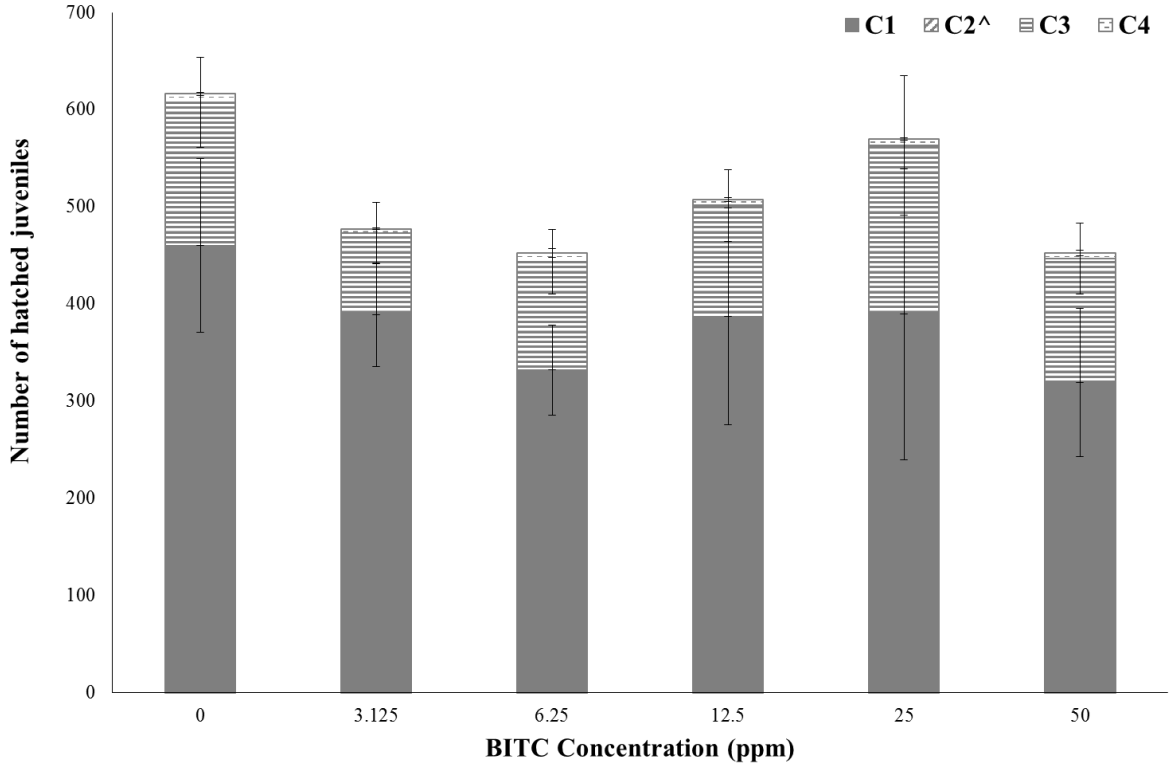


Figure 3.6. Hatch of *G. pallida* J2 when exposed to BITC for 7 days. C1-C4 indicate weekly counts and error bars represent the standard error. ^ C2 was not completed and C3 represents the count of both weeks.

MITC treatments did not result in a reduction in *G. pallida* hatch but 50ppm MITC after 1 days exposure increased hatch compared to the control (P<0.001; Figure 3.7). Several treatments delayed hatch. After 1 day exposure the majority of hatch was delayed by a week after all MITC treatments. After 7 days exposure, hatch delay increased as concentration increased (Figure 3.8); this pattern of hatch was similar for 4 and 10 days exposure (data not shown). Hatch was unaffected by MITC addition after 16 days exposure (data not shown).

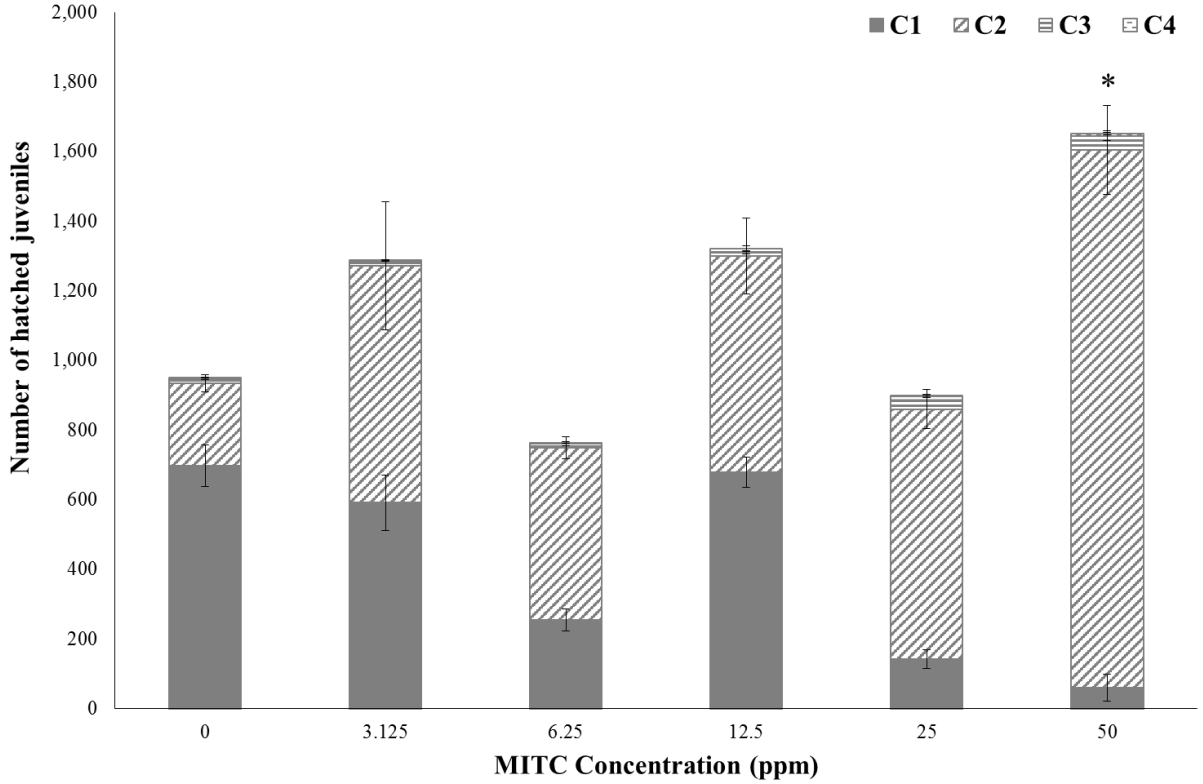


Figure 3.7. Hatch of *G. pallida* J2 when exposed to MITC for 1 day. C1-C4 indicate weekly counts and error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

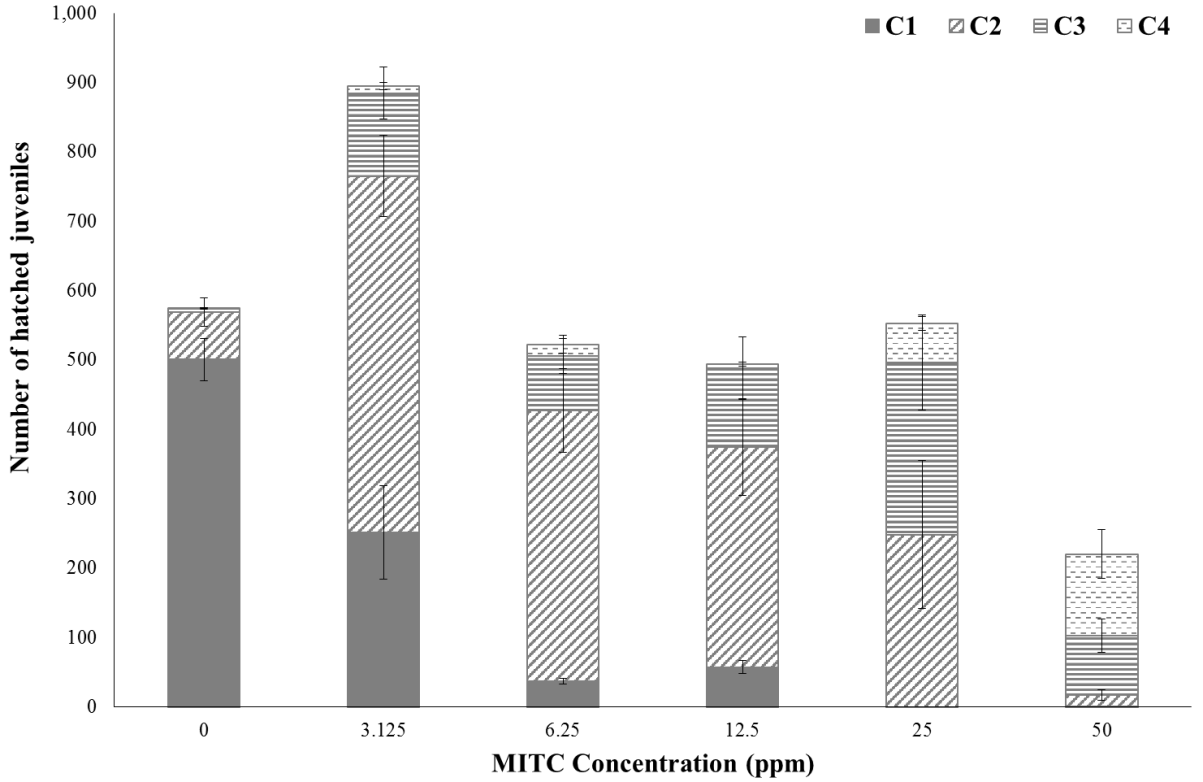


Figure 3.8. Hatch of *G. pallida* J2 when exposed to MITC for 7 days. C1-C4 indicate weekly counts and error bars represent the standard error.

EITC had no effect on total *G. pallida* J2 hatch. 25ppm and 50ppm EITC delayed hatch after 4 days exposure (Figure 3.9). This is representative of data collected after 1 and 7 days exposure where overall hatch was unaffected but hatch was delayed with increasing EITC concentration (data not shown).

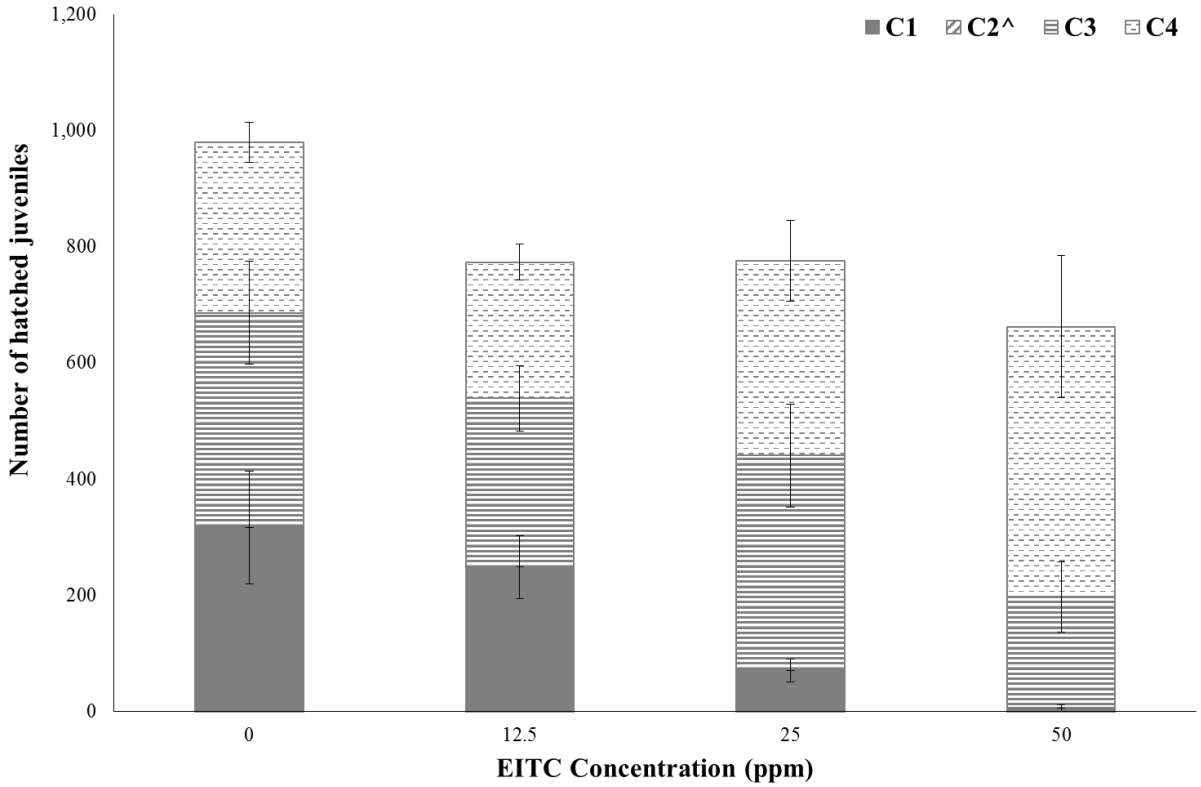


Figure 3.9. Hatch of *G. pallida* J2 when exposed to EITC for 4 days. C1-C4 indicate weekly counts and error bars represent the standard error. ^ C2 was not completed and C3 represents the count of both weeks.

SUL had no effect on overall *G. pallida* J2 hatch (Table 3.3) and rate of hatch was unaffected (data not shown).

Concentration (ppm)	Exposure Period		
	1 Day	4 Days	7 Days
0	470.50 (±168.63)	778.25 (±173.65)	445.50 (±92.77)
25	345.00 (±65.30)	507.50 (±122.06)	660.75 (±87.58)
50	325.50 (±171.52)	233.50 (±82.72)	447.25 (±207.21)

Table 3.3. Overall hatch of *G. pallida* J2 when exposed to SUL. The standard errors are indicated within brackets.

3.3.4. Isothiocyanates and encysted G. rostochiensis *hatch*

Globodera rostochiensis cysts were exposed to AITC, PEITC, BITC and MITC for 1-16 days at concentrations ranging between 3.125-50ppm. Two of the ITCs screened in the toxicity

assay (PITC and IITC) were excluded from the hatching assay due to an absence of activity in the mortality assays.

There was no effect on overall *G. rostochiensis* hatch when exposed to AITC. When exposed to 25ppm and 50ppm AITC for 7 days, J2 emergence occurred later compared to the control (Figure 3.10); this is representative of the data collected after 1 and 4 days exposure periods (data not shown). When the exposure period increased to 10 or 16 days, differences in rate of hatch were reduced; none of the treatments had an effect (data not shown).

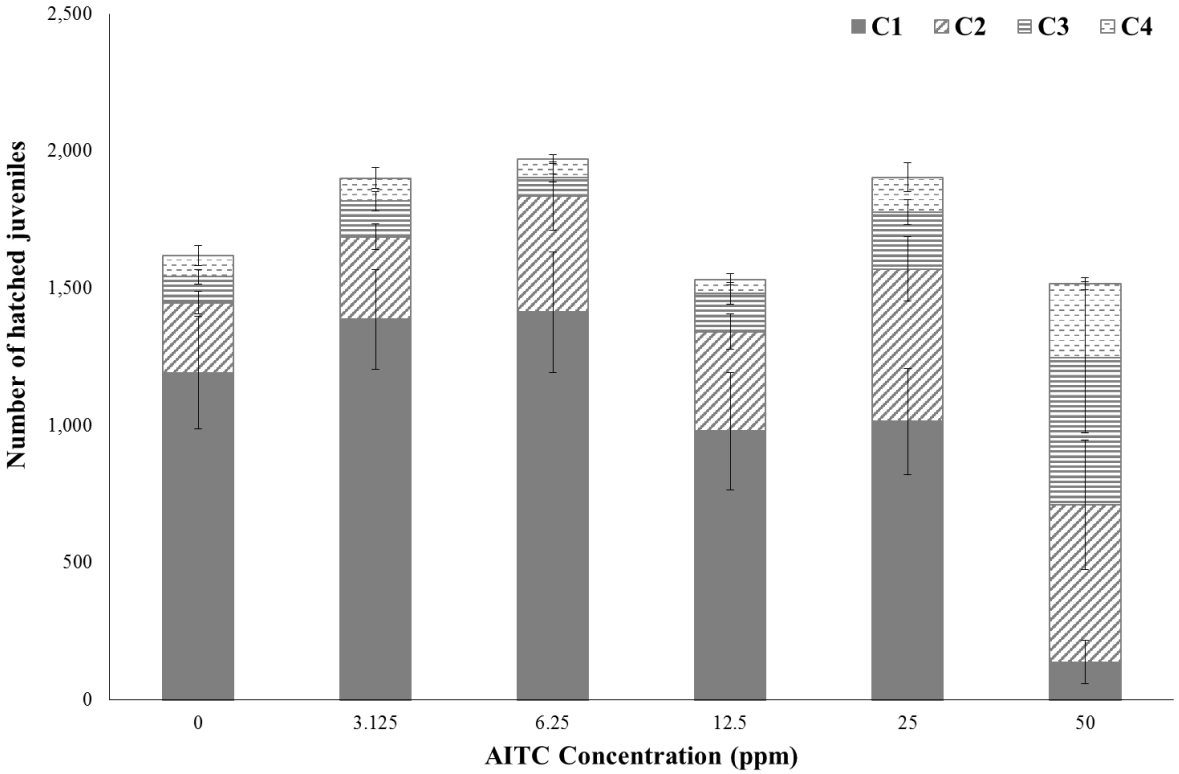


Figure 3.10. Hatch of *G. rostochiensis* J2 when exposed to AITC for 7 days. C1-C4 indicate weekly counts and error bars represent the standard error.

PEITC was not consistently effective at reducing *G. rostochiensis* hatch. After 7 days exposure, 3.125ppm and 50ppm significantly reduced hatch (P=0.026; Figure 3.11). After 10 days exposure, all PEITC concentrations except 6.25ppm significantly reduced hatch compared to the control (P=0.022; Figure 3.12). None of the other treatments had an effect on overall hatch or rate of hatch after 1, 4 and 16 days exposure (data not shown).

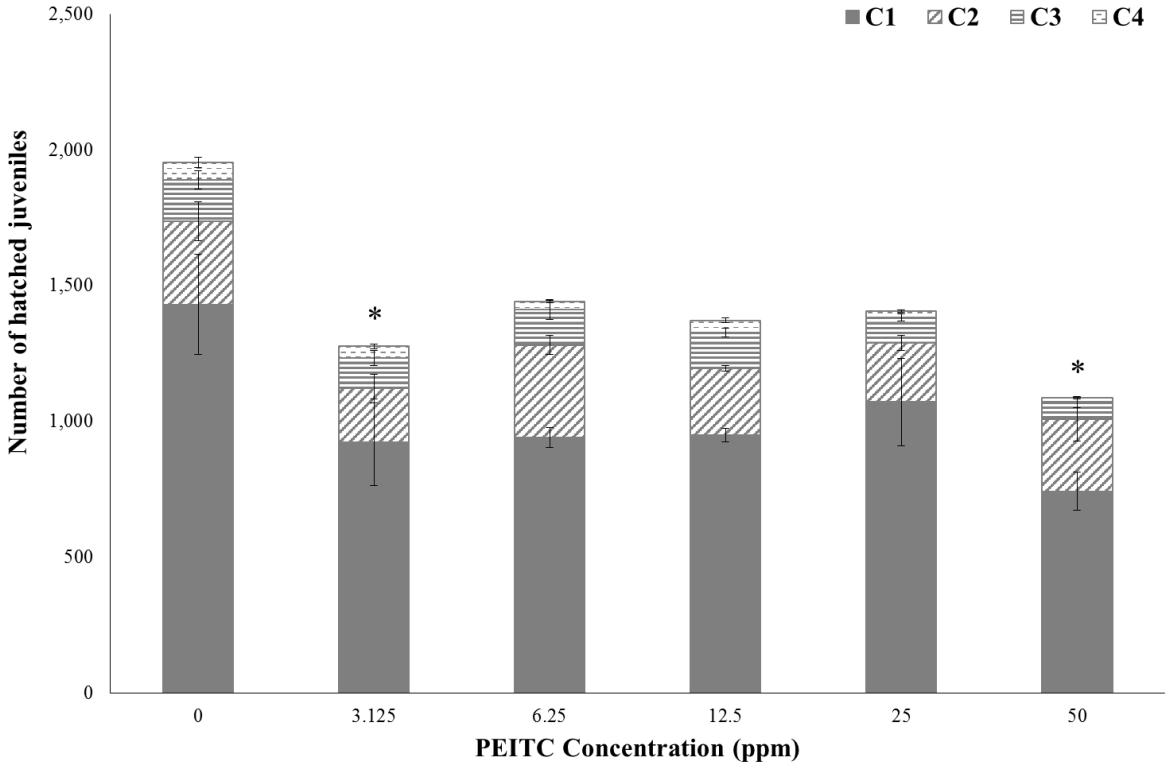


Figure 3.11. Hatch of *G. rostochiensis* J2 when exposed to PEITC for 7 days. C1-C4 indicate weekly counts and error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

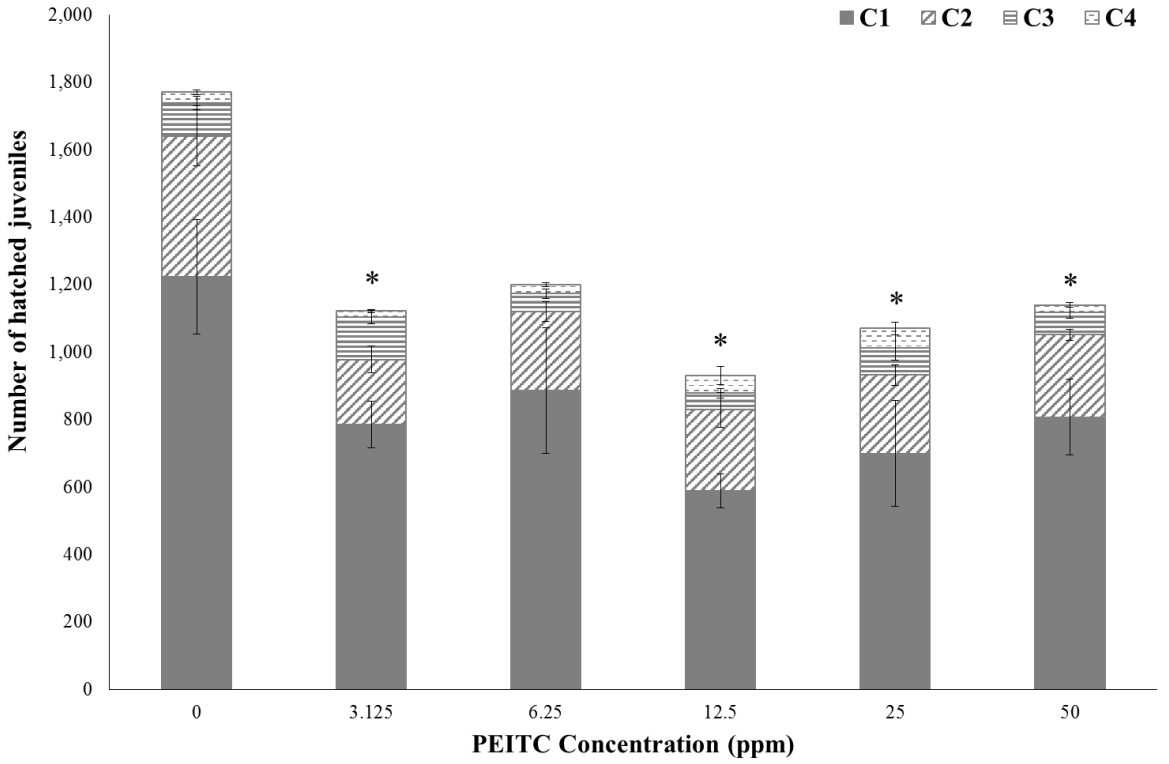


Figure 3.12. Hatch of *G. rostochiensis* J2 when exposed to PEITC for 10 days. C1-C4 indicate weekly counts and error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

BITC had no effect on *G. rostochiensis* hatch and rate of hatch was unaffected. Data for 7 days exposure is shown (Figure 3.13) and is representative of the results from the other exposure periods (data not shown).

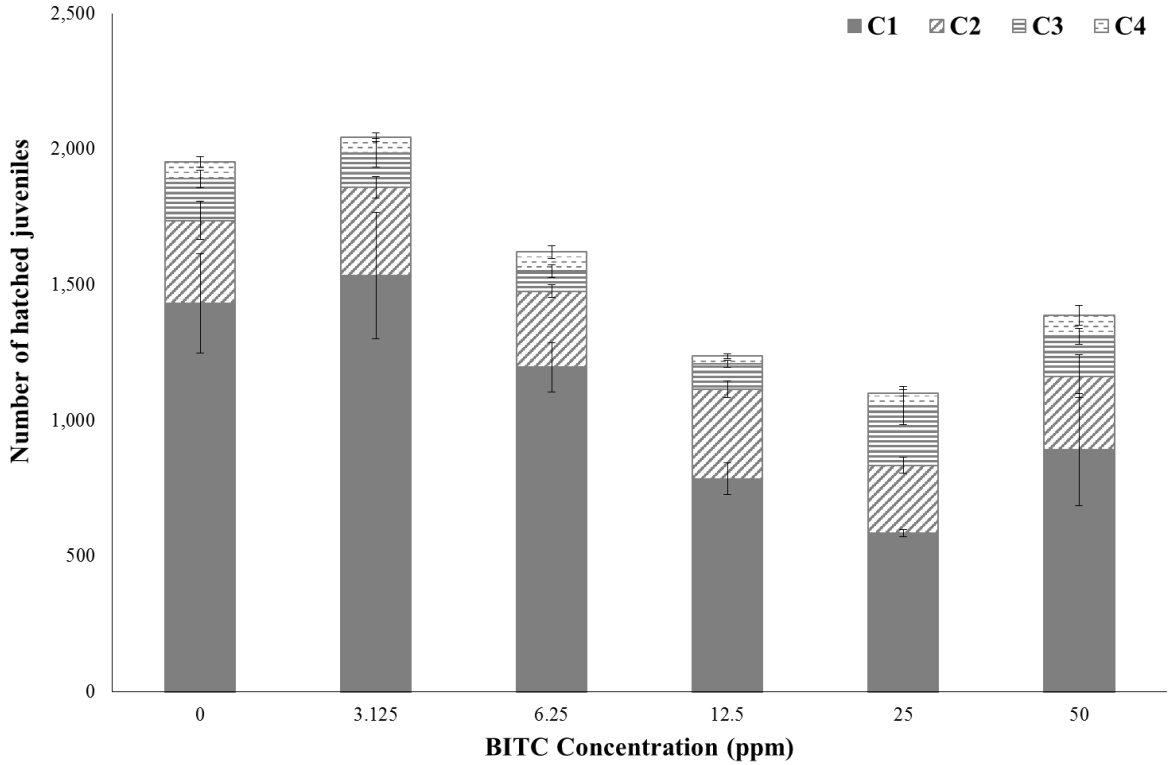


Figure 3.13. Hatch of *G. rostochiensis* J2 when exposed to BITC for 7 days. C1-C4 indicate weekly counts and error bars represent the standard error.

MITC treatments significantly affected hatch compared to the control (Table 3.4). After 1 day exposure, 6.25ppm and 50ppm MITC increased hatch. After 4 days exposure only 6.25ppm MITC significantly increased hatch. After 7 days exposure, 3.125ppm and 12.5ppm MITC treatments increased total hatch compared to the control; 50ppm MITC reduced hatch significantly. When exposure was increased to 10 and 16 days, all treatments, except 12.5ppm and 50ppm MITC, increased hatch compared to the water control. Rate of hatch was also affected by MITC exposure (Table 3.5); 25 and 50ppm MITC delayed hatch independent of exposure period and 12.5ppm MITC delayed hatch after 1 and 4 days exposure.

Concentration (ppm)	Exposure Period				
	1 Day	**4 Days**	**7 Days**	**10 Days**	**16 Days**
0	874.50 (±222.81)	891.50 (±130.33)	1042.00 (±239.33)	520.50 (±177.43)	447.75 (±172.04)
3.125	1179.00 (±69.51)	1447.00 (±192.90)	**1800.50 (±63.32)**	**1502.50 (±156.57)**	**1309.50 (±149.66)**
6.25	**2480.00 (±265.78)**	**1567.00 (±187.12)**	1455.00 (±80.46)	**1437.50 (±195.93)**	**1776.50 (±313.12)**
12.5	1240.50 (±212.34)	1553.00 (±190.01)	**1814.00 (±298.81)**	1083.00 (±134.08)	1152.50 (±106.81)
25	1675.50 (±266.25)	1367.50 (±147.38)	1274.50 (±68.54)	**1260.00 (±260.94)**	**1732.00 (±258.73)**
50	**1884.00 (±122.96)**	703.00 (±166.07)	**306.50 (±109.88)**	250.00 (±68.71)	125.75 (±33.22)
	ANOVA P-values				
Concentration	**<0.001**	**0.007**	**<0.001**	**<0.001**	**<0.001**

Table 3.4. Overall hatch of *G. rostochiensis* J2 when exposed to MITC and associated ANOVA P-values. The standard error is indicated within brackets. Concentrations which were significantly different to the control ($P<0.05$; within columns) are in bold.

Exposure Period	Concentration (ppm)	Percentage Total Hatch (%) C1	C2	C3	C4
1 Day	**0**	**41.17**	33.96	17.04	7.83
	3.125	**46.44**	36.90	13.02	3.65
	6.25	**40.04**	39.74	12.70	7.52
	12.5	22.37	**54.41**	19.39	3.83
	25	0.00	**70.73**	23.01	6.27
	50	0.00	4.91	**77.49**	17.60
4 Days	**0**	**74.93**	20.19	2.97	1.91
	3.125	**69.18**	21.42	3.63	5.77
	6.25	**51.15**	40.71	6.99	1.15
	12.5	22.89	**54.96**	18.74	3.41
	25	1.61	**54.11**	37.07	7.20
	50	0.00	0.00	48.72	**51.28**
7 Days	**0**	**87.48**	8.97	2.35	1.20
	3.125	**73.26**	19.80	5.19	1.75
	6.25	**73.54**	13.95	9.38	3.13
	12.5	**48.21**	39.28	11.19	1.32
	25	5.10	32.84	**50.80**	11.26
	50	0.00	0.00	18.11	**81.89**
10 Days	**0**	**90.20**	4.61	3.31	1.87
	3.125	**85.96**	12.21	1.30	0.53
	6.25	**76.73**	20.70	2.09	0.49
	12.5	**69.11**	26.45	2.72	1.71
	25	12.26	**55.71**	20.99	11.03
	50	0.00	0.00	22.00	**78.00**
16 Days	**0**	**84.42**	13.57	1.34	0.67
	3.125	**49.18**	43.07	6.38	1.37
	6.25	**80.61**	16.44	2.56	0.39
	12.5	**61.43**	30.46	6.90	1.21
	25	13.19	**58.00**	23.96	4.85
	50	0.00	0.20	27.44	**72.37**

Table 3.5. *Globodera rostochiensis* hatch at each count as a percentage of total hatch after MITC exposure. The count where the majority of hatch occurred is in bold. Data presented is the average of four replicates.

3.4. Discussion

3.4.1. Isothiocyanate concentrations

Maximum doses used within this study are thought to be at concentrations achievable in the field as GSL concentrations in various Brassicaceae cultivars are high enough to release comparable ITC levels. The parent GSL of AITC, sinigrin, has been identified at concentrations as high as 90µmol g^{-1} dry weight (DW) in *B. juncea* leaves (Ngala, Woods and Back, 2015a) and 100µmol g^{-1} DW in *B. nigra* reproductive tissue (Bellostas, Sørensen and Sørensen, 2007). A number of other studies have noted lower concentrations between 15-35µmol g^{-1} DW depending on plant species and tissue type (Kirkegaard and Sarwar, 1998; Gimsing and Kirkegaard, 2006; Lord *et al.*, 2011; Neubauer, Heitmann and Müller, 2014).

Although there is a large range in sinigrin concentration between cultivars and studies, GSL concentrations above 13 μmol g^{-1} DW would produce a minimum of 50ppm AITC assuming a 1% GSL to ITC conversion (Morra and Kirkegaard, 2002). Gluconasturtiin has also been detected at concentrations able to release a minimum of 50ppm of its breakdown product, PEITC; this GSL is present between 14-53μmol g^{-1} DW in *R. sativus, B. napus, B. carinata, B. nigra* and *B. juncea* root tissue (Gimsing and Kirkegaard, 2006; Bellostas, Sørensen and Sørensen, 2007; Ngala, Woods and Back, 2015a). Glucoraphanin, the parent GSL of SUL, has been identified in one study at 25.4μmol g^{-1} DW in *R. sativus* leaf samples (Ngala, Woods and Back, 2015a) however an earlier study identified it in *E. sativa* cultivars at concentrations <3 μmol g^{-1} DW (Lord *et al.*, 2011) suggesting that the ability to release SUL levels comparable to those in the current study depends on the plant species. There is little information on the parent GSLs of several ITCs in this study therefore further work is required to determine if the concentrations tested can be released from biofumigants.

3.4.2. Isothiocyanates and juvenile mortality

The ITCs tested had varying effects on *G. pallida* and *G. rostochiensis* J2 mortality. AITC was found to be the most toxic as it resulted in significant levels of mortality at all concentrations; total J2 mortality occurred for both species after only 24hrs exposure to 50ppm AITC. This is consistent with previous work performed with the AITC precursor, sinigrin, whose breakdown products had a similar effect on *G. rostochiensis* J2 *in vitro* (Buskov *et al.*, 2002).

PEITC increased *G. pallida* and *G. rostochiensis* J2 mortality, independent of concentration, suggesting that its release could control PCN levels across this range of concentrations. These results are similar to a previous study where comparable concentrations of 2-phenylethyl GSL, the precursor to PEITC, released toxic breakdown products which led to 80% mortality of *G. rostochiensis* J2 after 72hrs exposure (Serra *et al.*, 2002). When concentration was increased to 300ppm and 1000ppm, mortality increased to 90% and 100% respectively suggesting that increasing PEITC concentration to higher doses than used in this study would have a much greater effect on PCN mortality.

EITC, BITC, MITC and SUL increased in effectiveness against *G. pallida* as concentration increased so high levels in the soil would be required for successful control. MITC also increased *G. rostochiensis* mortality as concentration increased suggesting a dose-response. In comparison to *G. pallida*, when *G. rostochiensis* J2 were exposed to BITC, mortality was increased but it was not dose-dependent. In a previous study 50ppm benzyl GSL, in the presence of myrosinase, only led to 25% *G. rostochiensis* mortality after 72hrs (Buskov *et al.*,

2002) compared to the 76% mortality in the current study. This is likely due to inefficient GSL to ITC conversion breakdown leading to lower BITC release so a direct comparison cannot be made in the absence of BITC concentration data.

With the exception of the previously discussed study regarding potential SUL release from *R. sativus* leaves (Ngala, Woods and Back, 2015a), common biofumigant cultivars do not contain high enough concentrations of GSLs to release over 50ppm of EITC, BITC, MITC or SUL (Kirkegaard and Sarwar, 1998; Lord *et al.*, 2011; Pasini *et al.*, 2012; Bell, Oruna-Concha and Wagstaff, 2015). Due to this, the use of these ITCs as PCN biofumigants is limited and different plant species containing high concentrations of the parent GSLs would need to be identified if wanting to utilise their toxic abilities.

PHITC and PITC were only effective against *G. pallida* at 50ppm and the resulting mortality was not as high as after other ITC exposures. PITC had no effect on *G. rostochiensis* mortality. Only the highest BUITC concentration increasing *G. pallida* mortality and none of the IITC treatments had an effect on either species. This is the first study looking at the effect of these pure ITCs on PCN mortality. The effect of BUITC and IITC on fungal pathogens have been studied. Pure BUITC suppressed *Fusarium oxysporum* (Smolinska *et al.*, 2003) and butyl GSL-containing biofumigant material suppressed *Sclerotinia sclerotiorum in vitro* (Ojaghian *et al.*, 2012). Results from the current study indicate that BUITC is unlikely to be effective against encysted PCN but previous research suggests it may play a role in fungal suppression. In contrast, IITC was ineffective at reducing radial growth of various fungal potato pathogens *in vitro* (Taylor, Kenyon and Rosser, 2014) implying that IITC is not an effective biofumigant compound against common soil-borne potato pests. Due to the lack of positive results, in conjunction with the knowledge that these ITCs are not commonly released from biofumigant Brassicaceae cultivars (Fahey, Zalcmann and Talalay, 2001), they were discounted from further consideration as PCN biofumigants.

ITCs are known to be volatile and breakdown quickly (Brown *et al.*, 1991; Gardiner *et al.*, 1999; Morra and Kirkegaard, 2002; Gimsing and Kirkegaard, 2006), consequently higher control would be achieved by ITCs that affect mortality rapidly. In the mortality assays, there were variations in the time required to induce high rates of juvenile mortality, with dose in some cases impacting on this period. As a general rule, *G. pallida* mortality occurred earlier at higher doses; several ITCs deviated from this depending on their overall effectiveness. In contrast, rate of *G. rostochiensis* mortality was less affected by ITC treatment and dose with only the two most effective ITCs displaying dose-dependent mortality rate changes. AITC

showed the most promise as a control agent as it resulted in complete mortality of PCN J2 after only one day exposure at doses which can be achieved in field situations.

3.4.3. Isothiocyanates and encysted juvenile hatch

The cyst remains an effective protective structure for PCN. In comparison to the toxicity assays, the hatching assays indicated that ITCs were not effective at suppressing encysted J2.

With respect to *G. pallida* juvenile hatch, 25ppm and 50ppm AITC after 7 days exposure were the only treatments able to reduce hatch. *Globodera rostochiensis* hatch was only suppressed by PEITC at select concentrations after 7 and 10 days exposure and 50ppm MITC after 7 days exposure. AITC has previously been shown to reduce *G. pallida* hatch *in vitro* (Brolsma *et al.*, 2014) and plant material containing the parent GSLs of AITC and PEITC were identified as able to reduce *G. pallida* hatch *in vitro* (Ngala, Woods and Back, 2015a); the corresponding ITCs were not as successful in this study. Differences compared to Ngala *et al.*, (2015) is likely due to concentration differences as they used high concentrations (25-100% (w/v)) of freeze-dried plant extracts whilst the discrepancy between the study by Brolsma *et al.*, (2014) and the current study is potentially due to different exposure and hatching protocols.

Although unable to consistently reduce overall hatch, AITC and MITC did delay both *G. pallida* and *G. rostochiensis* hatch depending on exposure period. In general, high concentrations of AITC consistently delayed hatch (with the exception of 16 days exposure) whereas the higher the MITC concentration the later hatch occurred with exposure period playing a part in the extent of the delay. EITC also delayed *G. pallida* hatch independent of exposure period at high concentrations. Although the underlying cause of this transient nematostatic effect has not been investigated in this study, a delay in hatch could be attributed to direct paralysis of J2 or an indirect effect on the cyst or eggshell permeability.

The paralysis of free J2 has been noted in previous biofumigant studies after exposure to low concentrations (Ngala, Woods and Back, 2015a) or short exposure periods (Fatemy and Sepideh, 2016) of ITC-releasing plant material. There is the possibility that the effect of high concentrations which paralyse encysted J2 at long exposure periods may be reversible when the ITC presence is removed or when the paralysed J2 are exposed to stimulants in PRD which initiates processes that overcome paralysis. ITCs may also act indirectly on the cyst or eggshell permeability leading to a reduction in hatch. ITCs are known to interact with proteins by covalently modifying or bonding with amines, in particular cysteines (Drobnica, Kristián and Augustín, 1977; Brown and Hampton, 2011), and since the PCN cyst wall and eggshell both contain a large percentage of protein (72% and 59%, respectively; Clarke, Cox and

Shepherd, 1967; Clarke, 1968) they are vulnerable to attack by free ITCs leading to alterations in permeability. Further to this, it has been shown that protein modification by ITC interaction is reversible (Hinman *et al.*, 2006) and that ITC-cysteine complexes are water-soluble (Zheng, Kenney and Lam, 1992), allowing the release of the parent ITC (Brown and Hampton, 2011; Karlsson *et al.*, 2016) when stored in water for an extended period of time. This could explain a non-permanent effect on the cyst wall permeability through the disassociation of ITCs from proteins over time either due to extended time in water or due to the natural and rapid breakdown of ITCs. Although this delay in hatch didn't significantly reduce final hatch at these concentrations, it does suggest that certain ITCs have a nematostatic effect on PCN and this is explored further in Chapter 4.

This is the first study investigating the effect of BITC on PCN hatch. BITC had no effect on overall hatch of either species suggesting that it would be an ineffective encysted PCN hatch suppressor. Similarly, SUL had no effect on encysted *G. pallida*. Although no studies have been completed investigating the effect of pure SUL on PCN mortality and hatch, this result is inconsistent with an *in vitro* trial where *R. sativus* leaf material containing the parent GSL reduced *G. pallida* hatch (Ngala, Woods and Back, 2015a); it should be noted that high concentrations of material were used compared to the current study and complete hatch inhibition was not achieved. An earlier glasshouse trial determined that incorporating a *B. oleracea* cultivar containing a high concentration of the parent GSL for SUL significantly reduced the formation of new *G. rostochiensis* cysts (Aires *et al.*, 2009). Inconsistencies between results are most likely due to the initial SUL concentrations, the presence of other GSLs in the plant material, and the PCN species investigated. Hatching assay results for BITC and SUL contradict the toxicity assay suggesting an inability of the ITCs to pass through the cyst wall and eggshell. This is most likely due to the ITCs structures (see Figure 2.1), where BITC contains an aromatic ring and SUL has a long side chain; these attributes would restrict their movement into the cyst. Furthermore, neither ITC affected rate of hatch implying that they do not have a nematostatic effect at these concentrations.

A number of treatments increased hatch significantly compared to the controls. PEITC increased *G. pallida* hatch after 7 days exposure independent of concentration and MITC concentrations between 3.125-25ppm increased *G. rostochiensis* hatch after various exposure periods. This is consistent with a previous study which identified an increase in *G. rostochiensis* hatch when exposed to various green manures *in vitro* (Valdes *et al.*, 2011); the authors hypothesised that this was due to a change in eggshell permeability or priming of juveniles for hatch induced by ITC release. Due to the ability of these ITCs to induce free J2

mortality the dual capability of ITCs to prime J2 for hatching seems unlikely therefore the more probable reasoning is an interaction between these ITCs and the cyst wall proteins (as previously discussed) which alters the permeability in such a way that allows enhanced hatch. Although PEITC increased hatch, an effect was not noted at any other exposure period suggesting that between 4-7 days is required for this effect to be induced after initial exposure. In addition, the effect is likely to be transient as it was not noted after 10 days exposure implying that enhanced hatch is unlikely to occur in response to PEITC release from biofumigation in practice. Although MITC increased hatch, shorter exposure periods had less of an effect. This suggests that, unlike PEITC, the effect on *G. rostochiensis* is not transient and that extended time is required after initial exposure to enhance hatch. Due to this, an increase in hatch may not be seen under field conditions where the ITC would volatise into the atmosphere quickly (Brown *et al.*, 1991; Gardiner *et al.*, 1999) reducing contact time. Further studies are needed to confirm if this would indeed happen and if MITC release would have a negative impact on PCN control in the field.

Certain ITCs may have a bi-modal effect on encysted PCN. Enhanced *G. rostochiensis* hatch by MITC increased as concentration and exposure period increased indicating that MITC influences the cyst wall permeability long-term; in contrast, MITC also delayed hatch suggesting temporary paralysis of encysted J2. In the presence of MITC, J2 paralysis and cyst wall protein modifications could occur simultaneously. The removal of ITCs, or simulation of hatch, could then lead to a reversal of J2 paralysis and increased hatch due to the longer lasting cyst wall permeability alterations. MITC did not induce the same hatch enhancement in *G. pallida* with only a temporary delay in hatch suggesting differences in the cyst wall composition between species. Although the bi-modal effect in this case was disadvantageous, other ITCs able to paralyse J2 and alter the cyst wall permeability may interact with proteins in a way that suppresses hatch.

There were two instances where AITC and MITC treatment significantly increased *G. pallida* hatch as an isolated event. In the possibility that the increases were not related to an ITC effect, results could have been false positives. Cysts are known to have a high variation in the proportion of eggs ready to hatch between cysts (Jones and Jones, 1974; Antoniou, 1989). The significant increase in hatch with the AITC treatment could be due to low hatch from the control and high hatch from the treatment. The increase in hatch due to MITC addition would likely be due to a high number of viable eggs in the treated sample as the control hatch was not adversely low. A repeat of these treatments would be beneficial to distinguish between a true effect of ITC addition and a false positive due to variable cyst content.

Differing effects between treatments would suggest that ITCs vary in their ability and mode of action when interacting with encysted PCN. The majority of effects were transient with very little suppression still apparent four weeks after ITCs were removed. This would imply that concentrations lower than 50ppm are unlikely to have a long term effect on PCN in the field. In saying this, biofumigation is not applied directly before potato crops in practice therefore the *in vitro* and field conditions are not openly comparable and further experimentation is required.

3.4.4. Differences between encysted species

There were a few differences in overall ITC effectiveness between the two encysted PCN species. MITC had a greater effect on *G. rostochiensis* compared to *G. pallida*, albeit as a hatch enhancer rather than a hatch suppressor, and the effect of PEITC on PCN varied with an increase in *G. pallida* hatch compared to a decrease in *G. rostochiensis* hatch at select exposure periods. In addition to overall hatch, the delayed hatch effect of ITCs differed between species: high AITC concentrations affected the rate of *G. pallida* hatch at longer exposure periods than *G. rostochiensis*, whilst high MITC concentrations affected *G. rostochiensis* hatch at longer exposure periods than *G. pallida*. Although differences in overall ITC effectiveness and hatch delay would suggest that ITC toxicity is dependent upon the target species, neither encysted species were effectively controlled by ITC exposure. To date no studies have been completed comparing the effects of biofumigation and ITCs between PCN species although it has been noted that *G. rostochiensis* is more sensitive than *G. pallida* when exposed to organic soil amendments (Renčo and Kováčik, 2015) and the pesticide, oxamyl (Whitehead *et al.*, 1984). Further studies are required to confirm the species-specific ITC effects noted.

3.4.5. Concluding remarks

Several of the ITCs screened in the *G. pallida* toxicity assay have not been previously tested for PCN toxicity therefore this study was important in identifying ITCs that may not have previously been considered for control. Although ITCs increased free J2 mortality, none of the ITCs were consistently effective against encysted eggs. Further work is required to both understand and increase the suppressive effect of ITCs on PCN as these results, in particular the AITC and PEITC results, contradict previous studies. Several treatments enhanced PCN hatch which would be disadvantageous in the field and additional research is needed to determine the persistence of this negative effect. These outcomes have been further investigated in Chapter 4 where assay conditions have been: altered to more accurately reflect field conditions and optimised for maximum ITC toxicity.

Chapter 4. Further Investigating the Effect of Isothiocyanates on *G. pallida* Viability

Part of this chapter is included in a manuscript published in Nematology:

'Allyl isothiocyanate shows promise as a naturally produced suppressant of the potato cyst nematode, *Globodera pallida*, in biofumigation systems' (Wood, Kenyon and Cooper, 2017)

4.1. Introduction

Results from Chapter 3 demonstrated that although several isothiocyanates (ITCs) were able to induce potato cyst nematode (PCN) juvenile (J2) mortality when in the form of free J2, this effect was not maintained when J2 were encysted. Hatch was not suppressed effectively with only select AITC, PEITC and MITC treatments reducing *G. pallida* and *G. rostochiensis* hatch. This contradicts previous ITC and biofumigation *in vitro* studies which noted significant reductions in *G. pallida* hatch (Lord *et al.*, 2011; Brolsma *et al.*, 2014; Ngala, Woods and Back, 2015a).

Due to the lack of consistent PCN suppression it was decided that a number of experiments, both *in vitro* and glasshouse, were required in order to determine if altered assay conditions had an impact on the effectiveness of ITCs on encysted PCN. There was a focus on AITC as it had the greatest effect on PCN with respect to rate of hatch in Chapter 3 and has been extensively studied on a number of soil pests with positive results (Lazzeri, Tacconi and Palmieri, 1993; Donkin, Eiteman and Williams, 1995; Harvey, Hannahan and Sams, 2002; Zasada and Ferris, 2003; Yu *et al.*, 2005). In several of the experiments, BITC, PEITC or MITC were included in order to determine if the changed assay conditions impacted their ability to suppress PCN. As *G. pallida* is currently the predominant infective species in the UK, due to the introduction of *G. rostochiensis* resistant potato cultivars (Minnis *et al.*, 2002; Trudgill *et al.*, 2003), experiments were performed with this species alone.

The aims of this study were to:

- Determine if ITCs affect *G. pallida* hatch *in vitro* during hatch stimulation
- Understand how AITC affects *G. pallida* hatch and egg mortality when applied multiple times
- Investigate the effect of ITCs on *G. pallida* hatch when a delay is introduced between treatment and hatch stimulation to more accurately reflect the biofumigation process
- Study the effect of high AITC concentrations on encysted *G. pallida* hatch and mortality

- Examine the influence of temperature on AITC effectiveness on encysted *G. pallida* hatch and mortality in soil
- Determine if a combination of ITCs in soil increases their ability to reduce *G. pallida* hatch and multiplication in pot trials
- Investigate the effect of different soil types on the ability of AITC to reduce hatch, increase encysted egg mortality and reduce multiplication of *G. pallida* in a pot trial

4.2. Materials and Methods

4.2.1. Cyst hatching assays

Hatching assays were performed with batches of five *G. pallida* cysts as described in 2.3.2. Treatments consisted of AITC, BITC, PEITC or MITC at concentrations of 3.125-1500ppm for time periods of 1-16 days. Four replicates of each treatment were completed in each assay. A summary of alterations from the standard protocol is described in Table 4.1.

Section	ITC	Concentration (ppm)	Exposure (days)	Deviations
4.3.1	AITC BITC PEITC	12.5, 25, 50	1, 4	Exposed in PRD
4.3.2	AITC	3.125, 6.25, 12.5, 25, 50	4, 7, 10, 16	Cysts pre-soaked in H_2O for variable lengths of time[a] Treatments replenished every three days Stored for four months before staining
4.3.3	AITC	12.5, 25, 50	4, 7, 10	Initial batch of ten cysts Treatments replenished every three days Stored for four months before further analysis[b]
4.3.4	AITC BITC PEITC MITC	3.125, 6.25, 12.5, 25, 50	1, 4, 7	Stored for four weeks between exposure and counting
4.3.5	AITC BITC	3.125, 6.25, 12.5, 25, 50	1, 4, 7	Stored for four weeks in H_2O between treatment and counting
4.3.6	AITC	50, 100, 250, 500, 750, 1000, 1250, 1500	1	Stored for four weeks in H_2O between treatment and counting

Table 4.1. Deviations from the standard hatching assay protocol for each experiment. [a]Pre-soaked based on length of time in liquid before hatch stimulation (pre-soaking + ITC exposure period) so that total time was 20 days. [b]Cysts from the first hatching assay were split into two batches so half were stained and half were subjected to a second hatching assay.

4.2.2. Egg viability assays

After hatching assays in 4.3.2, 4.3.3 and 4.3.6, a Meldola's Blue Dye (MB) stain was used to determine the viability of unhatched J2 as described in 2.3.3. Four replicates of batches of five cysts were completed for each treatment. Due to an extended period of dry storage of cysts prior to staining of 4.3.2 and 4.3.3 cysts, these samples were pre-soaked in distilled water (dH_2O) for seven days before staining. The percentage of dead (unhatched nonviable J2), hatched (viable J2) and viable unhatched J2 were calculated. The number of hatched J2 in 4.3.2 was divided by two before calculating the percentage.

4.2.3. Microcosm assay

A soil microcosm experiment investigating the effect of temperature on AITC efficiency was set up in 50 mL Falcon tubes filled with 15g John Innes No. 2 soil. Batches of ten *G. pallida* cysts were exposed to 100-1500ppm AITC treatments in 1.5 mL volumes. Due to high levels of variation in the *in vitro* assays, six replicates of each treatment were completed when soil was introduced as a factor. Tubes were shaken to evenly distribute ITCs in the soil. Treated cysts were incubated at three temperatures: 10°C, 13°C and 17°C in the dark for four weeks. Cysts were removed and stored in dH_2O for four weeks prior to viability analysis. Hatching assays followed by MB staining as described in 2.3.2 and 2.3.3 were completed. The percentage of dead (unhatched nonviable J2), hatched (viable J2) and viable unhatched J2 were calculated.

4.2.4. Isothiocyanate combination pot trial

A pot trial investigating the effect of exposing encysted *G. pallida* to a mixture of AITC, BITC and PEITC was completed. Two muslin bags containing thirteen cysts each were placed in 2 L pots three-quarter filled with John Innes No. 2 soil at 5-10cm depths. Thirteen cysts were chosen to ensure a large number of newly formed cysts were collected in the control post-harvest. Treatments (Table 4.2) were incorporated in combinations at high (100ppm) and low (5ppm) concentrations in 50 mL volumes and pots were sealed with plastic wrap. Six replicates of each treatment were included and pots were set up in a randomised block design layout. After four weeks one cyst bag was removed and five cysts were subjected to a hatching assay as described in 2.3.2. Desiree potato tubers were planted in each pot containing the remaining cyst bag and grown to maturity as 2.4.3 to allow PCN multiplication. Newly formed cysts were collected as described in 2.6.1; new cysts were counted for each sample and an MB stain (2.3.3) was applied to a subsample of ten cysts to determine total number of new eggs. Due to issues with low hatch in the water controls the trial was completed twice.

Treatment Identifier	ITC Combination
C	H_2O
A	100ppm AITC
B	100ppm BITC
PE	100ppm PEITC
Abpe	100ppm AITC + 5ppm BITC + 5ppm PEITC
aBpe	5ppm AITC + 100ppm BITC + 5ppm PEITC
abPE	5ppm AITC + 5ppm BITC + 100ppm PEITC

Table 4.2. ITC treatments applied in the first ITC pot trial.

4.2.5. Isothiocyanate and soil type pot trial

A pot trial investigating the effect of AITC in three different soil types on *G. pallida* viability and multiplication was completed similar to 4.2.4. Two muslin bags containing ten cysts each were placed in 2 L pots filled with dry sandy silt loam, clay loam or sandy loam soil prepared as described in 2.4.4. AITC treatments (100, 500, 1000 and 1500ppm) were incorporated into each soil type in 50 mL volumes and pots were sealed. Six replicates of each treatment were included and pots were set up in a randomised block design layout. After four weeks one cyst bag was removed and subjected to a hatching assay and MB stain (2.3.2 and 2.3.3). Desiree potato tubers were planted in each pot containing the remaining cyst bag and grown to maturity as 2.4.3. Newly formed cysts were collected as described in 2.6.1. New cysts were counted and a hatching assay and MB stain was completed on a subsample of ten cysts to determine total number of new eggs and viability; when less than ten cysts were present the entire sample was analysed.

4.2.6. Data analysis

Data was analysed with one- or two-way ANOVA as in 2.10.1. Factors investigated and means comparison test used are listed in Table 4.3. Tukey's was applied when there were multiple factors involved or when a concentration effect required further analysis. Dunnett's was applied when results were discussed in relation to the control alone and a concentration effect was not evident.

Section	Factors	Means Test
4.3.1	Concentration	Dunnett's
4.3.2	Concentration	Tukey's
4.3.3	Concentration x Exposure Period	Tukey's & Dunnett's
4.3.4	Concentration	Dunnett's
4.3.5	Concentration	Dunnett's
4.3.6	Concentration	Tukey's
4.3.7	Concentration x Temperature	Tukey's
4.3.8	Treatment	Tukey's
4.3.9	Concentration x Soil Type	Tukey's

Table 4.3. Factors analysed and means comparison test applied in each experiment.

4.3. Results

4.3.1. Isothiocyanates and G. pallida *hatch during hatch stimulation*

The effect of AITC, BITC and PEITC on encysted *G. pallida* J2 when stimulated to hatch in conjunction with ITC exposure was investigated. After 1 day exposure none of the treatments significantly reduced hatch; 50ppm AITC delayed hatch (Figure 4.1). After 4 days exposure 50ppm AITC significantly reduced total hatch compared to the PRD control ($P<0.001$; Figure 4.2). The 25ppm AITC treatment delayed initial hatch by a week. BITC and PEITC treatments had no effect on hatch compared to the control at either exposure period.

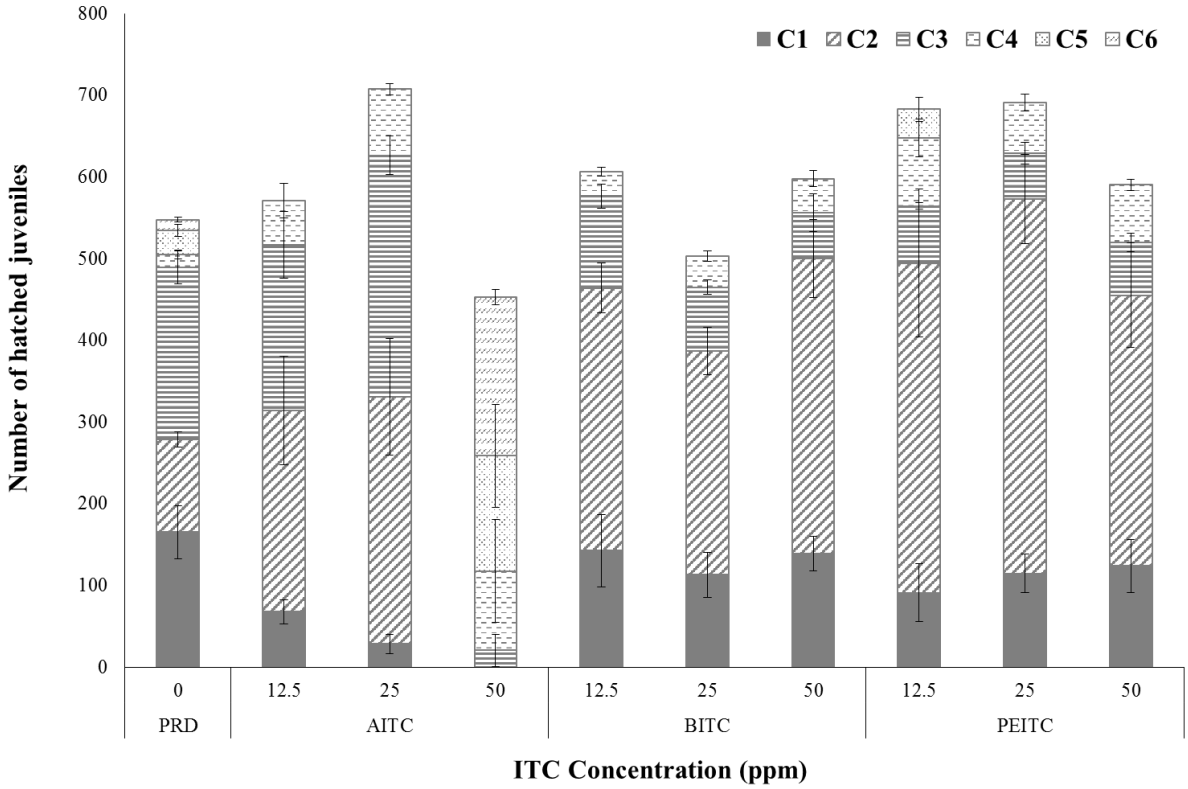


Figure 4.1. Hatch of *G. pallida* J2 when exposed to ITCs for 1 day during hatch stimulation. C1-C6 indicate weekly counts and error bars represent the standard error.

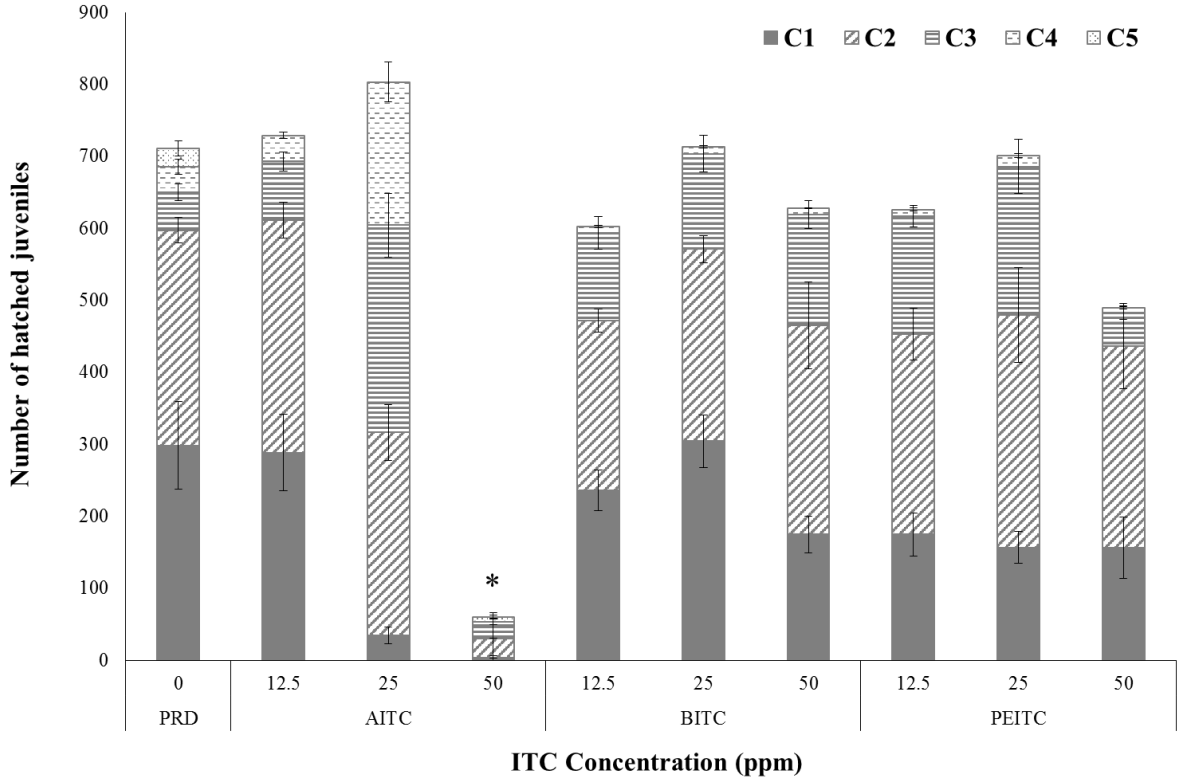


Figure 4.2. Hatch of *G. pallida* J2 when exposed to ITCs for 4 days during hatch stimulation. C1-C5 indicate weekly counts and error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

4.3.2. Replenishing AITC and encysted G. pallida *viability*

The effect of 3.125-50ppm AITC, replenished every 3 days during exposure, on *G. pallida* hatch and viability was investigated (Table 4.4).

After 4 days exposure (replenished on day 3) there were no differences between treatments with respect to the percentage of dead J2; 25ppm and 50ppm AITC significantly reduced the percentage of hatched J2 compared to the control (P<0.001). In addition, 50ppm treatment reduced hatched J2 compared to all other treatments except 25ppm AITC. When studying the percentage of unhatched viable J2, 25ppm and 50ppm treatments significantly increased the percentage compared to all other treatments (P<0.001).

After 7 days exposure (replenished on day 3 and 6) there were differences with respect to dead, hatched and unhatched viable J2 (P<0.001 for all three classifications). 12.5-50ppm AITC significantly increased the percentage of dead J2 compared to the control. In addition the percentage of dead J2 was higher in 50ppm treatments compared to the 3.125ppm and 6.25ppm AITC treated cysts. Hatch was significantly reduced when exposed to 12.5ppm and 25ppm AITC; 50ppm AITC completely inhibited hatch. Hatch from cysts exposed to 25ppm and 50ppm AITC was significantly lower than all other AITC treated cysts. The percentage of

unhatched viable J2 was higher after 12.5-50ppm exposure compared to the control. There were no differences in unhatched viable J2 between AITC treatments.

After 10 days exposure (replenished on day 3, 6, and 9) there was no effect of AITC on percentage of dead J2; there were significant differences in the percentage of hatched (P<0.001) and unhatched viable J2 (P=0.002). 25ppm AITC significantly reduced hatch compared to the control and 50ppm AITC completely inhibited hatch. In addition, hatch after exposure to these treatments was significantly lower than hatch after exposure to 3.125-12.5ppm AITC. With respect to the unhatched viable J2, percentages were significantly higher after treatment with 12.5-50ppm AITC compared to the control. There were no differences in unhatched viable J2 between AITC treatments.

After 16 days exposure (replenished on days 3, 6, 9, 12 and 15) there were significant differences in dead (P=0.021), hatched (P<0.001) and unhatched viable J2 (P=0.02) percentages between treatments and the water control but not between AITC treatments. 25ppm and 50ppm AITC increased mortality compared to the control. Hatch was significantly reduced after treatment with 3.125-25ppm AITC and 50ppm AITC completely inhibited hatch. The only treatments to show a significant difference in unhatched viable J2 compared to the control was 6.25ppm and 12.5ppm AITC.

Exposure Period	Concentration (ppm)	Percentage Total J2 (%)		
		Dead	Hatched	Unhatched
4 Days (replenished day 3)	**0**	8.10 (±2.20)	67.44 (±4.78)[a]	24.46 (±2.74)[a]
	3.125	12.76 (±3.38)	61.72 (±2.75)[a]	25.52 (±1.32)[a]
	6.25	8.13 (±2.21)	47.59 (±6.15)[ab]	44.28 (±5.07)[a]
	12.5	10.23 (±3.34)	45.36 (±14.67)[ab]	44.41 (±11.77)[a]
	25	11.03 (±1.44)	**15.84 (±7.16)[bc]**	**73.13 (±5.82)[b]**
	50	10.76 (±1.96)	**1.15 (±1.15)[c]**	**88.10 (±2.93)[b]**
7 Days (replenished day 3 and 6)	**0**	4.64 (±1.79)[a]	82.07 (±10.72)[a]	13.29 (±10.19)[a]
	3.125	9.66 (±5.69)[ab]	58.76 (±11.05)[ab]	31.57 (±10.45)[ab]
	6.25	12.63 (±1.52)[ab]	48.45 (±8.67)[ab]	38.91 (±8.26)[abc]
	12.5	**22.68 (±1.66)[bc]**	**26.21 (±11.74)[b]**	**51.11 (±10.43)[bc]**
	25	**22.69 (±4.06)[bc]**	**0.70 (±0.29)[c]**	**76.60 (±3.94)[bc]**
	50	**32.99 (±4.20)[c]**	**0.00 (±0.00)[c]**	**67.01 (±4.20)[bc]**
10 Days (replenished day 3,6 and 9)	**0**	23.02 (±13.38)	60.89 (±16.09)[a]	16.08 (±4.23)[a]
	3.125	38.61 (±3.62)	20.71 (±5.15)[a]	40.68 (±1.67)[ab]
	6.25	40.20 (±11.18)	16.16 (±8.30)[a]	43.64 (±10.86)[ab]
	12.5	35.32 (±5.94)	19.03 (±4.93)[a]	**45.65 (±5.46)[b]**
	25	37.96 (±7.21)	**0.04 (±0.04)[b]**	**62.00 (±7.20)[b]**
	50	47.24 (±2.67)	**0.00 (±0.00)[b]**	**52.76 (±2.67)[b]**
16 Days (replenished day 3, 6, 9, 12 and 15)	**0**	24.72 (±2.42)[a]	39.91 (±9.50)[a]	35.37 (±8.10)[a]
	3.125	34.08 (±2.88)[ab]	**7.17 (±5.63)[b]**	58.75 (±7.37)[ab]
	6.25	38.09 (±2.65)[ab]	**2.18 (±0.94)[b]**	**59.73 (±2.31)[b]**
	12.5	35.28 (±2.28)[ab]	**0.73 (±0.62)[b]**	**63.99 (±2.08)[b]**
	25	**43.38 (±5.40)[b]**	**0.05 (±0.05)[b]**	56.57 (±5.36)[ab]
	50	**40.47 (±3.94)[b]**	**0.00 (±0.00)[b]**	59.53 (±3.94)[ab]

Table 4.4. Percentage of *G. pallida* J2 that were; dead, hatched and unhatched viable after exposure to AITC for various exposure periods replenished every 3 days. The standard error is stated within brackets. Within columns and exposure periods, means followed by the same letter are not significantly different ($P<0.05$). Treatments statistically different to the exposure period control are highlighted in bold.

During the hatching assay, which preceded the application of the viability dye, differences in rate of hatch occurred depending on treatment and exposure period (Table 4.5). After 4 days exposure all AITC treatments delayed peak hatch by one week. After 7 days exposure there was a delay in peak hatch (where it occurred) after exposure to all AITC treatments; 3.125ppm AITC delayed hatch by a week and 6.25-25ppm AITC delayed hatch by three weeks. When exposure was increased to 10 days, majority hatch was delayed after exposure to the treatments which did not affect overall hatch. After exposure for 16 days the majority of hatch when exposed to 3.125 and 6.25ppm AITC was delayed.

Exposure Period	Concentration (ppm)	Percentage Total Hatch (%) C1	C2	C3	C4	C5
4 Days (replenished day 3)	**0**	**46.04**	42.13	7.29	4.54	-
	3.125	17.80	**59.91**	14.05	8.24	-
	6.25	5.30	**59.74**	17.83	17.13	-
	12.5	22.37	**57.45**	12.88	7.30	-
	25	2.12	**40.89**	20.81	36.18	-
	50	4.17	**66.67**	4.17	25.00	-
7 Days (replenished day 3 and 6)	**0**	**41.01**	34.96	18.20	5.83	-
	3.125	26.41	**30.40**	28.45	14.74	-
	6.25	11.96	29.82	20.30	**37.92**	-
	12.5	20.21	20.26	27.91	**31.63**	-
	25	42.50	5.00	7.14	**45.36**	-
	50	*	*	*	*	-
10 Days (replenished day 3,6 and 9)	**0**	**24.80**	24.74	15.27	20.79	14.40
	3.125	7.81	9.83	24.58	**32.94**	24.84
	6.25	9.32	20.47	**25.78**	21.68	22.76
	12.5	17.89	17.27	14.64	**32.69**	17.51
	25	**100.00**	0.00	0.00	0.00	0.00
	50	*	*	*	*	*
16 Days (replenished day 3, 6, 9, 12 and 15)	**0**	6.32	16.96	**33.46**	21.66	21.59
	3.125	3.62	7.97	17.54	**43.91**	26.96
	6.25	0.00	20.36	15.04	29.25	**35.36**
	12.5	**83.33**	16.67	0.00	0.00	0.00
	25	**100.00**	0.00	0.00	0.00	0.00
	50	*	*	*	*	*

Table 4.5. *Globodera pallida* hatch at each weekly count as a percentage of total hatch after exposure to AITC when replenished every 3 days. C1-C5 represents weekly counts. The count where peak hatch occurred is in bold. Dashed lines specify where no count was completed and asterisks fill treatments where no J2 hatched.

4.3.3. Is G. pallida *hatch suppression after replenishing AITC transient?*

Following on from the experiment in 4.3.2, where hatch was suppressed but a large number of unhatched J2 remained viable after treatment, an experiment was set up to investigate whether this hatch suppression was transient or permanent. Cysts were exposed to 12.5-50ppm AITC treatments replenished every 3 days. A hatching assay was performed, cysts were stored for four months and a viability stain or second hatching assay occurred.

During the initial hatching assay, there was a significant effect of each factor individually but there was no interaction between the two (Concentration $P<0.001$, Exposure Period $P=0.011$). 50ppm AITC at all exposure periods and 25ppm AITC after 7 days exposure significantly reduced hatch compared to the controls (Figure 4.3). Hatch after exposure to 50ppm AITC was significantly lower than after exposure to all other AITC treatments when comparing within the same exposure period.

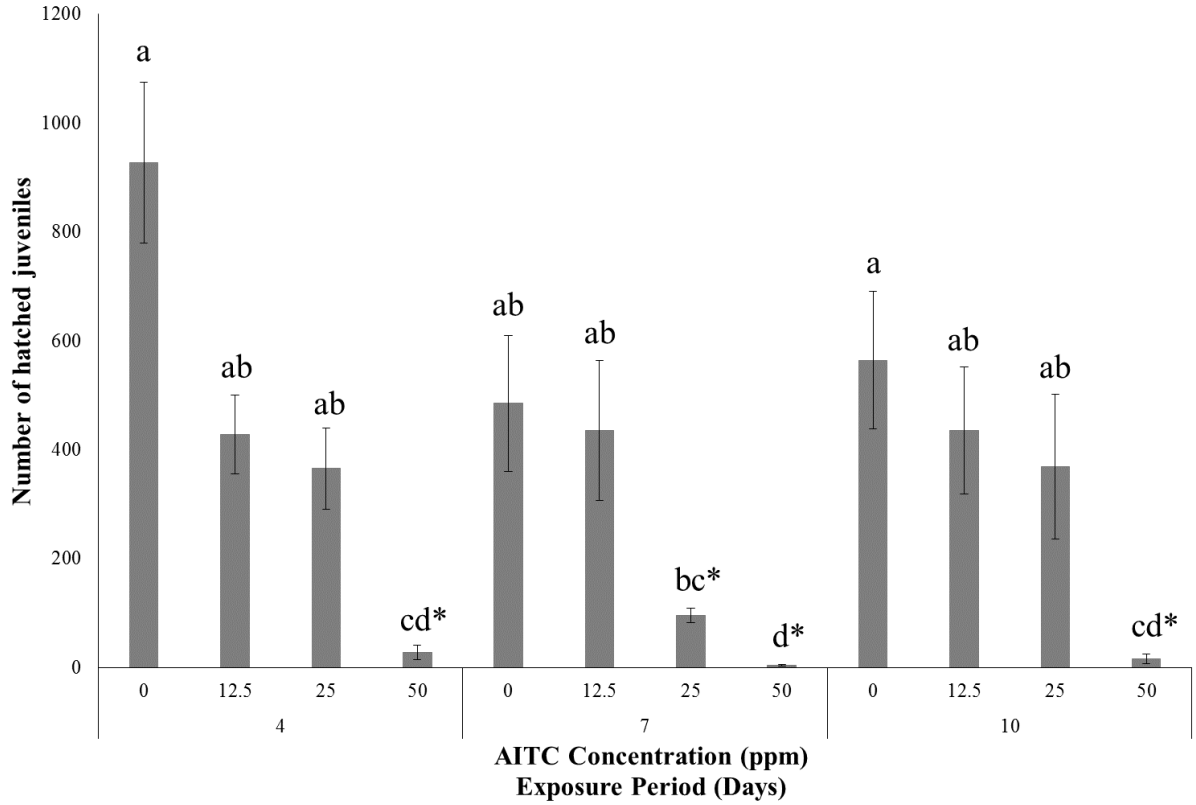


Figure 4.3. Total hatch of *G. pallida* J2 when exposed to AITC for various exposure periods replenished every 3 days. Error bars represent the standard error. Significant differences (P<0.05) compared to the exposure period control are indicated by an asterisk. Significant differences between treatments are shown by different letters.

The viability of treated cysts following the hatching assay indicated that there were significant differences in the percentages of dead, hatched and unhatched viable J2 with respect to AITC concentration and in the percentage of unhatched J2 with respect to exposure period (Table 4.6). The percentage of dead J2 after treatment with 50ppm AITC for 4 days was significantly higher than the control. The percentage of hatched J2 was significantly lower after 50ppm AITC treatment at every exposure period. 25ppm AITC significantly reduced the percentage of hatched J2 after 4 and 7 days exposure (replenished every 3 days). No significant differences were noted within exposure periods when considering changes in the percentage of unhatched viable J2 in spite of an overall concentration and exposure period effect.

Exposure Period	Concentration (ppm)	Percentage Total J2 (%)		
		Dead	Hatched	Unhatched
4 Days (replenished day 3)	0	28.49 (±2.60)^{a}	42.40 (±7.29)^{a}	29.08 (±6.36)^{a}
	12.5	40.80 (±2.94)^{ab}	17.90 (±3.30)^{abc}	41.32 (±2.43)^{ab}
	25	45.78 (±4.69)^{ab}	**12.50 (±3.85)^{bc}**	41.69 (±4.73)^{ab}
	50	**55.54 (±1.31)^{b}**	**2.10 (±1.18)^{c}**	42.37 (±2.03)^{ab}
7 Days (replenished day 3 and 6)	0	27.28 (±9.13)^{a}	32.40 (±10.61)^{ab}	40.34 (±3.95)^{ab}
	12.5	41.74 (±4.29)^{ab}	19.10 (±4.68)^{abc}	39.16 (±4.14)^{ab}
	25	39.79 (±4.92)^{ab}	**5.60 (±0.97)^{c}**	54.64 (±5.67)^{b}
	50	42.73 (±4.45)^{ab}	**0.30 (±0.09)^{c}**	57.00 (±4.44)^{b}
10 Days (replenished day 3,6 and 9)	0	36.21 (±1.72)^{ab}	31.70 (±3.97)^{ab}	32.06 (±3.98)^{ab}
	12.5	34.45 (±3.92)^{ab}	19.10 (±3.75)^{abc}	46.47 (±1.93)^{ab}
	25	25.97 (±4.40)^{a}	23.70 (±9.35)^{abc}	50.32 (±6.99)^{ab}
	50	44.95 (±8.60)^{ab}	**1.10 (±0.65)^{c}**	53.97 (±9.16)^{ab}
ANOVA P-values				
Concentration		**0.002**	**<0.001**	**<0.001**
Exposure Period		0.125	0.120	**0.039**
C x EP		0.123	0.510	0.614

Table 4.6. Percentage of *G. pallida* J2 that were; dead, hatched and unhatched viable four months post-AITC exposure, replenished every 3 days, and associated ANOVA P-values. The standard errors are stated within brackets. Within columns, means followed by the same letter are not significantly different (P<0.05). Treatments statistically different to the exposure period control are highlighted in bold.

The addition of the hatched J2 from the second hatching assay to the initial number of hatched J2 showed that the hatch suppression was not transient (Figure 4.4). When considering the total number of hatched J2 over both hatching assays, there was a significant effect of each factor individually but no interaction between the two (Concentration P<0.001, Exposure Period P=0.002). 50ppm AITC at all exposure periods and 25ppm AITC after 7 days exposure significantly reduced total hatch compared to the controls. Hatch after exposure to 50ppm AITC was significantly lower than all other AITC treatments, except 25ppm AITC after 7 days exposure, when comparing within the same exposure period; this is the only difference in total hatch which differs from the initial hatching assay alone.

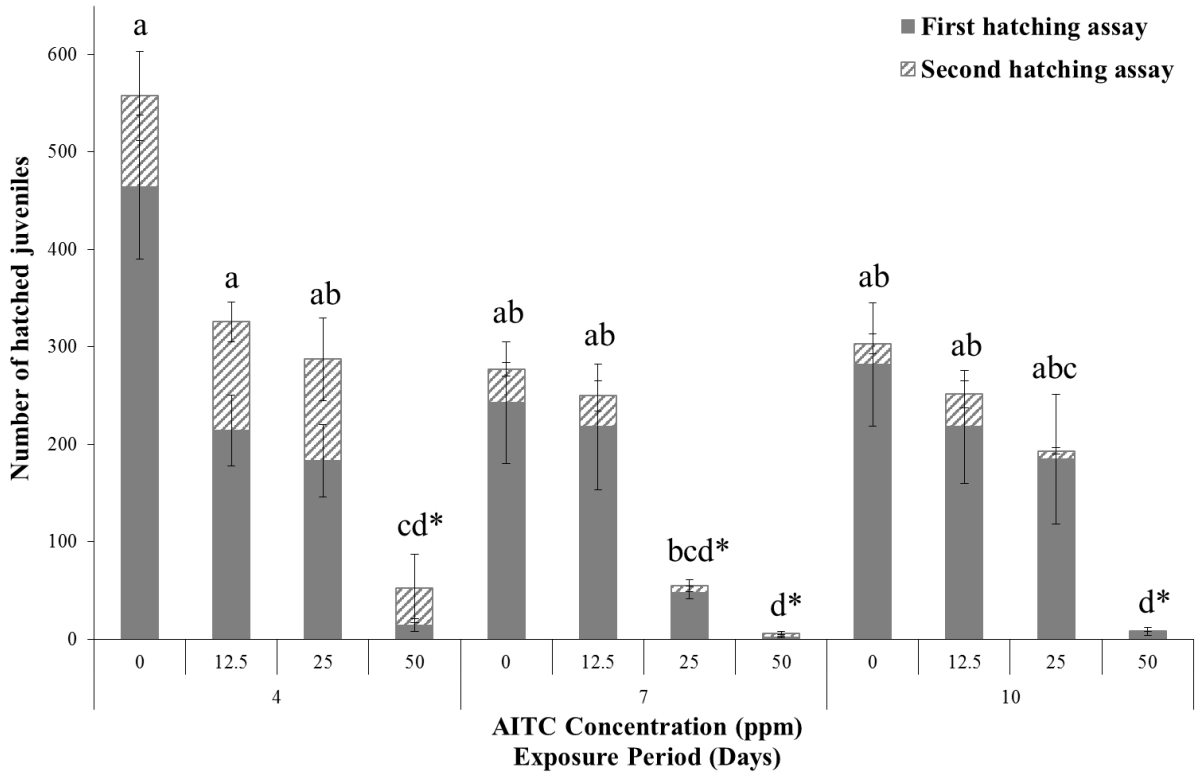


Figure 4.4. Total hatch of *G. pallida* J2 from two hatching assays combined when exposed to AITC replenished every 3 days. The first hatching assay was completed immediately after exposure and the second was completed four months later. Error bars represent the standard error. Significant differences (P<0.05) compared to the exposure period control are indicated by an asterisk. Significant differences (P<0.05) between treatments are shown by different letters.

4.3.4. Delaying G. pallida *hatch stimulation*

Hatching assays were performed with AITC, BITC, PEITC and MITC with a delay of four weeks introduced before hatching to determine if extended time after ITC exposure increased ITC-related *G. pallida* hatch suppression.

AITC had no effect on total hatch after 1 day exposure (Figure 4.5). Treating cysts with 25ppm and 50ppm AITC delayed initial hatch until the third count. After 4 days exposure the only treatment to reduce hatch was 50ppm AITC (P<0.001; Figure 4.6); 25ppm and 50ppm AITC delayed hatch. After 7 days exposure, 50ppm AITC completely inhibited hatch (P<0.001; Figure 4.7) and 25ppm AITC delayed hatch.

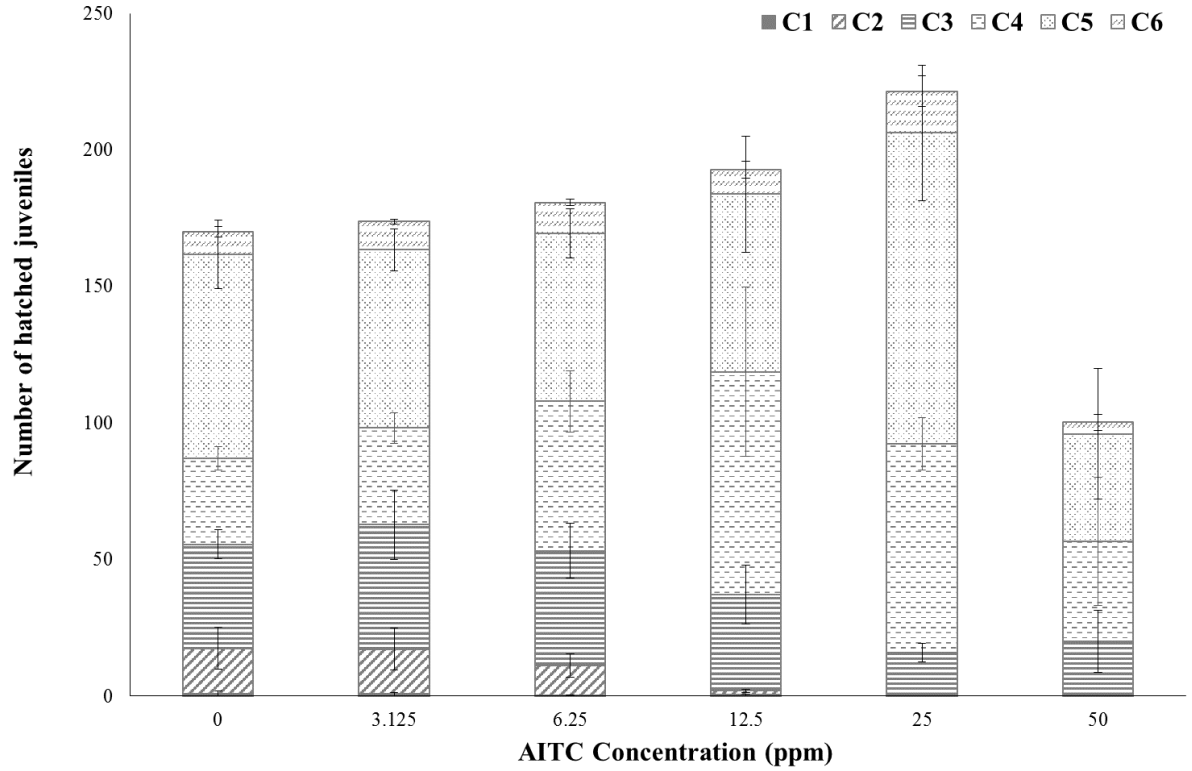


Figure 4.5. Hatch of *G. pallida* J2 following exposure to AITC for 1 day and storage for four weeks. C1-C6 indicates weekly counts and error bars represent the standard error.

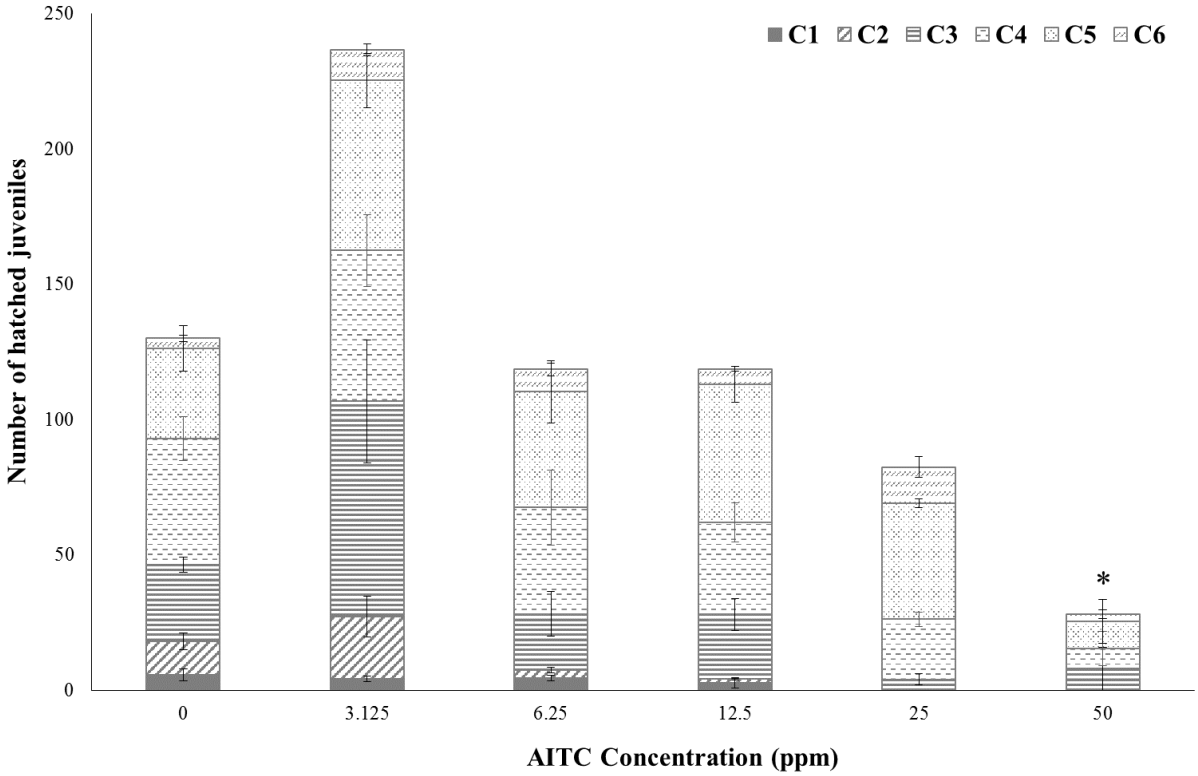


Figure 4.6. Hatch of *G. pallida* J2 following exposure to AITC for 4 days and storage for 4 weeks. C1-C6 indicates weekly counts. Error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

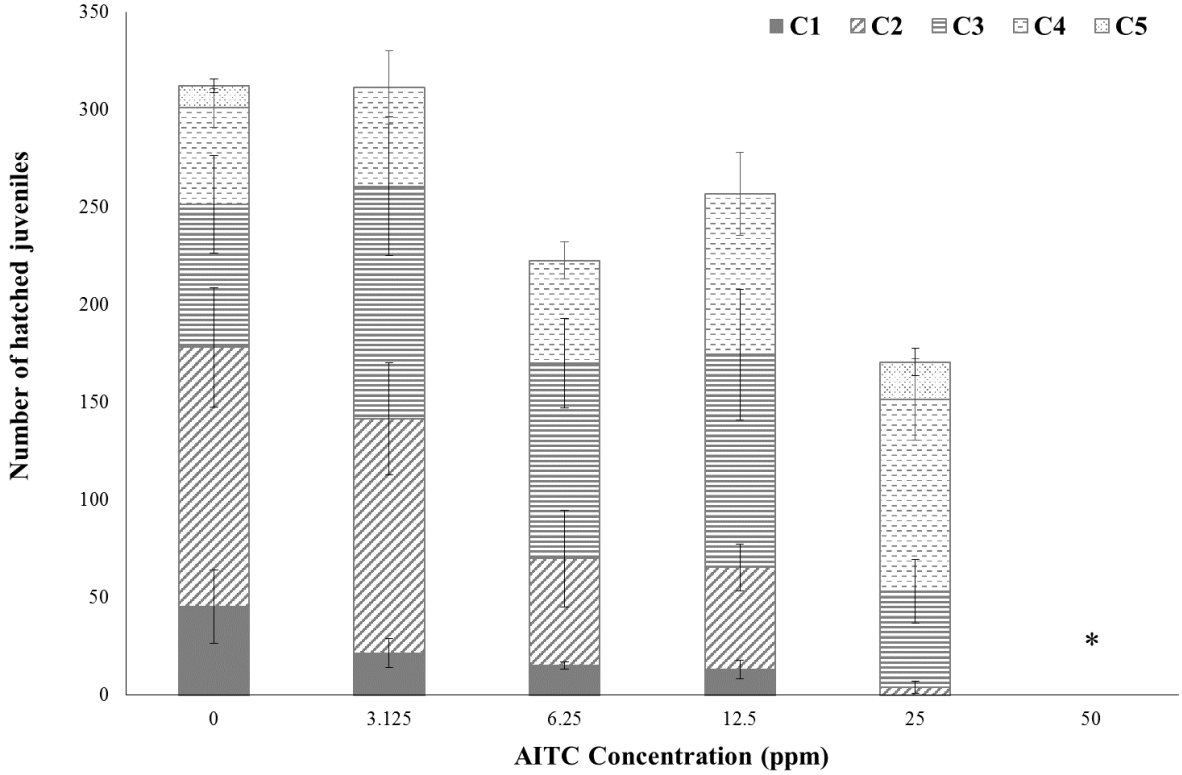


Figure 4.7. Hatch of *G. pallida* J2 following exposure to AITC for 7 days and storage for 4 weeks. C1-C5 indicates weekly counts. Error bars represent the standard error. Significant differences ($P<0.05$) in total hatch compared to the control are indicated by an asterisk.

BITC was not consistently effective at reducing hatch when introducing a delay before hatch stimulation. After 1 day exposure, only 3.125ppm BITC reduced hatch (P=0.035; Figure 4.8). When the exposure period was increased to 4 and 7 days, none of treatments had an effect on hatch (data not shown). BITC had no effect on rate of hatch.

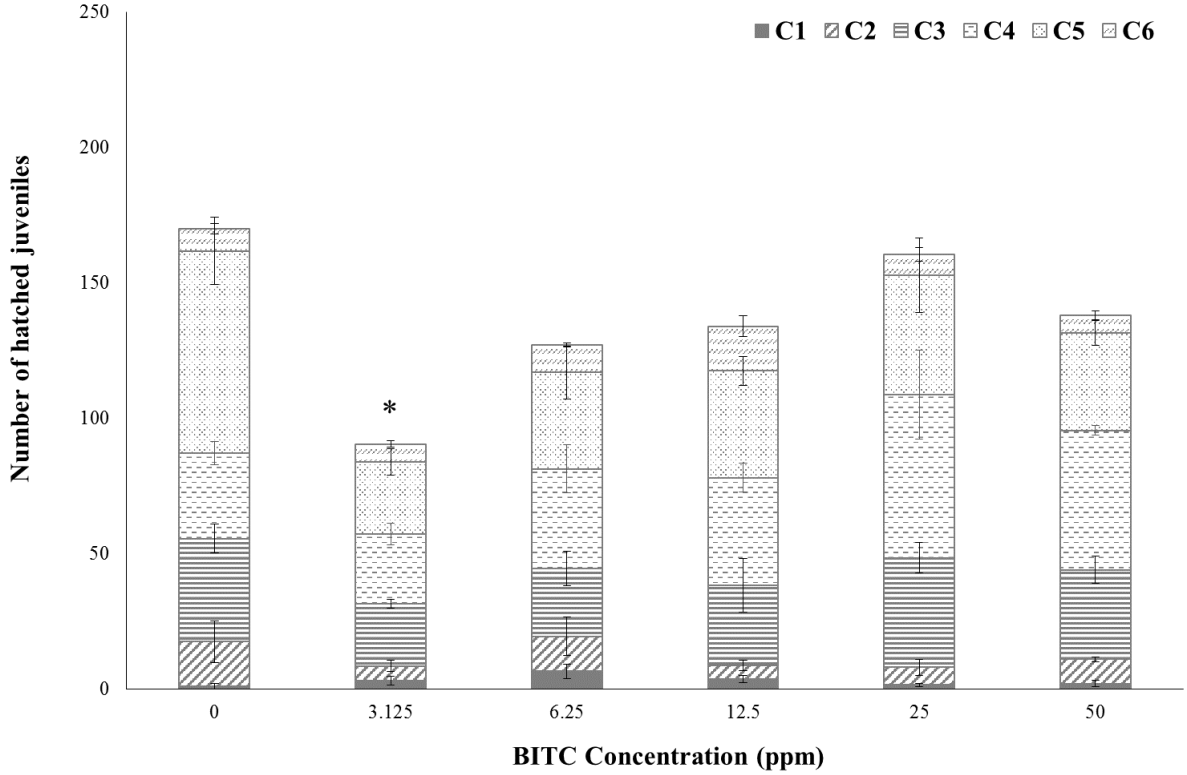


Figure 4.8. Hatch of *G. pallida* J2 following exposure to BITC for 1 day and storage for 4 weeks. C1-C6 indicates weekly counts. Error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

PEITC had no effect on *G. pallida* hatch when a delay was introduced between treatment and hatch stimulation. Rate of hatch was unaffected. Data for 7 days exposure is shown (Figure 4.9) which is representative of the data (not shown) obtained from the 1 and 4 days exposure periods.

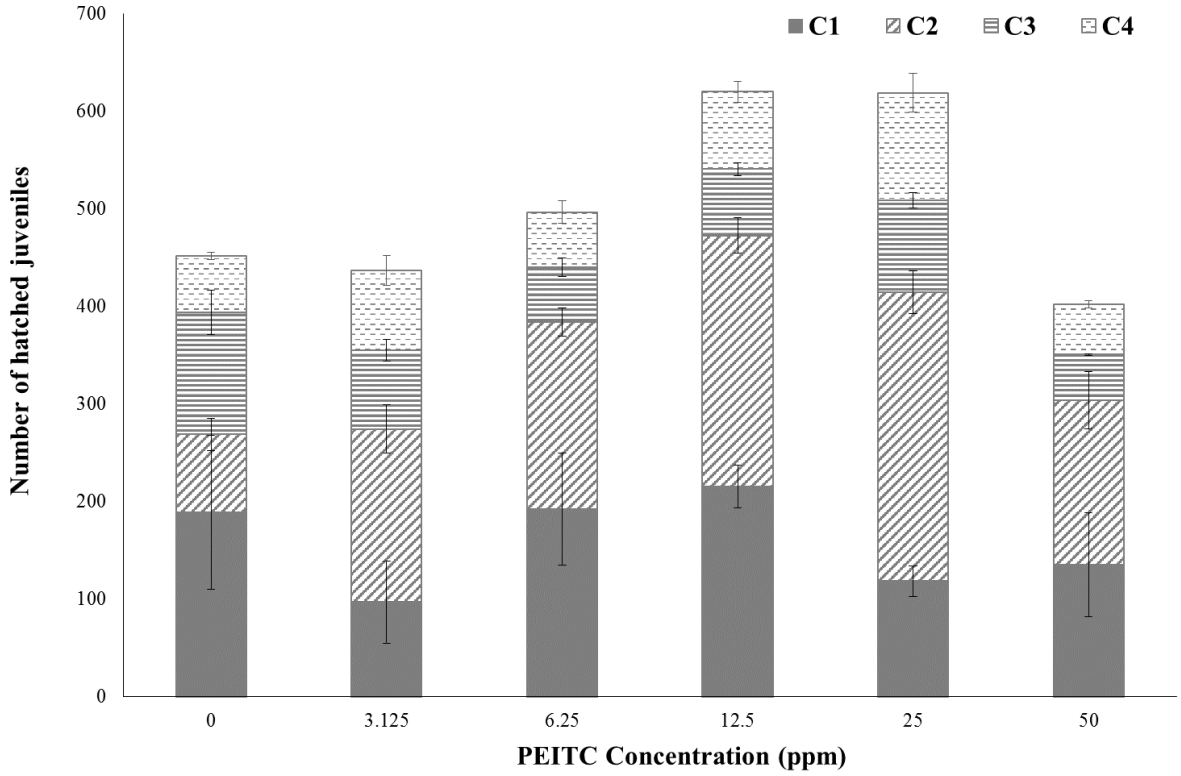


Figure 4.9. Hatch of *G. pallida* J2 following exposure to PEITC for 7 days and storage for 4 weeks. C1-C4 indicates weekly counts. Error bars represent the standard error.

Treatment with MITC did not result in a reduction in hatch from *G. pallida* cysts when a delay was introduced between exposure and hatch stimulation. Several treatments did significantly increase hatch compared to the control. After 1 day exposure, 12.5ppm MITC increased hatch compared to the control ($P<0.001$; Figure 4.10); exposure to 25ppm and 50ppm MITC resulted in delayed hatch. When the exposure period was increased to 4 days, 12.5ppm and 25ppm increased hatch compared to the control ($P<0.001$; Figure 4.11); hatch was delayed when cysts were exposed to 25ppm and 50ppm MITC. After 7 days exposure 25ppm MITC significantly increased overall hatch compared to the control ($P<0.001$; Figure 4.12). Treating cysts with 12.5-50ppm MITC affected rate of hatch at this exposure period.

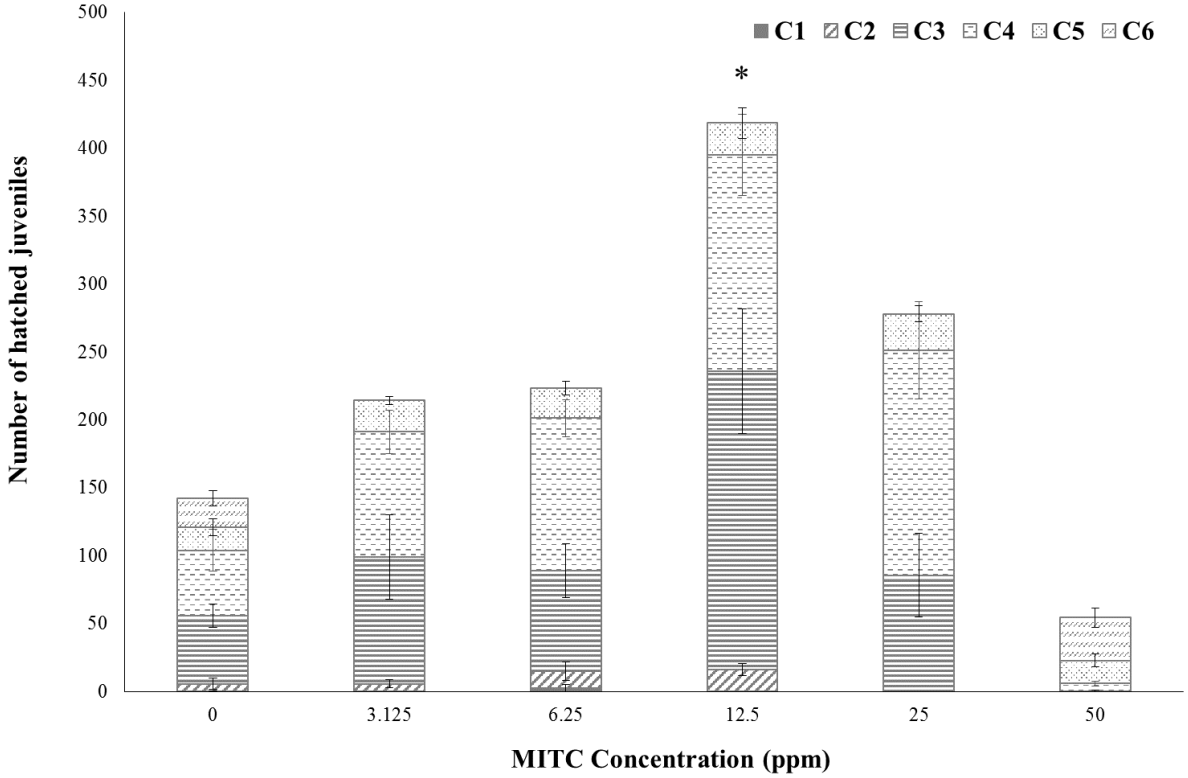


Figure 4.10. Hatch of *G. pallida* J2 following exposure to MITC for 1 day and storage for 4 weeks. C1-C6 indicates weekly counts. Error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

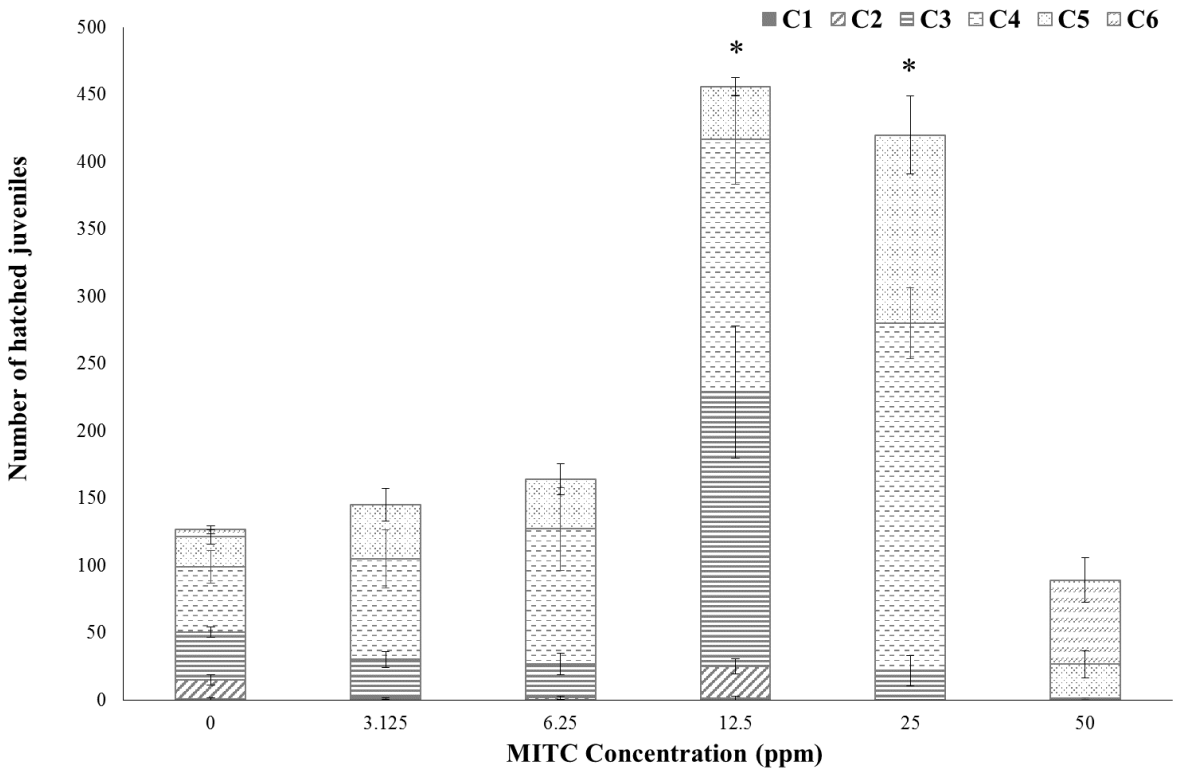


Figure 4.11. Hatch of *G. pallida* J2 following exposure to MITC for 4 days and storage for 4 weeks. C1-C6 indicates weekly counts. Error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

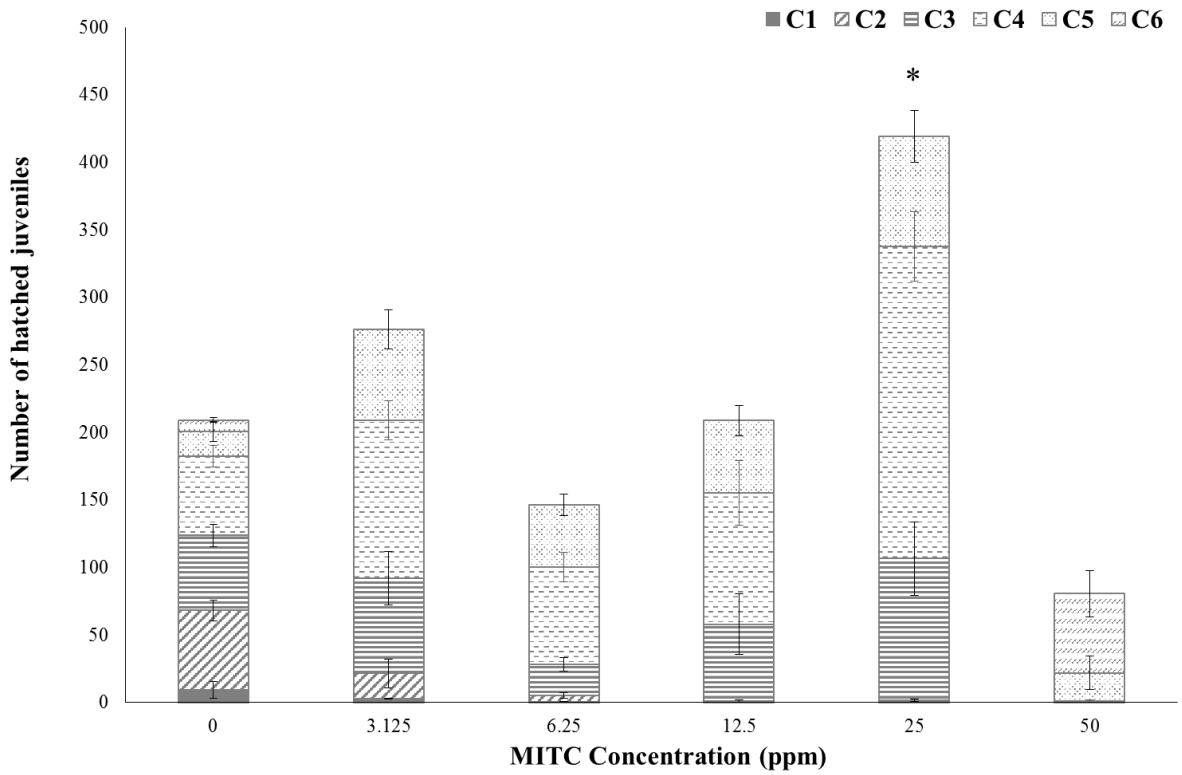


Figure 4.12. Hatch of *G. pallida* J2 following exposure to MITC for 7 days and storage for 4 weeks. C1-C6 indicates weekly counts. Error bars represent the standard error. Significant differences ($P<0.05$) in total hatch compared to the control are indicated by an asterisk.

4.3.5. Delaying G. pallida *hatch stimulation in water*

Following on from the previous experiment, hatching assays were performed with AITC and BITC with a delay of four weeks in water introduced between exposure and hatching in order to determine if the extended time after ITC application and the continued storage in water increased the ability of the ITCs to suppress *G. pallida* hatch.

After exposure to AITC for 1 day, the only concentration to reduce hatch was 50ppm AITC ($P=0.009$; Figure 4.13); hatch was delayed when exposed to 25ppm and 50ppm AITC. After 4 days exposure, 50ppm AITC significantly reduced overall hatch and delayed hatch ($P=0.017$; Figure 4.14). Although there was an overall effect of concentration on hatch after 7 days exposure ($P=0.025$), none of the AITC treatments significantly affected final hatch compared to the control (Figure 4.15). Hatch from cysts was delayed when exposed to 12.5ppm and 25ppm AITC for 7 days.

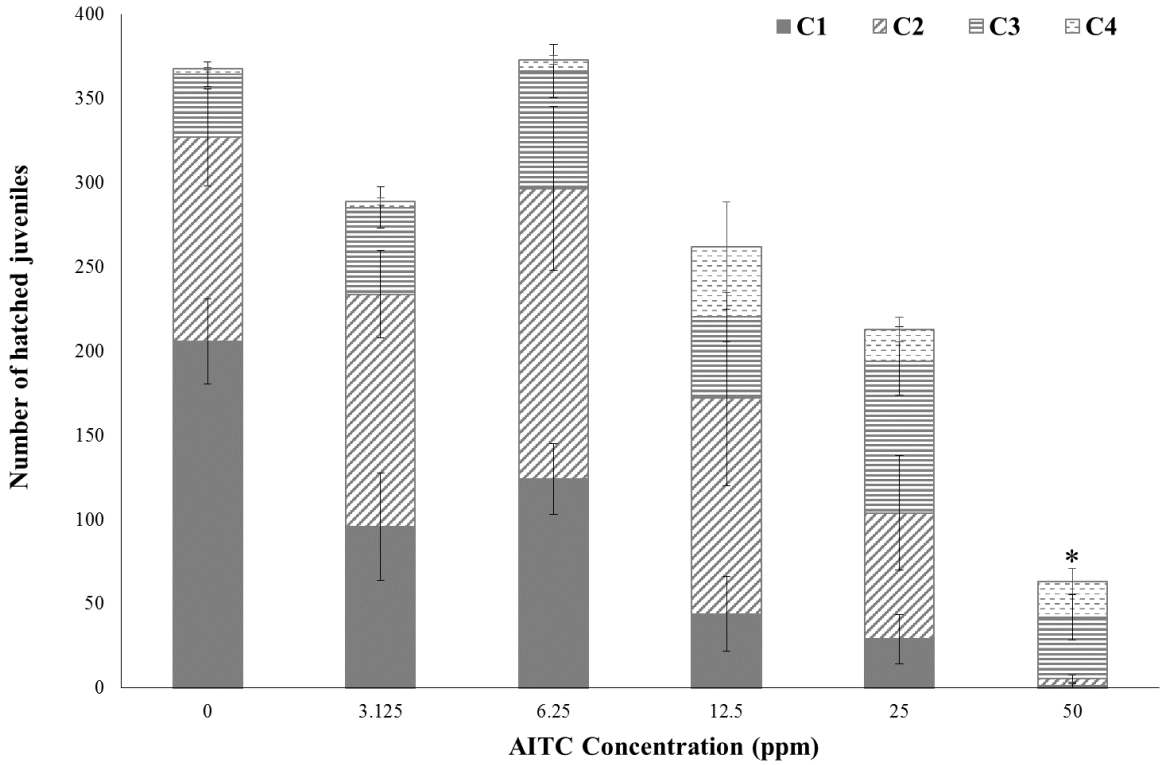


Figure 4.13. Hatch of *G. pallida* J2 following exposure to AITC for 1 day and storage in water for 4 weeks. C1-C4 indicates weekly counts. Error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

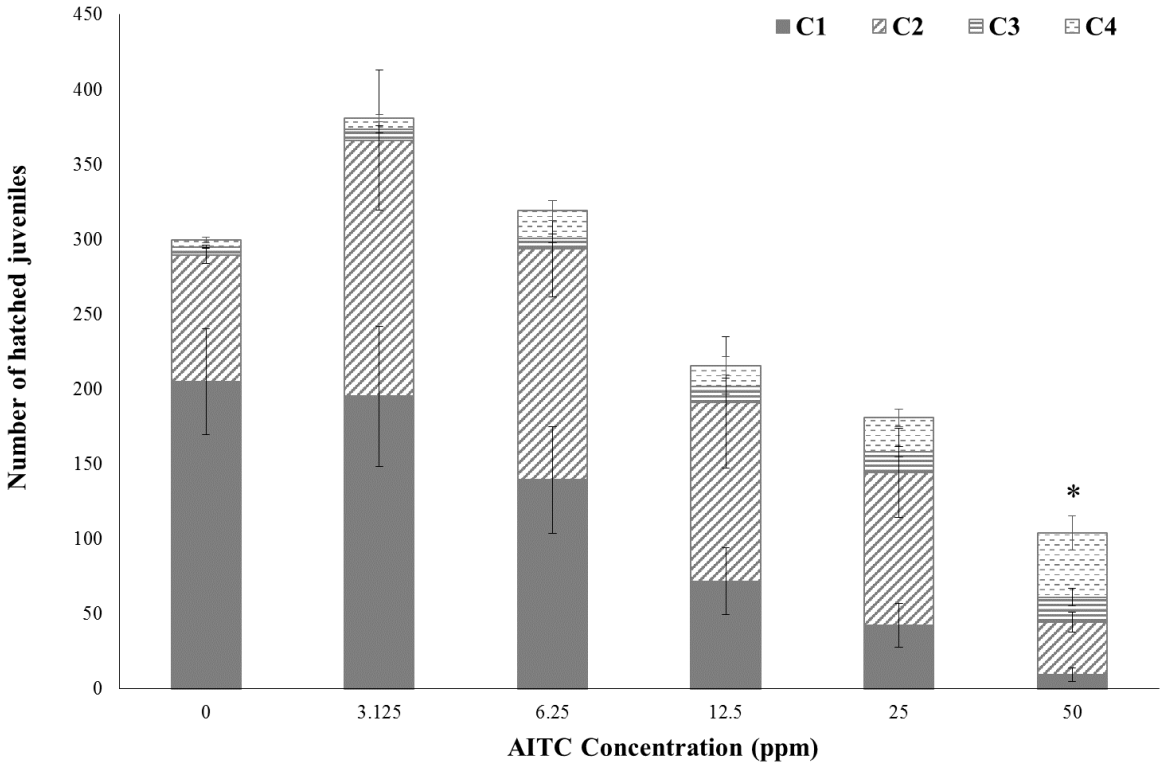


Figure 4.14. Hatch of *G. pallida* J2 following exposure to AITC for 4 days and storage in water for 4 weeks. C1-C4 indicates weekly counts. Error bars represent the standard error. Significant differences (P<0.05) in total hatch compared to the control are indicated by an asterisk.

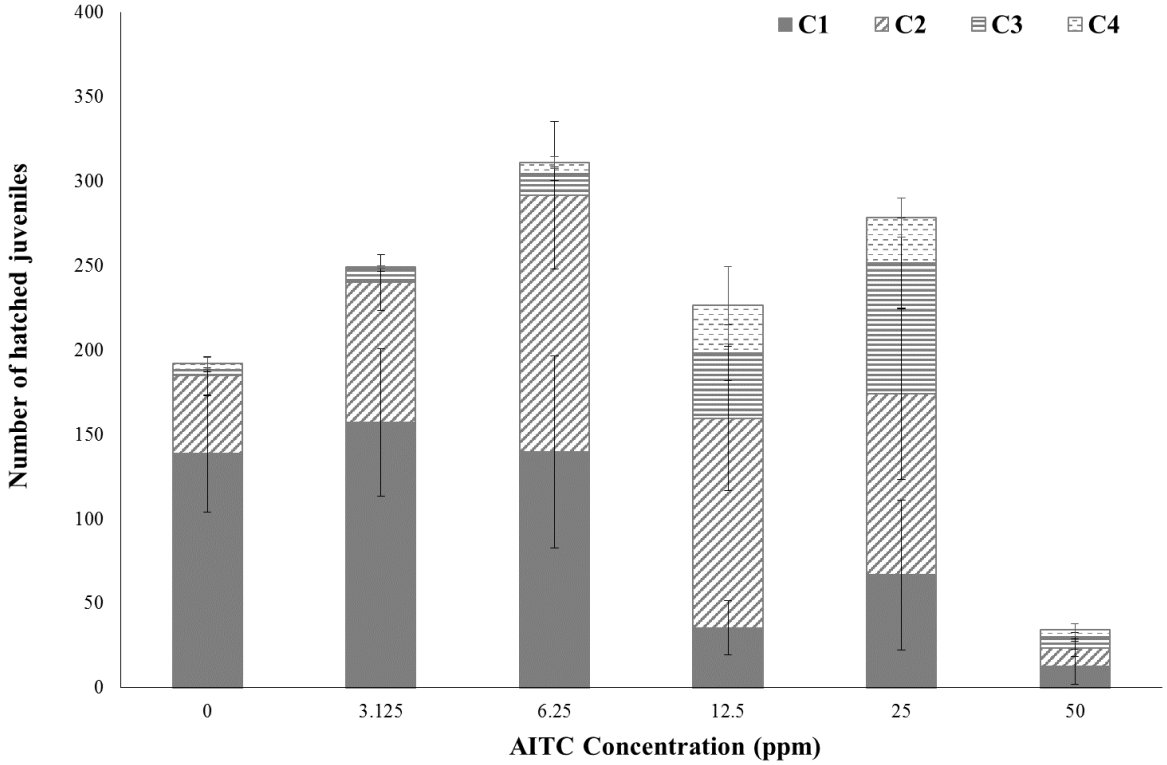


Figure 4.15. Hatch of *G. pallida* J2 following exposure to AITC for 7 days and storage in water for 4 weeks. C1-C4 indicates weekly counts. Error bars represent the standard error.

BITC had no effect on *G. pallida* hatch when cysts were stored in water between treatment and hatch stimulation. Data for 1 day exposure is shown (Figure 4.16) which is representative of the data (not shown) obtained from the later exposure periods of 4 and 7 days.

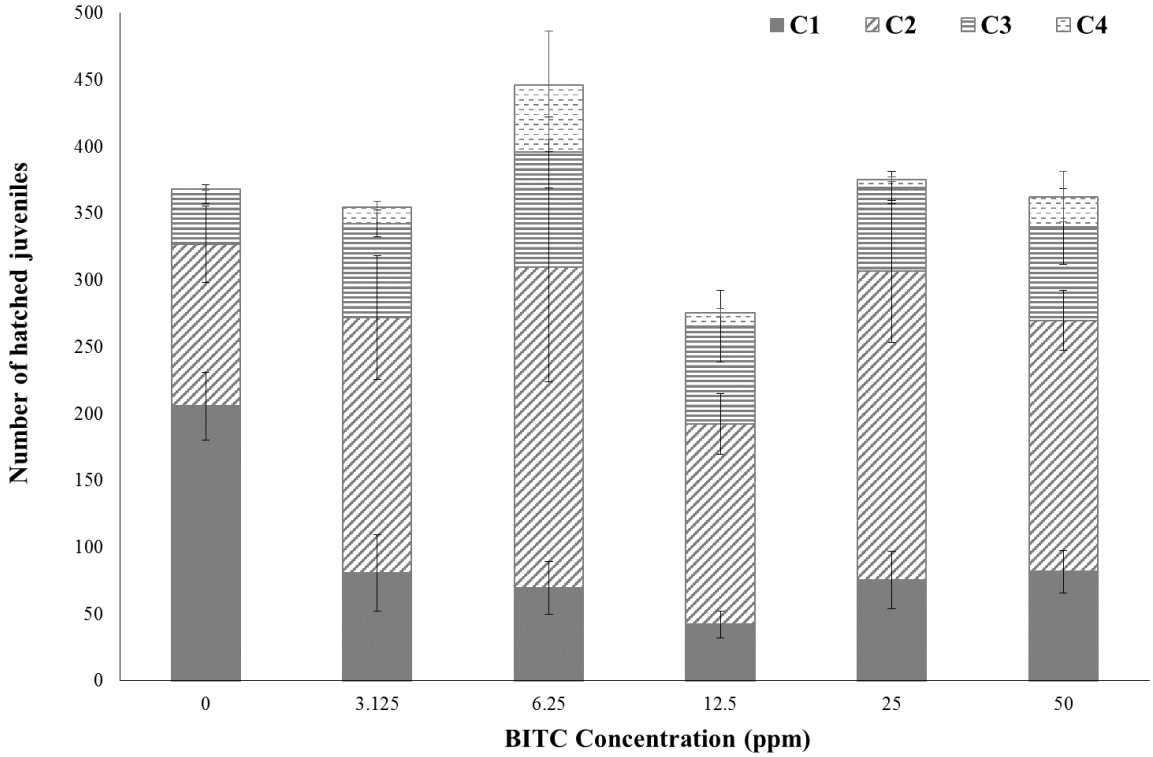


Figure 4.16. Hatch of *G. pallida* J2 following exposure to BITC for 1 day and storage in water for 4 weeks. C1-C4 indicates weekly counts. Error bars represent the standard error.

4.3.6. Increased AITC concentrations and encysted G. pallida *viability*

The effect of 50-1500ppm AITC on *G. pallida* hatch and viability after a 1 day exposure period was investigated. These concentrations were chosen due to previous experimentation which suggested that concentrations higher than those previously screened are required for consistent hatch suppression, especially at short exposure periods. Cysts were stored in water for four weeks between treatment and hatch stimulation. There were significant differences in dead, hatched and unhatched viable J2 percentages between treatments (P<0.001 for all classifications; Figure 4.17). All AITC concentrations, with the exception of 50ppm, significantly increased J2 mortality compared to the control. After exposure to 750ppm AITC the percentage of dead J2 was significantly higher than 250ppm AITC treated cysts. AITC concentrations of 1000-1500ppm significantly increased the percentage of dead J2 compared to 50-500ppm treatments. 50ppm AITC exposure significantly reduced hatch compared to the control and 100-1500ppm AITC exposure completely inhibited hatch. All AITC treatments above 100ppm significantly decreased hatch compared to the 50ppm treatment. AITC treatments of 50-500ppm significantly increased the percentage of unhatched viable J2 compared to the control and 50-250ppm AITC treatments significantly increased the percentage of unhatched viable J2 compared to 1000-1500ppm AITC treated cysts.

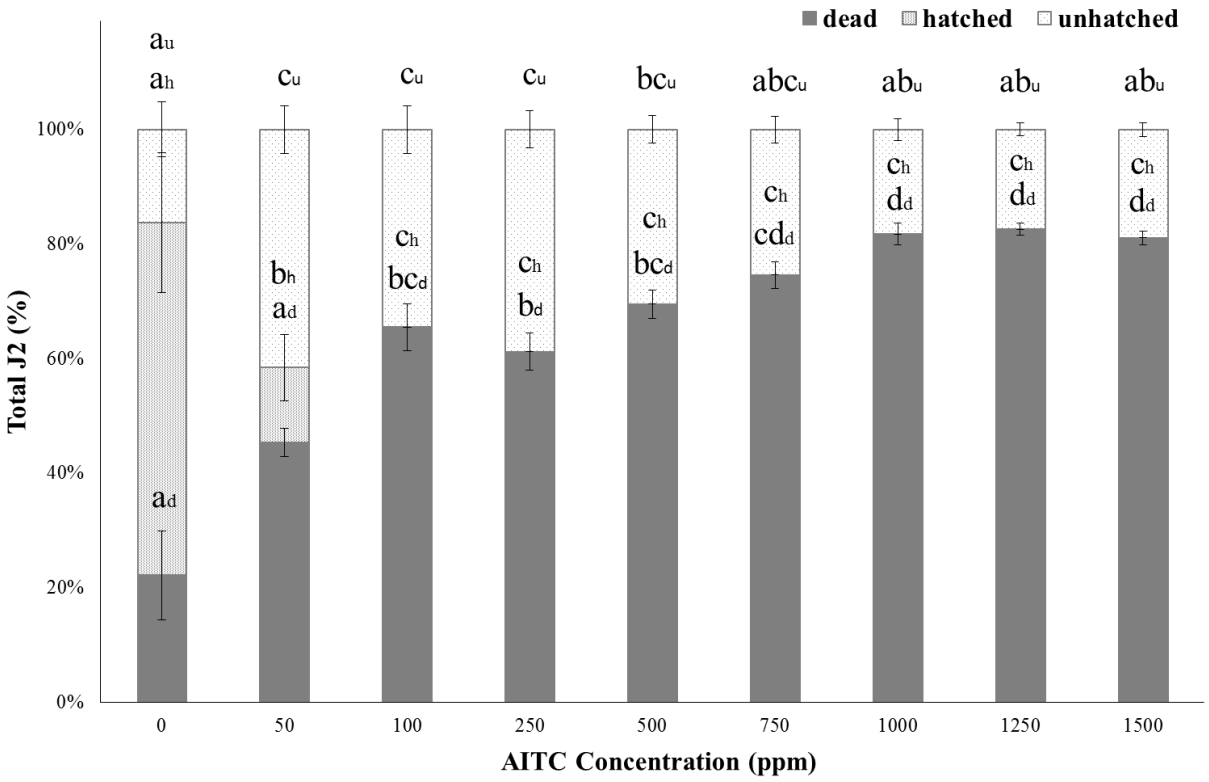


Figure 4.17. Percentage of *G. pallida* J2 that were: dead, hatched and unhatched viable after exposure to AITC for 1 day. Error bars represent the standard error. Significant differences (P<0.05) are indicated by different letters; the classification these are related to are indicated by the subscript letters: d (dead) h (hatched viable) u (unhatched viable).

4.3.7. The influence of temperature on AITC efficiency and encysted G. pallida *viability in soil*

AITC concentrations between 100-1500ppm were included in a soil microcosm assay examining the effect of AITC on encysted *G. pallida* viability after four weeks exposure in soil at three temperatures. There were significant differences in the percentage of dead, hatched and unhatched viable J2 when considering both concentration and temperature individually as well as their interaction (Table 4.7); the exception to this was the percentage of hatched J2, which was unaffected by temperature alone. In general, as AITC concentration increased, hatch decreased with a corresponding increase in mortality.

All AITC treatments significantly increased the percentage of dead J2 compared to the water controls, independent of temperature. In addition, there were significant differences in the percentage of dead J2 between AITC treatments depending on incubation temperature. When exposed at 10°C and 17°C, the percentage of nonviable J2 after 100ppm AITC treatment was lower than after exposure to all higher treatments. Cysts exposed at 13°C to 100ppm AITC displayed a lower percentage of dead J2 compared to 1000ppm and 1500ppm AITC treated cysts. Incubation temperature had little impact on AITC efficiency when comparing within

concentration although exposure of 500ppm AITC at 17°C led to significantly higher mortality than 500ppm AITC treatments at the lower temperatures.

Temperature had little influence on the impact of AITC when considering the percentage of hatched J2. When cysts were exposed to 100ppm AITC, only those exposed at 13°C showed a significant reduction in hatch compared to the control. When exposed to 500-1500ppm AITC, hatch was completely inhibited independent of temperature.

Cysts exposed to 500ppm AITC at 17°C displayed a significant reduction in unhatched viable J2 compared to the control and the 500ppm AITC treatments at 10 and 13°C. There were no other differences between temperatures within concentrations. There were no significant differences between AITC treatments when exposed at 10°C. When exposed at 13°C the percentage of unhatched viable J2 was lower in 1000ppm and 1500ppm treated cysts than in 100ppm and 500ppm AITC treated cysts. When the temperature was increased to 17°C, the only difference between treatments in the percentage of unhatched viable J2 was between 500ppm treated cysts and 100ppm treated cysts.

Concentration (ppm)	Temperature (°C)	Percentage Total J2 (%)		
		Dead	Hatched	Unhatched
0	**10**	33.67 (±3.44)^{a}	48.76 (±5.18)^{a}	17.58 (±2.20)^{abc}
	13	28.51 (±4.08)^{a}	46.91 (±4.91)^{a}	24.58 (±2.62)^{bc}
	17	34.41 (±3.90)^{a}	47.14 (±4.57)^{a}	18.45 (±2.71)^{abc}
100	**10**	**55.25 (±5.38)^{b}**	22.05 (±4.31)^{a}	22.70 (±4.53)^{abc}
	13	**62.17 (±5.30)^{b}**	**7.78 (±3.48)^{b}**	30.05 (±4.23)^{a}
	17	**70.72 (±3.82)^{bcd}**	13.43 (±4.17)^{a}	15.64 (±0.98)^{bc}
500	**10**	**79.38 (±1.60)^{cde}**	**0.00 (±0.00)^{c}**	20.62 (±1.60)^{abc}
	13	**69.40 (±3.89)^{bc}**	**0.00 (±0.00)^{c}**	30.60 (±3.89)^{a}
	17	**95.80 (±1.25)^{f}**	**0.00 (±0.00)^{c}**	**4.20 (±1.25)^{d}**
1000	**10**	**86.06 (±1.23)^{def}**	**0.00 (±0.00)^{c}**	13.94 (±1.23)^{bc}
	13	**84.37 (±1.46)^{cdef}**	**0.00 (±0.00)^{c}**	15.63 (±1.46)^{bc}
	17	**89.38 (±2.32)^{ef}**	**0.00 (±0.00)^{c}**	10.62 (±2.32)^{cd}
1500	**10**	**81.87 (±1.60)^{cdef}**	**0.00 (±0.00)^{c}**	18.13 (±1.60)^{abc}
	13	**83.98 (±3.58)^{cdef}**	**0.00 (±0.00)^{c}**	16.02 (±3.58)^{bc}
	17	**89.73 (±1.91)^{ef}**	**0.00 (±0.00)^{c}**	10.27 (±1.91)^{cd}
		ANOVA P-values		
Concentration		**<0.001**	**<0.001**	**<0.001**
Temperature		**<0.001**	0.060	**<0.001**
C x T		**0.012**	**0.01**	**<0.001**

Table 4.7. Percentage of *G. pallida* J2 that were; dead, hatched and unhatched viable post-AITC exposure for four weeks at different temperatures in a soil microcosm experiment and associated ANOVA P-values. The standard errors are stated within brackets. Within columns, means followed by the same letter are not significantly different (P<0.05). Treatments statistically different to the temperature control are highlighted in bold.

4.3.8. Isothiocyanates and encysted G. pallida *hatch and multiplication in soil*

A pot trial was performed in order to determine the effect of applying 100ppm AITC, BITC and PEITC, on their own or in combination with 5ppm of the other two ITCs, on encysted *G. pallida* in soil. Due to issues with hatch in the controls, the pot trial was duplicated.

None of the treatments significantly affected hatch (Figure 4.18). Data shown is from the second trial as cysts from the first trial exhibited little hatch so data could not be analysed (Appendix B).

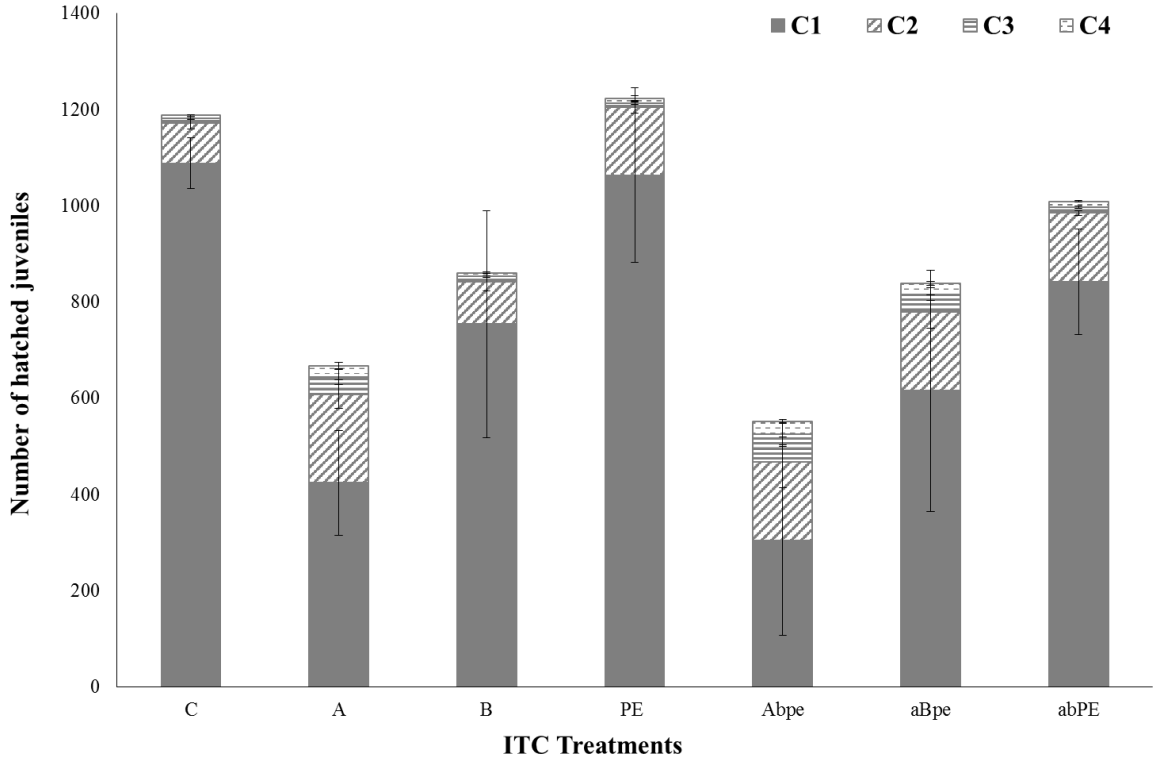


Figure 4.18. Hatch of *G. pallida* J2 following exposure to ITCs for four weeks in soil. Water control (C), AITC (A), BITC (B) and PEITC (PE). Upper case = 100ppm, lower case = 5ppm. C1-C4 indicates weekly counts. Error bars represent the standard error.

None of the treatments had an effect on the number of newly formed cysts sample^{-1}, eggs sample^{-1} or eggs cyst^{-1} post-multiplication (Table 4.8). Due to similarities in results only data from the first trial is shown; results from the replicate trial are in Appendix B.

Treatment	Cysts Sample^{-1}	Eggs Sample^{-1}	Eggs Cyst^{-1}
C	279.67 (±85.58)	40812.33 (±13609.55)	136.00 (±27.71)
A	164.50 (±46.94)	25842.00 (±7422.28)	143.00 (±22.88)
B	184.00 (±43.40)	33292.67 (±8402.2)	164.00 (±29.62)
PE	177.83 (±33.37)	27105.33 (±5775.99)	149.67 (±17.61)
Abpe	307.33 (±40.73)	53877.67 (±8787.39)	172.67 (±10.58)
aBpe	293.67 (±57.58)	46187.33 (±8092.84)	160.67 (±8.03)
abPE	122.83 (±42.92)	22711.60 (±8465.65)	163.20 (±42.08)

Table 4.8. The number of new cysts sample^{-1}, eggs sample^{-1} and eggs cyst^{-1} post-multiplication after ITC exposure. Water control (C), AITC (A), BITC (B) and PEITC (PE). Upper case = 100ppm, Lower case = 5ppm. The standard errors are indicated within brackets.

4.3.9. The influence of soil composition on AITC efficiency and encysted **G. pallida** *viability and multiplication*

A pot trial was completed in order to determine the effect of exposing cysts to 100-1500ppm AITC in three soil types: sandy silt loam, sandy loam or clay loam soil.

In general, as AITC concentration increased, hatch decreased with a corresponding increase in mortality (Table 4.9). Soil type had no overall effect on AITC efficiency and there was no effect of the interaction between concentration and soil type.

Several AITC treatments increased the percentage of dead J2; 500ppm AITC treatments in sandy and clay loam soil led to significantly higher mortality compared to the controls. After exposure to 1000ppm and 1500ppm AITC in all soil types, J2 mortality increased significantly. Cysts exposed to 1000ppm AITC in sandy loam soil contained a higher percentage of dead J2 compared to 100ppm AITC treated cysts. 1500ppm AITC in clay loam soil led to significantly higher mortality than cysts exposed to 100ppm AITC in clay loam soil. Soil type had no effect on the percentage of dead J2 within concentration.

Hatch was significantly reduced after treatment with 500-1500ppm AITC in all three soil types compared to the water control. In addition, AITC treatments between 500-1500ppm significantly reduced hatch compared to 100ppm AITC in sandy silt and sandy loam type soil. In clay loam soil, 500ppm and 1000ppm AITC treatments reduced hatch compared to 100ppm AITC treatments; 1500ppm AITC did not. Soil type had no effect on AITC-related hatch suppression within concentration.

The percentage of unhatched viable J2 in each sample was unaffected by both soil type and AITC concentration.

Concentration (ppm)	Soil Type	Percentage Total J2 (%)		
		Dead	Hatched	Unhatched
0	**Sandy Silt Loam**	63.44 (±9.03)^{ab}	21.90 (±8.68)^{a}	14.66 (±3.12)
	Sandy Loam	52.79 (±4.70)^{a}	33.61 (±4.33)^{a}	13.60 (±1.56)
	Clay Loam	56.23 (±4.27)^{a}	27.67 (±4.18)^{a}	16.11 (±3.09)
100	**Sandy Silt Loam**	75.96 (±5.72)^{abcde}	14.88 (±5.89)^{a}	10.14 (±2.08)
	Sandy Loam	61.42 (±12.22)^{abc}	22.72 (±15.14)^{a}	15.86 (±5.33)
	Clay Loam	70.66 (±4.99)^{abcd}	13.62 (±6.32)^{ab}	15.72 (±2.02)
500	**Sandy Silt Loam**	79.34 (±6.60)^{bcde}	**3.95 (±3.95)^{bc}**	16.72 (±4.37)
	Sandy Loam	**83.67 (±1.65)^{bcde}**	**0.02 (±0.02)^{bc}**	16.32 (±1.64)
	Clay Loam	**88.01 (±1.53)^{de}**	**0.00 (±0.00)^{c}**	11.99 (±1.53)
1000	**Sandy Silt Loam**	**86.86 (±0.81)^{cde}**	**0.00 (±0.00)^{c}**	13.14 (±0.81)
	Sandy Loam	**86.53 (±4.27)^{de}**	**0.01 (±0.01)^{c}**	13.46 (±4.27)
	Clay Loam	**82.26 (±4.83)^{bcde}**	**0.00 (±0.00)^{c}**	17.74 (±4.83)
1500	**Sandy Silt Loam**	**89.49 (±1.86)^{e}**	**0.00 (±0.00)^{c}**	10.51 (±1.86)
	Sandy Loam	**84.03 (±1.87)^{bcde}**	**1.50 (±1.50)^{bc}**	15.34 (±1.61)
	Clay Loam	**90.32 (±1.10)^{e}**	**0.02 (±0.02)^{bc}**	9.65 (±1.09)
		ANOVA P-values		
Concentration		**<0.001**	**<0.001**	0.483
Soil Type		0.197	0.533	0.561
C x ST		0.346	0.904	0.276

Table 4.9. Percentage of *G. pallida* J2 that were; dead, hatched and unhatched viable after exposure to AITC for four weeks in different soil types in a pot trial and associated ANOVA P-values. The standard errors are stated within brackets. Within columns, means followed by the same letter are not significantly different (P<0.05). Treatments statistically different to the soil type control are highlighted in bold.

In general, high concentrations of AITC significantly reduced the number of newly formed cysts and egg content post-multiplication (Table 4.10).

Concentration was the only factor to affect the number of cysts sample^{-1}. For all soil types, treatment with 500ppm and 1500ppm AITC led to a significant reduction in new cysts compared to the control. When treated with 1000ppm AITC, cyst numbers were significantly lower than the controls in the clay loam soil but not the sandy silt loam soil type. 500ppm and 1000ppm AITC in sandy loam soil completely inhibited the formation of new cysts. The number of cysts collected was significantly lower after treatment with 1500ppm AITC than after treatment with 100ppm AITC in sandy silt loam soil.

There was an effect of concentration and the interaction between concentration and soil type on egg number and an effect of concentration on viable egg number in each sample and cyst.

All cysts collected after exposure to 500-1500ppm AITC, with the exception of 500ppm AITC in clay loam soil and 1000ppm AITC in sandy soil loam soil, were empty. 500ppm AITC in clay loam soil significantly reduced the number of eggs sample^{-1}, eggs cyst^{-1}, viable eggs sample^{-1} and viable eggs cyst^{-1} compared to the controls. Empty cysts were significantly different to 100ppm AITC treated cysts, independent of soil type, with respect to egg content and viability. Cysts treated with 1000ppm AITC in sandy silt loam soil contained a lower egg content than the 100ppm AITC treated cysts in this soil type.

Concentration (ppm)	Soil Type	Cysts Sample^{-1}	Eggs Sample^{-1}	Eggs Cyst^{-1}	Viable Eggs Sample^{-1}	Viable Eggs Cyst^{-1}
0	**Sandy Silt**	52.00 (±16.12)^{a}	7837.20 (±2229.46)^{a} b	129.92 (±37.28)^{ab}	5424.40 (±1629.24)^{a} b	92.72 (±29.30)^{ab}
	Sandy Loam	73.67 (±28.17)^{a}	11178.80 (±4721.04)^{a}	130.17 (±15.95)^{a}	8083.13 (±3442.21)^{a}	87.83 (±18.32)^{a}
	Clay Loam	65.33 (±24.42)^{a}	13995.17 (±6286.96)^{a}	174.13 (±25.77)^{a}	11157.83 (±4730.87)^{a}	141.80 (±21.31)^{a}
100	**Sandy Silt**	10.67 (±2.68)^{ab}	1605.03 (±519.01)^{a}	147.18 (±23.46)^{a}	1223.37 (±466.80)^{a}	104.18 (±20.60)^{a}
	Sandy Loam	23.50 (±13.39)^{ab} c	4327.17 (±2348.07)^{a} b	125.87 (±42.31)^{ab}	3573.50 (±1974.88)^{a} b	101.54 (±33.55)^{ab}
	Clay Loam	4.67 (±2.82)^{abc}	779.97 (±481.72)^{abc}	70.03 (±34.64)^{ab} c	597.30 (±378.27)^{abc}	47.03 (±27.05) abc
500	**Sandy Silt**	**0.50 (±0.34)^{bc}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**
	Sandy Loam	**0.00 (±0.00)^{c}**	-	-	-	-
	Clay Loam	**0.17 (±0.17)^{c}**	**7.33 (±7.33)^{bc}**	**7.33 (±7.33)^{bc}**	**2.67 (±2.67)^{bc}**	**2.67 (±2.67) bc**
1000	**Sandy Silt**	0.67 (±0.33)^{abc}	28 (±28)^{bc}	28 (±28)^{bc}	25 (±25)^{bc}	25 (±25)^{bc}
	Sandy Loam	**0.00 (±0.00)^{c}**	-	-	-	-
	Clay Loam	**0.33 (±0.33)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**
1500	**Sandy Silt**	**0.33 (±0.33)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**
	Sandy Loam	**0.17 (±0.17)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**
	Clay Loam	**0.50 (±0.34)^{bc}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**	**0.00 (±0.00)^{c}**
ANOVA P-values						
Concentration		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Soil Type		0.071	0.870	0.860	0.746	0.694
C x ST		0.383	**0.025**	**0.026**	0.074	0.094

Table 4.10. New cysts sample^{-1}, eggs sample^{-1}, eggs cyst^{-1}, viable eggs sample^{-1} and viable eggs cyst^{-1} post-multiplication after exposure to AITC in three different soil types in a pot trial. The standard errors are indicated within brackets. Within columns, means followed by the same letter are not significantly different (P<0.05). Treatments statistically different to the soil type control are highlighted in bold.

4.4. Discussion

Results from this study show that AITC is able to suppress hatch and cause encysted J2 mortality both *in vitro* and in soil dependent upon concentration, exposure period and assay conditions.

4.4.1. AITC and delaying hatch stimulation

By including a delay between treatment and hatch stimulation, in order to more closely mimic the biofumigation method employed in the field, hatch was suppressed when exposed to 50ppm AITC. Hatch suppression was noted after 4 and 7 days exposure when cysts were left dry and after 1 and 4 days exposure when cysts were stored in water. Differences in AITC efficiency between dry and wet storage is potentially due to the effect of desiccation on encysted J2 after AITC exposure or differences in J2 state when stored dry or in moisture.

Increased desiccation of J2 can occur after eggshell permeability alteration or J2 activation during hatch stimulation (Ellenby, 1968; Perry, 1983). If AITC interferes with the eggshell permeability or attacks the J2 directly in a similar way as during hatch stimulation, desiccation could occur at a higher level than in the control leading to decreased hatch when stored in the absence of water for an extended period of time. This is evidenced after 4 and 7 days exposure of J2 to 50ppm AITC but not after 1 day exposure. Differences between exposure periods are likely to be due to differences in the states of the J2 before storage. J2 after 1 day exposure are likely to still be in diapause as this is not long enough to trigger the move into quiescence. As J2 in diapause are more resistant to toxic compounds then it is likely that these J2 were unaffected by the AITC exposure compared to the J2 in a more vulnerable state at longer liquid exposure periods.

Including a delay in water before PRD exposure suggests that ITC exposure can be short but extended time is needed in order for AITC to effectively suppress hatch. This can be explained by the mode of action of AITC where the chemical first induces paralysis before leading to J2 death as discussed in 4.4.2. The lack of permanent suppression after 16 days exposure in chapter 3.3.3 would suggest that a minimum of 3-4 weeks between exposure and hatch stimulation is required in order for permanent suppression to occur. In this study hatch was not significantly reduced after 7 days exposure. This is likely due to increased drowning in the control which reduced the significance of the lower hatch after 50ppm AITC exposure. This appears to be the first study to look at the effect of delaying hatch stimulation after exposure to AITC although in one previous study cysts were soaked for two days in water after AITC exposure and a reduction in hatch compared to a water control was found (Brolsma *et al.*, 2014). As fields infested with *G. pallida* are likely to contain moisture

(reducing the occurrence of desiccation), further experimentation included a delay in water after exposure even though a delay in the absence of water appeared to be more effective at longer exposure periods. AITC is known to breakdown quickly with a half-life of between 16-60hrs depending on soil type, water content and temperature (Brown *et al.*, 1991; Borek *et al.*, 1995; Gardiner *et al.*, 1999; Petersen *et al.*, 2001) therefore cyst storage which can increase AITC effectiveness at short exposure periods was considered advantageous. Although 50ppm AITC may be effective at suppressing hatch, this was the only concentration to affect encysted J2 when a delay was introduced between exposure and hatch stimulation and therefore higher concentrations should be considered.

4.4.2. Mode of AITC-mediated hatch suppression

Several assays provided evidence that AITC suppression of cyst hatch consisted of J2 paralysis followed by mortality. During AITC replenishment, AITC efficiency was increased the longer the exposure period and the more the AITC was replenished suggesting the need for extended contact time between ITC and target organism at concentrations below 50ppm. When studying concentrations over the exposure periods, a pattern was identified: initially hatch was delayed, and then total hatch was reduced with only the highest concentrations for the longest exposure period increasing mortality. This pattern was also noted during the first experiment and delayed stimulation experiments; several AITC treatments led to a delay in hatch with 50ppm AITC also reducing total hatch.

In general, concentrations lower than 50ppm AITC delayed hatch but didn't reduce hatch compared to a control suggesting the temporary paralysis of J2 by low AITC concentrations. This is consistent with the results from Chapter 3 where a temporary nematostatic effect was noted after exposure to a single dose of 25ppm and 50ppm AITC. In contrast, when the concentration was increased to 50ppm AITC in the current experiments, hatch was both delayed and reduced implying a more permanent form of suppression under the altered assay conditions, permanent paralysis being followed by J2 mortality.

Significant hatch suppression not attributed to mortality after 50ppm AITC replenishment was determined to last longer than four months, suggesting a permanent nematostatic effect. AITC concentrations not nematotoxic may still be able to contribute to *G. pallida* control by permanently paralysing a proportion of the encysted J2. It should be noted that ITC release in the field would only occur once therefore it is unlikely that this permanent suppression would occur after exposure to 50ppm AITC and a higher concentration would be required.

In 4.3.6, exposure to 50ppm AITC for 1 day reduced hatch but did not increase mortality; exposure to higher AITC concentrations between 100-1500ppm AITC completely suppressed

hatch. At concentrations between 50-500ppm, hatch suppression appeared to be due to a mixture of paralysis and mortality, whereas hatch suppression after exposure to the higher AITC concentrations was due to J2 mortality alone. This further supports the theory that AITC acts by first paralysing encysted J2 before inducing mortality, and that the efficiency of AITC is concentration dependent.

Previous studies have also shown that mustard root exudate containing low concentrations of ITCs can suppress hatch but not increase mortality *in vitro* (Forrest and Farrer, 1983; Forrest, 1989) and several toxicity assays studying the effect of *Brassica* spp. material on hatched J2 noted paralysis of a large proportion of free J2 after exposure (Ngala, Woods and Back, 2015a; Fatemy and Sepideh, 2016). These results suggest that AITC acts directly on the J2 in a concentration-dependent manner by interfering with J2 processes leading to paralysis followed by mortality.

4.4.3. AITC and G. pallida *in different states*

The effect of ITCs on encysted *G. pallida* in different states has not previously been investigated. One study did note that exposure of *G. rostochiensis* cysts to different *Brassica* leaves in the presence of PRD decreased hatch to <1% after 6 weeks exposure *in vitro* (Fatemy and Sepideh, 2016). As it is known that *G. pallida* encysted J2 enter dormancy in the absence of potatoes (Hominick, Forrest and Evans, 1985; Perry, 1989), and biofumigation involves the release of ITCs before diapause is broken by winter (Muhammad, 1994), it is therefore appropriate to examine whether J2 in dormancy are differentially affected compared to J2 which are in the process of hatching (quiescent; Perry and Moens, 2011). It has previously been shown that PCN in diapause can be more resistant to nematicides (Spears, 1968; Elling *et al.*, 2007; Palomares-Rius *et al.*, 2013) due to differentially regulated genes in PCN in diapause or quiescence (Palomares-Rius *et al.*, 2016) providing the possibility that *G. pallida* in dormancy could be less affected by other toxic compounds, such as ITCs.

Results from 4.3.1 demonstrate that 50ppm AITC significantly reduced hatch compared to the control when exposed in conjunction with hatch stimulants for 4 days. In Chapter 3, exposure to 50ppm AITC for 4 days had no effect on *G. pallida* hatch. This would suggest that exposing encysted J2 to AITC during hatch stimulation increases hatch suppression. This is most likely due to the physical changes that occur when *G. pallida* is exposed to PRD during hatch preparation, as discussed in 1.3.1, leaving the J2 more vulnerable to AITC.

Further evidence to support this theory can be found in several of the other assays in this study. Results from experiments which utilised a viability stain indicated that cysts treated with water contained a percentage of unhatched viable J2; these are most likely J2 still in

diapause. When cysts were exposed to concentrations of AITC which suppressed hatch and increased mortality, the percentage of dead J2 after AITC exposure did not exceed the percentage of dead and hatched J2 combined in the controls, leaving the percentage of unhatched viable J2, thought to be in diapause, unaffected by AITC treatment. This has occurred in all experiments where a viability stain was used.

These results suggest that the current practice of incorporating biofumigants in autumn may not be effective against encysted *G. pallida* due to the unique ability of PCN to enter diapause in the absence of potatoes. This is discussed in more detail in Chapter 8.

4.4.4. AITC and G. pallida *control in soil*

Previous studies have not studied the effect of pure AITC in soil on *G. pallida*, making several of the experiments performed here the first of their kind. These studies provide valuable information with respect to the effect of AITC in soil and the potential influence of various factors on AITC efficiency, specifically: the presence of soil, ITC combination, AITC concentration, soil composition, and temperature.

The presence of soil decreased the efficiency of AITC as a higher AITC concentration was required for complete hatch suppression and an increase in mortality compared to *in vitro* experiments. *In vitro*, 100ppm AITC suppressed *G. pallida* whereas in the pot trials, 100ppm AITC had no effect on PCN hatch or multiplication. In the soil microcosm and soil type pot trial, 500ppm AITC was the lowest concentration able to completely inhibit hatch and increase mortality. It should be noted that concentrations between 100ppm and 500ppm were not included in the soil trials so there is the potential that AITC release within this range would be effective. The decrease in AITC efficiency in soil compared to *in vitro* is likely due to several factors: increased surface area, increased headspace, and contact interference. AITC would volatize into the environment quicker, reducing the direct contact of AITC to cysts. In addition, AITC would be interacting with the organic material portion of soil (Drobnica, Kristián and Augustín, 1977; Borek *et al.*, 1995; Brown and Hampton, 2011). Due to the decreased efficiency noted, biofumigant cultivars containing a high concentration of sinigrin would be required for successful *G. pallida* control in soil.

It is known that Brassicaceae spp. contain a combination of GSLs which produce a number of different ITCs (Cole, 1976; Fahey, Zalcmann and Talalay, 2001). Due to this a small trial was set up to better mimic the field application of multiple ITCs and to explore the possibility of ITCs mixed with low background concentrations of other ITCs having a greater suppressive effect on *G. pallida* in soil. As AITC is thought to alter the cyst wall or eggshell permeability the idea that low ITC concentrations could allow entry of other more prevalent ITCs was

considered in this trial. Exposing cysts to 100ppm AITC in soil has been shown to have little effect on hatch and multiplication and results from this experiment show that combining 100ppm AITC with low concentrations of BITC and PEITC did not appear to provide any added benefit. As this lack of effect is potentially to be expected when considering the highest concentration of AITC applied, there is still the possibility that mixing and applying ITCs in combination would have an added effect at increased concentrations.

Although soil reduced AITC efficiency and 100ppm AITC was shown to not be effective against *G. pallida* in soil, higher concentrations in the soil type experiment were able to reduce hatch and multiplication. Generally, concentrations above 500ppm AITC significantly reduced hatch which in turn led to a reduction in newly formed cysts post-potato harvest. As well as reducing the number of new cysts, the AITC treatments reduced the number of new eggs which were within these cysts with a large number of cysts being empty. This suggests a further advantage of applying AITC to the soil before potato planting; in addition to inhibiting the initial infection of the potato crop, the production of new generations would be reduced limiting the effects of *G. pallida* on subsequent potato crops.

Soil composition had little impact on the ability of AITC to suppress *G. pallida* hatch or multiplication when comparing within AITC concentrations although there were a few differences when comparing between different treatments. In sandy silt loam soil, 500ppm AITC exposure did not increase egg mortality compared to the control whereas exposure in sandy and clay loam soil did increase mortality. This is most likely a false negative effect due to a high percentage of dead J2 in the sandy silt loam control combined with a high level of variation between repeats for both the control and the 500ppm AITC treatment. The number of newly formed cysts and cyst content after 1000ppm AITC treatment in sandy silt loam was not significantly different to the control whilst 1000ppm AITC in the other two soil types was effective at reducing the formation of new cysts and egg number. If not a false negative effect, these results would suggest that a higher concentration of AITC is required to consistently control *G. pallida* in soil characterised as sandy silt loam although control can still be achieved. This is potentially due to the high iron and organic matter content of silt loam soil (Hanschen *et al.*, 2015) interfering with the direct contact of AITC to cysts in this soil type. The main results from this experiment show that AITC is effective in three different agricultural soil types and provides evidence to suggest that biofumigation would be effective in the field, independent of soil composition, if Brassicaceae tissue containing a high concentration of sinigrin was incorporated as the biofumigant.

There were little differences in the ability of AITC to suppress *G. pallida* in soil when temperature was explored as an influencing factor. Hatch was suppressed more by 100ppm AITC at 13°C compared to the other temperatures suggesting extended persistence of AITC in the soil at this temperature leading to higher J2 paralysis. 500ppm AITC at 17°C increased the percentage of dead J2 compared to the lower temperatures which implies an advantage for warmer climates with respect to increasing *G. pallida* mortality. Aside from these two differences, temperature did not influence AITC efficiency between 10, 13 and 17°C. These temperatures were chosen based on the average temperatures for Lincolnshire, England during August - October (Appendix C). This is the period of time *Brassica* spp. are grown and incorporated to release suppressing ITCs and it is the location of the field trials completed in Chapters 6 and 7. Implementing biofumigation involving AITC release should be effective in climates where temperatures are within the 10-17°C range.

4.4.5. The effect of other isothiocyanates on encysted G. pallida

Altering assay conditions did not increase the efficiency of PEITC, BITC or MITC on *G. pallida* hatch *in vitro* or in soil compared to the results in Chapter 3. Concentrations ≤100ppm were ineffective at suppressing encysted *G. pallida*. Higher concentrations were not tested as it is unlikely that concentrations higher than 100ppm can be achieved in the field for these ITCs, with several common biofumigants not containing the parent GSL at all (Kirkegaard and Sarwar, 1998; Fahey, Zalcmann and Talalay, 2001; Gimsing and Kirkegaard, 2006; Bellostas, Sørensen and Sørensen, 2007).

PEITC was ineffective at influencing J2 hatch when: encysted J2 were in diapause, a delay was introduced before hatch stimulation or when applied at 100ppm on its own or in combination with 5ppm AITC and BITC in soil. Experiments in Chapter 3 noted a significant but transient increase in *G. pallida* hatch after exposure to certain PEITC treatments. The absence of an increase when assay conditions were altered to include a delay before hatch stimulation would confirm this transience and suggests that enhanced hatch would not be noted under field conditions. These results contradict those by Ngala *et al.* (2015), where *R. sativus* root material containing a high concentration of the 2-phenylethyl GSL (theoretically able to release over 100ppm PEITC) significantly affected hatch and mortality *in vitro*; the actual concentration of PEITC produced is unknown so a direct comparison cannot be made. The results from the current study would imply that PEITC is ineffective as a *G. pallida* control ITC at these concentrations and previous studies have noted that higher concentrations are unlikely to be released therefore PEITC is not considered a desirable ITC produced from biofumigants.

No previous studies have studied the effect of pure BITC on PCN *in vitro* and in soil. BITC was ineffective on cysts: in diapause, when a delay in water was introduced and when applied to cysts in soil. When a delay in the absence of water was introduced, 3.125ppm BITC led to a decrease in hatch after 1 day compared to the control. This effect was inconsistent and variable, with no effect on hatch at longer exposure periods or higher concentrations, therefore this is likely to be a false positive due to lower egg numbers in this batch of cysts. These results are consistent with those from Chapter 3 providing further evidence that biofumigation with BITC-releasing cultivars is unlikely to be effective at controlling *G. pallida* populations.

MITC significantly affected *G. pallida* hatch when a delay was introduced after treatment. There was an increase in hatch when exposed to 12.5ppm and 25ppm MITC for different exposure periods. There was no effect of 50ppm MITC on overall hatch although a delay in hatch was apparent as concentration and exposure period increased. An increase in hatch after exposure to MITC was also noted and discussed in Chapter 3; the effect was more pronounced when a delay in the absence of water was introduced providing evidence that MITC requires time to affect the encysted *G. pallida* after its presence has been removed. As discussed in Chapter 3, MITC could prime J2 for hatch or alter the eggshell permeability in such a way that hatch is enhanced. The simultaneous hatch delay would suggest a bi-modal effect of MITC on PCN where the J2 are temporarily paralysed and the eggshell permeability is altered long-term leading to delayed but increased hatch. Although MITC treatments enhanced *G. pallida* hatch, common biofumigants do not contain this ITC (Fahey, Zalcmann and Talalay, 2001) therefore it should not present a problem when considering biofumigation for PCN control. In spite of this, potential PCN biofumigants should be screened for MITC before consideration in order to reduce the risk of increasing populations.

4.4.6. Concluding remarks

It is useful to determine the concentration of AITC required to completely suppress hatch and increase mortality *in vitro* and in soil, in order to select biofumigant cultivars able to produce enough sinigrin to suppress PCN. Replenishing AITC *in vitro* showed that AITC affects J2 by suppressing hatch, most likely by paralysing the J2, before causing mortality. Although 50ppm AITC can reduce hatch and permanently paralyse encysted J2 after repeated application, only one application would occur in the field therefore a higher concentration is required for efficient control.

When cysts were kept in water prior to the induction of hatch, the minimum concentration required to completely suppress hatch was 100ppm AITC. In a further experiment, 750ppm

AITC was required to kill all encysted eggs which would have otherwise hatched in the control after 1 day exposure. This suggests that AITC is fast acting at high concentrations and suppression can be achieved after a short exposure period.

When applying AITC to soil, the concentration required to suppress hatch was 500ppm AITC; this was also the lowest concentration where an increase in mortality explained hatch suppression entirely. Therefore, 500ppm AITC is the concentration required to be released from Brassicaceae spp. incorporation for effective control of *G. pallida* in the field. It is believed that the effective AITC concentrations used in these experiments can be achieved in the field. Further investigation into the concentration of sinigrin present in plant material is required and has been explored in Chapter 5. Temperature and soil composition had very little impact on AITC efficiency above 500ppm suggesting that as long as a high concentration can be achieved in the soil these additional factors, which can vary in the field, should not negatively impact the effect AITC-releasing biofumigants have on PCN. Aside from AITC, none of the other ITCs used in this study were able to suppress *G. pallida* therefore Brassicaceae spp. containing the parent GSLs releasing these ITCs and concentrations are considered inadequate for PCN control.

Chapter 5. The Analysis of Brassicaceae Glucosinolate Profiles at Different Growth Stages

5.1. Introduction

Over 130 glucosinolates (GSLs) have been identified in the order Brassicales (Fenwick, Heaney and Mullin, 1983; Daxenbichler *et al.*, 1991; Fahey, Zalcmann and Talalay, 2001), which contain the family of interest: Brassicaceae. The type, concentration and diversity of these GSLs vary greatly both amongst and within species as discussed in 1.5.1. In addition, GSL content is known to vary at different stages of plant development and between organs (Brown *et al.*, 2003; Bellostas, Sørensen and Sørensen, 2007; Velasco *et al.*, 2007) further increasing the complexity of Brassicaceae spp. GSL profiles.

It is thought that there is a higher concentration and greater diversity of GSLs in the roots of Brassicaceae material compared to the shoots (Angus *et al.*, 1994; Dam, Tytgat and Kirkegaard, 2009) with studies focussing on above-ground tissue underrepresenting total plant GSL profiles. Although GSL concentration can be higher in Brassicaceae roots, their contribution with respect to biofumigation is limited by their low biomass (Kirkegaard and Sarwar, 1998). The combination of GSL concentration and plant biomass in biofumigation is essential, with desirable cultivars being at a developmental stage where there is both a high GSL content and a large biomass at incorporation.

It is important to determine the GSL profiles of different cultivars in order to select effective biofumigant crops, as the desirable type and concentration of GSL will differ depending on the target pest in question. In addition, determining the optimum plant growth stage for GSL production will provide important information on the timing of plant incorporation during biofumigation. With respect to this study, results from Chapter 4 suggest that Brassicaceae cultivars at a developmental stage containing high concentrations of sinigrin, the parent GSL of AITC, should be selected for potato cyst nematode (PCN) suppression.

The GSL profiles of above-ground plant material of five Brassicaceae cultivars were determined at five developmental stages using liquid chromatography-mass spectrometry (LC-MS). Brassicaceae cultivars were selected based on their use as commercial biofumigants and their inclusion in previous GSL profile studies (Taylor, 2013; Ngala *et al.*, 2014). Although it has been noted that GSL content can differ between root and shoot material, only above-ground plant tissue was analysed due to the difficulty associated with collecting and analysing root tissue from plants grown in soil. Above-ground material was analysed in combination, as during the biofumigation process all plant material is incorporated into the

soil therefore separating out different plant organs was unnecessary. Plant growth stages were chosen which were both distinct from each other and covered the entire lifecycle of the plant (Bellostas, Sørensen and Sørensen, 2007; Taylor, 2013). LC-MS utilises two commonly used chemical analysis methods in one process: liquid chromatography separates a sample into its constituent molecules according to polarity, and mass spectrometry analyses the ions based on their mass to charge ratio. When the two methods are combined, they can provide detailed structural information on compounds of interest allowing both identification and quantification of a number of metabolites within a single sample. A protocol for the LC-MS analysis of GSLs in Brassicaceae material has been developed by the University of Reading, where the analysis was performed (Bell, Oruna-Concha and Wagstaff, 2015).

The main aim of this study was to:

- Analyse the GSL profiles of biofumigant cultivars in order to determine differences in GSL content between cultivars and developmental growth stages.

5.2. Materials and Methods

5.2.1. Brassicaceae pot trial

Five Brassicaceae cultivars (Table 5.1; Barworth Agriculture, UK) were grown from seed in 2 L pots three-quarter filled with John Innes No. 2 soil, with 2 seeds per pot, until plants had reached one of the five assigned growth stages (Table 5.2). At this time above-ground plant material was harvested (2.4.5) and freeze-dried in preparation for LC-MS analysis. Four experimental replicates of each cultivar and growth stage were included. Pots were set up in a randomised design layout.

Cultivar	Species
Bento	*Raphanus sativus*
Ida Gold	*Sinapis alba*
ISCI 99	*Brassica juncea*
Nemat	*Eruca sativa*
Scala	*Brassica juncea*

Table 5.1. Cultivars included in the glasshouse trial.

Growth Stage	Physical Plant Attribute
1	First leaf
2	Initial flowering
3	70% flowering
4	Seed development
5	Plant browning

Table 5.2. Plant developmental growth stages that plant material was harvested from. Growth stages were chosen based on physical changes that occur throughout the plants life cycle.

5.2.2. LC-MS analysis of Brassicaceae species

After plant material was grown and collected, the tissue was prepared (2.8.1), GSLs extracted (2.8.2) and LC-MS analysis (2.8.3) run on two technical replicates of each sample by Dr Luke Bell, Department of Food & Nutritional Sciences, University of Reading.

5.2.3. Analysis of LC-MS results

Compounds were identified and quantified as described in 2.8.4. See Appendix D for a list of GSLs identified, retention times, primary ions and relative response factors. GSL concentrations (mg g^{-1} dry weight (DW)) were determined and used to analyse differences in GSL profiles between cultivars and growth stages.

5.2.4. Data analysis

Data was analysed as in 2.10.1. Two-way ANOVA analysis was performed with Cultivar x Growth Stage as factors in 5.3.1 for each GSL individually. Significant ANOVA P-values (P<0.05) were further investigated using the means comparison test, Tukey's HSD test.

Multivariate analysis was completed when studying differences between overall GSL profiles in 5.3.2 as described in detail in 2.10.2. Canonical variate (CV) analysis was performed three times with data grouped by; Cultivar, Growth Stage or Cultivar x Growth Stage. All GSLs were used as variates during the analysis. The significance of distances from the CV analyses were investigated using Analysis of Distance with the treatment structure Cultivar x Growth Stage. Where this produced a significant probability (Pr<0.05), scatter plots of the first and second CV ordinates were formed with data sets grouped by the factor displaying significance and Tukey's HSD test was performed on the first and second CV ordinates.

5.3. Results

5.3.1. Glucosinolate identification and concentration

Twenty-one GSLs were identified across cultivars where the presence and concentration of each varied between cultivar and growth stage (Table 5.3).

Nemat displayed the highest diversity of GSLs with twelve GSLs identified across the growth stages; of these, initial flowering contained the highest number. Nine GSLs were identified in ISCI 99 and all were present at 70% flowering. Eight GSLs were detected in Scala with all present at initial flowering. Nine GSLs were in Ida Gold samples with the highest number identified at initial flowering. Bento displayed the lowest diversity with a total of seven GSLs: all were detected at initial flowering and plant browning.

There was a significant effect of the interaction between cultivar and growth stage on the concentration of fourteen identified GSLs.

4-hydroxyglucobrassicin was measured in Bento, ISCI 99 and Scala at concentrations between 0.01-0.87mg g^{-1} DW although it was not present at all growth stages. In most cases values of 4-hydroxyglucobrassicin were equally low, except for in ISCI 99 and Scala at the plant browning stage when levels were significantly higher than other measured values. Gluconapin was detected at low concentrations in ISCI 99 and Scala at all growth stages and Ida Gold at selected growth stages. The concentration was consistently low in the Ida Gold samples. Gluconapin levels in ISCI 99 were highest at first leaf and then decreased as the plant matured. In contrast, levels in Scala samples peaked at the initial flowering stage.

Glucotropaeolin was identified in Ida Gold, ISCI 99 and Scala at generally low concentrations. Ida Gold initial flowering and 70% flowering samples contained significantly higher levels compared to all other measured samples. Concentrations of glucobrassicin were identified in all cultivars and were consistently low across all growth stages for Bento, ISCI 99, Nemat and Scala, but varied with growth stage for Ida Gold. For this cultivar glucobrassicin concentrations were highest at the initial flowering and seed development stages and lowest at plant browning.

4-methoxyglucobrassicin was not a major GSL. It was detected at low concentrations, 0.004-0.11mg g^{-1} DW, in one or two growth stages of Ida Gold, ISCI 99 and Nemat. The concentration was consistently low across samples although Nemat at initial flowering did contain higher levels compared to all other samples, except ISCI 99 at first leaf. Gluconasturtiin was detected between 0.02-1.68mg g^{-1} DW in all cultivars except Bento; its concentration did not vary between growth stages in Ida Gold, Nemat or Scala. Levels in ISCI 99 at plant browning were significantly higher than in ISCI 99 at seed development.

Neoglucobrassicin was measured in all cultivars. There were no significant differences in concentration within cultivars. ISCI 99 first leaf samples had the highest concentrations while Bento had very low or non-detectable levels. Glucoraphanin was identified at between 2.15-20.14mg g^{-1} DW in Bento and Nemat at all growth stages. There were no differences in levels amongst Bento growth stages, whilst for Nemat highest values were recorded at the seed development stage with lowest values (similar to those measured in Bento) at the first leaf and initial flowering stages.

Progoitrin was measured in Ida Gold and Nemat. Levels were consistently low in Nemat, whereas levels in Ida Gold were highest at the initial flowering growth stage then decreased as the plant aged. Glucoraphenin was identified in Bento alone, throughout development, where the concentration at first leaf stage was significantly lower than at 70% flowering and plant

browning. Glucosinalbin was only detected in Ida Gold samples. Levels were highest in 70% flowering samples and lowest at the beginning and end of the plants development.

Glucosativin, dimeric glucosativin (DMB) and diglucothiobeinin were identified in Nemat alone at all growth stages. Glucosativin levels were highest at initial flowering and 70% flowering growth stages and lowest at the plant browning growth stage. DMB was measured at high concentrations which varied significantly over growth stages: levels were low at first leaf, increased as the plant matured to 70% flowering then decreased with further plant ageing. Diglucothiobeinin was identified at low levels where concentration was highest at the initial flowering growth stage then decreased as the plant developed.

In the absence of a significant effect of the interaction between cultivar and growth stage on GSL concentration, growth stage did not influence GSL concentration. Cultivar had a significant effect on the concentration of four GSLs. Glucoerucin was measured in Bento and Nemat where concentration in the Nemat cultivar (1.56 ±0.45 mg g^{-1} DW) was significantly higher than in Bento (0.08 ±0.02 mg g^{-1} DW). ISCI 99 and Scala contained high concentrations of sinigrin independent of growth stage. Bento contained glucoraphasatin at an average concentration of 5.47 mg g^{-1} DW. Glucoalyssin was identified in Nemat alone.

There were three GSLs where there was no significant effect of cultivar, growth stage or their interaction. Glucobrassicanapin was detected at low concentrations in ISCI 99 at the 70% flowering growth stage and in Scala at the initial flowering growth stage. Glucocapparin was identified in Ida Gold at plant browning and glucolepidiin was detected in Nemat first leaf samples; both GSLs were found at low concentrations.

Total GSL concentration ranged between 13.89-59.35mg g^{-1} DW across samples. There was no significant effect of the cultivar or the interaction between cultivar and growth stage but growth stage did have a significant effect on total GSL concentration (Figure 5.1). The highest concentration of GSLs was produced from the initial flowering and 70% flowering samples which was significantly higher than the GSL concentration at the first leaf growth stage, which produced the lowest concentration of GSLs.

Cultivar	Growth Stage	4-hydroxyglucobrassicin	Gluconapin	Glucotropaeolin	Glucobrassicin	4-methoxyglucobrassicin
Bento	First leaf	0.13 (±0.08)^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}
	Initial flowering	0.01 (±0.01)^{a}	ND^{a}	ND^{a}	0.04 (±0.01)^{a}	ND^{a}
	70% flowering	0.02 (±0.02)^{a}	ND^{a}	ND^{a}	0.02 (±0.02)^{a}	ND^{a}
	Seed development	ND^{a}	ND^{a}	ND^{a}	0.02 (±0.02)^{a}	ND^{a}
	Plant browning	0.11 (±0.07)^{a}	ND^{a}	ND^{a}	0.03 (±0.03)^{a}	ND^{a}
Ida Gold	First leaf	ND^{a}	ND^{a}	0.007 (±0.007)^{a}	1.91 (±0.68)^{ab}	ND^{a}
	Initial flowering	ND^{a}	0.10 (±0.09)^{ab}	3.86 (±1.77)^{b}	5.57 (±1.62)^{c}	0.006 (±0.006)^{a}
	70% flowering	ND^{a}	0.04 (±0.04)^{ab}	3.63 (±0.70)^{b}	4.03 (±0.88)^{bc}	ND^{a}
	Seed development	ND^{a}	ND^{a}	0.94 (±0.39)^{a}	4.93 (±1.08)^{c}	ND^{a}
	Plant browning	ND^{a}	ND^{a}	0.01 (±0.01)^{a}	0.72 (±0.17)^{a}	ND^{a}
ISCI 99	First leaf	ND^{a}	0.38 (±0.04)^{de}	ND^{a}	ND^{a}	0.05 (±0.05)^{ab}
	Initial flowering	0.05 (±0.05)^{a}	0.37 (±0.04)^{cde}	0.72 (±0.72)^{a}	0.04 (±0.03)^{a}	ND^{a}
	70% flowering	0.05 (±0.05)^{a}	0.23 (±0.08)^{abcde}	0.58 (±0.58)^{a}	0.11 (±0.03)^{a}	0.004 (±0.004)^{a}
	Seed development	0.02 (±0.02)^{a}	0.19 (±0.05)^{abcd}	0.06 (±0.06)^{a}	0.01 (±0.01)^{a}	ND^{a}
	Plant browning	0.76 (±0.40)^{b}	0.10 (±0.06)^{ab}	0.02 (±0.02)^{a}	ND^{a}	ND^{a}
Nemat	First leaf	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}
	Initial flowering	ND^{a}	ND^{a}	ND^{a}	0.003 (±0.003)^{a}	0.11 (±0.05)^{b}
	70% flowering	ND^{a}	ND^{a}	ND^{a}	0.03 (±0.01)^{a}	ND^{a}
	Seed development	ND^{a}	ND^{a}	ND^{a}	0.05 (±0.01)^{a}	ND^{a}
	Plant browning	ND^{a}	ND^{a}	ND^{a}	0.21 (±0.15)^{a}	0.02 (±0.02)^{a}
Scala	First leaf	ND^{a}	0.14 (±0.09)^{abc}	ND^{a}	ND^{a}	ND^{a}
	Initial flowering	0.12 (±0.07)^{a}	0.45 (±0.11)^{e}	0.14 (±0.14)^{a}	0.06 (±0.02)^{a}	ND^{a}
	70% flowering	0.10 (±0.03)^{a}	0.27 (±0.05)^{bcde}	ND^{a}	0.05 (±0.02)^{a}	ND^{a}
	Seed development	ND^{a}	0.21 (±0.05)^{abcd}	0.05 (±0.05)^{a}	0.01 (±0.01)^{a}	ND^{a}
	Plant browning	0.87 (±0.07)^{b}	0.04 (±0.03)^{ab}	ND^{a}	ND^{a}	ND^{a}
				ANOVA P-values		
Cultivar		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.031**
Growth Stage		**<0.001**	**<0.001**	**<0.001**	**0.005**	0.115
Cultivar x Growth Stage		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.005**

Table 5.3. Average GSL concentrations (mg g^{-1} DW) and associated ANOVA P-values. ND= not detected; values reported as ND were treated as equal to 0 during analysis. Standard errors are stated within brackets. Within columns, means followed by the same letter are not significantly different.

Cultivar	Growth Stage	Gluconasturtiin	Neoglucobrassicin	Glucoraphanin	Progoitrin	Glucoraphenin
Bento	First leaf	ND[a]	ND[a]	9.33 (±7.84)[abc]	ND[a]	14.81 (±7.02)[ab]
	Initial flowering	ND[a]	0.003 (±0.003)[a]	5.02 (±1.94)[ab]	ND[a]	35.52 (±6.56)[bc]
	70% flowering	ND[a]	ND[a]	6.16 (±1.74)[ab]	ND[a]	43.56 (±13.64)[c]
	Seed development	ND[a]	0.004 (±0.004)[a]	2.15 (±1.10)[ab]	ND[a]	36.24 (±10.43)[bc]
	Plant browning	ND[a]	0.006 (±0.006)[a]	5.52 (±0.95)[ab]	ND[a]	47.99 (±4.43)[c]
Ida Gold	First leaf	0.02 (±0.02)[a]	ND[a]	ND[a]	ND[a]	ND[a]
	Initial flowering	0.64 (±0.09)[abc]	0.12 (±0.09)[ab]	ND[a]	3.14 (±1.75)[b]	ND[a]
	70% flowering	0.63 (±0.08)[abc]	0.28 (±0.10)[ab]	ND[a]	1.05 (±0.48)[a]	ND[a]
	Seed development	0.27 (±0.20)[ab]	0.26 (±0.15)[ab]	ND[a]	0.85 (±0.48)[a]	ND[a]
	Plant browning	0.02 (±0.02)[a]	0.04 (±0.04)[ab]	ND[a]	0.55 (±0.11)[a]	ND[a]
ISCI 99	First leaf	0.66 (±0.08)[abc]	0.32 (±0.12)[b]	ND[a]	ND[a]	ND[a]
	Initial flowering	1.01 (±0.46)[abc]	0.08 (±0.05)[ab]	ND[a]	ND[a]	ND[a]
	70% flowering	0.72 (±0.24)[abc]	0.18 (±0.10)[ab]	ND[a]	ND[a]	ND[a]
	Seed development	0.31 (±0.14)[ab]	0.06 (±0.01)[ab]	ND[a]	ND[a]	ND[a]
	Plant browning	1.68 (±0.84)[c]	0.19 (±0.02)[ab]	ND[a]	ND[a]	ND[a]
Nemat	First leaf	0.23 (±0.04)[ab]	ND[a]	3.45 (±0.24)[ab]	ND[a]	ND[a]
	Initial flowering	0.10 (±0.10)[ab]	0.004 (±0.004)[a]	6.58 (±2.57)[ab]	0.03 (±0.02)[a]	ND[a]
	70% flowering	0.03 (±0.03)[ab]	0.03 (±0.01)[ab]	14.36 (±2.98)[bc]	0.11 (±0.11)[a]	ND[a]
	Seed development	0.04 (±0.02)[ab]	0.03 (±0.01)[ab]	20.14 (±6.48)[c]	ND[a]	ND[a]
	Plant browning	ND[a]	ND[a]	10.52 (±2.31)[abc]	0.39 (±0.26)[a]	ND[a]
Scala	First leaf	0.06 (±0.06)[ab]	0.16 (±0.07)[ab]	ND[a]	ND[a]	ND[a]
	Initial flowering	0.66 (±0.22)[abc]	0.08 (±0.05)[ab]	ND[a]	ND[a]	ND[a]
	70% flowering	1.24 (±0.18)[bc]	0.07 (±0.01)[ab]	ND[a]	ND[a]	ND[a]
	Seed development	0.83 (±0.33)[abc]	0.10 (±0.05)[ab]	ND[a]	ND[a]	ND[a]
	Plant browning	0.27 (±0.13)[ab]	0.23 (±0.05)[ab]	ND[a]	ND[a]	ND[a]
				ANOVA P-values		
Cultivar		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Growth Stage		0.137	0.673	0.526	0.118	0.113
Cultivar x Growth Stage		**0.006**	**0.009**	**0.019**	**0.020**	**0.030**

Table 5.3. continued.

Cultivar	Growth Stage	Glucosinalbin	Glucosativin	DMB	Diglucothiobeinin	Glucoerucin	Sinigrin
Bento	First leaf	ND[a]	ND[a]	ND[a]	ND[a]	0.15 (±0.01)	ND
	Initial flowering	ND[a]	ND[a]	ND[a]	ND[a]	0.06 (±0.02)	ND
	70% flowering	ND[a]	ND[a]	ND[a]	ND[a]	0.03 (±0.03)	ND
	Seed development	ND[a]	ND[a]	ND[a]	ND[a]	0.08 (±0.06)	ND
	Plant browning	ND[a]	ND[a]	ND[a]	ND[a]	0.09 (±0.06)	ND
Ida Gold	First leaf	19.51 (±2.63)[bc]	ND[a]	ND[a]	ND[a]	ND	ND
	Initial flowering	33.20 (±10.58)[cd]	ND[a]	ND[a]	ND[a]	ND	ND
	70% flowering	34.48 (±2.46)[d]	ND[a]	ND[a]	ND[a]	ND	ND
	Seed development	29.29 (±1.20)[bcd]	ND[a]	ND[a]	ND[a]	ND	ND
	Plant browning	16.38 (±7.37)[b]	ND[a]	ND[a]	ND[a]	ND	ND
ISCI 99	First leaf	ND[a]	ND[a]	ND[a]	ND[a]	ND	28.24 (±2.02)
	Initial flowering	ND[a]	ND[a]	ND[a]	ND[a]	ND	39.43 (±9.84)
	70% flowering	ND[a]	ND[a]	ND[a]	ND[a]	ND	32.05 (±10.46)
	Seed development	ND[a]	ND[a]	ND[a]	ND[a]	ND	45.40 (±10.24)
	Plant browning	ND[a]	ND[a]	ND[a]	ND[a]	ND	19.98 (±11.71)
Nemat	First leaf	ND[a]	2.25 (±0.36)[bc]	6.47 (±1.07)[a]	0.65 (±0.11)[cd]	0.78 (±0.21)	ND
	Initial flowering	ND[a]	2.63 (±0.59)[c]	26.45 (±2.05)[cd]	0.70 (±0.11)[d]	3.24 (±1.98)	ND
	70% flowering	ND[a]	2.55 (±0.86)[c]	32.64 (±4.62)[d]	0.58 (±0.10)[cd]	2.24 (±0.78)	ND
	Seed development	ND[a]	1.09 (±0.32)[ab]	18.22 (±4.23)[bc]	0.45 (±0.01)[bc]	0.75 (±0.30)	ND
	Plant browning	ND[a]	0.65 (±0.23)[a]	16.97 (±4.92)[b]	0.36 (±0.07)[b]	0.79 (±0.26)	ND
Scala	First leaf	ND[a]	ND[a]	ND[a]	ND[a]	ND	23.67 (±1.99)
	Initial flowering	ND[a]	ND[a]	ND[a]	ND[a]	ND	45.26 (±10.77)
	70% flowering	ND[a]	ND[a]	ND[a]	ND[a]	ND	34.68 (±6.39)
	Seed development	ND[a]	ND[a]	ND[a]	ND[a]	ND	28.41 (±5.92)
	Plant browning	ND[a]	ND[a]	ND[a]	ND[a]	ND	22.47 (±7.24)
				ANOVA P-values			
Cultivar		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Growth Stage		0.130	**0.021**	**<0.001**	**0.047**	0.277	0.104
Cultivar x Growth Stage		**0.041**	**<0.001**	**<0.001**	**0.004**	0.174	0.358

Table 5.3. continued.

Cultivar	Growth Stage	Glucoraphasatin	Glucoalyssin	Glucobrassicanapin	Glucocapparin	Glucolepidiin	Total
Bento	First leaf	6.56 (±2.74)	ND	ND	ND	ND	30.98 (±14.48)
	Initial flowering	10.12 (±3.44)	ND	ND	ND	ND	50.76 (±11.72)
	70% flowering	0.73 (±0.36)	ND	ND	ND	ND	50.51 (±14.18)
	Seed development	4.33 (±3.63)	ND	ND	ND	ND	42.82 (±9.89)
	Plant browning	5.60 (±4.51)	ND	ND	ND	ND	59.35 (±6.51)
Ida Gold	First leaf	ND	ND	ND	ND	ND	21.46 (±2.16)
	Initial flowering	ND	ND	ND	ND	ND	46.66 (±15.24)
	70% flowering	ND	ND	ND	ND	ND	44.13 (±1.85)
	Seed development	ND	ND	ND	ND	ND	36.54 (±1.51)
	Plant browning	ND	ND	ND	0.08 (±0.08)	ND	17.79 (±7.22)
ISCI 99	First leaf	ND	ND	ND	ND	ND	29.65 (±2.06)
	Initial flowering	ND	ND	ND	ND	ND	41.71 (±9.79)
	70% flowering	ND	ND	0.02 (±0.02)	ND	ND	33.94 (±10.10)
	Seed development	ND	ND	ND	ND	ND	46.05 (±10.34)
	Plant browning	ND	ND	ND	ND	ND	22.74 (±11.55)
Nemat	First leaf	ND	ND	ND	ND	0.06 (±0.06)	13.89 (±1.26)
	Initial flowering	ND	0.06 (±0.04)	ND	ND	ND	39.90 (±2.40)
	70% flowering	ND	0.07 (±0.07)	ND	ND	ND	52.65 (±6.45)
	Seed development	ND	0.13 (±0.10)	ND	ND	ND	40.89 (±9.52)
	Plant browning	ND	0.06 (±0.04)	ND	ND	ND	29.96 (±6.42)
Scala	First leaf	ND	ND	ND	ND	ND	24.03 (±2.12)
	Initial flowering	ND	ND	0.03 (±0.03)	ND	ND	46.79 (±10.87)
	70% flowering	ND	ND	ND	ND	ND	36.39 (±6.34)
	Seed development	ND	ND	ND	ND	ND	29.60 (±6.06)
	Plant browning	ND	ND	ND	ND	ND	23.87 (±7.40)
				ANOVA P-values			
Cultivar		**<0.001**	**<0.001**	0.529	0.413	0.413	0.064
Growth Stage		0.345	0.646	0.529	0.413	0.413	**<0.001**
Cultivar x Growth Stage		0.338	0.853	0.430	0.467	0.467	0.566

Table 5.3. continued.

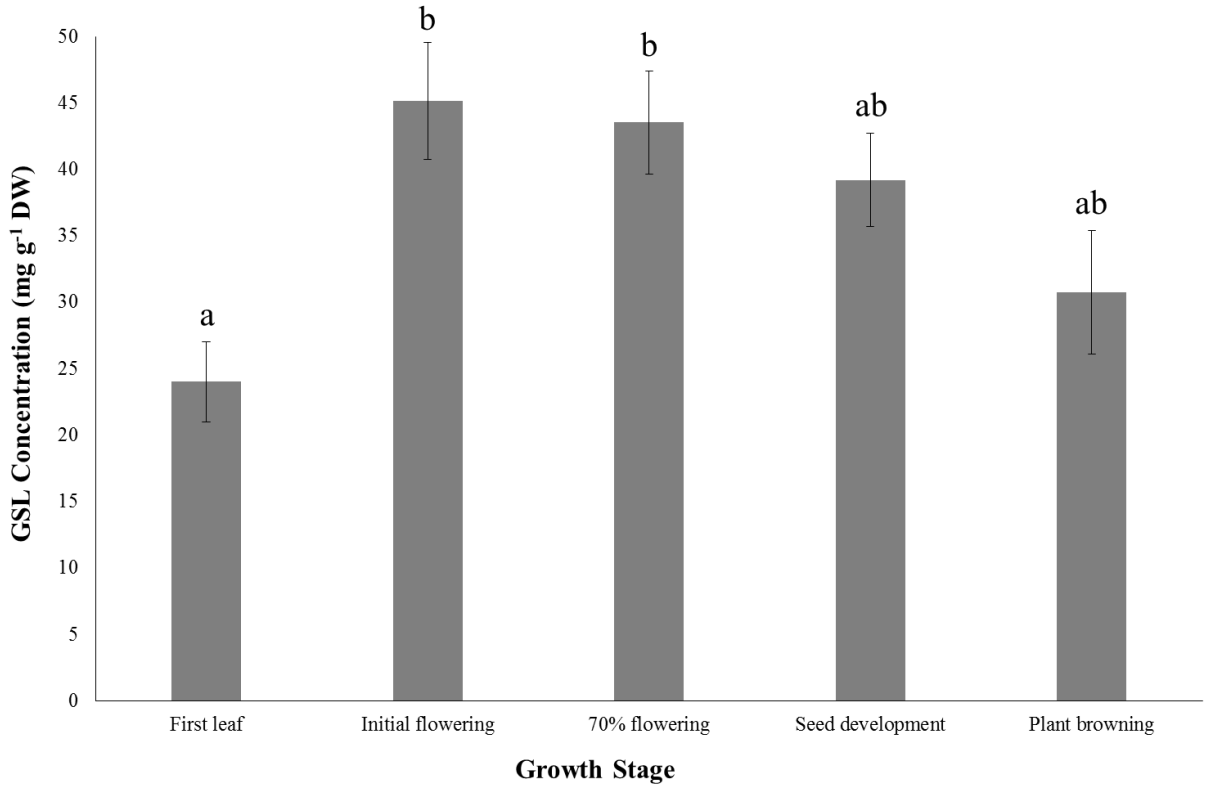


Figure 5.1. Total GSL concentration (mg g^{-1} DW) of samples over five growth stages. Error bars represent the standard error. Significant differences (P<0.05) are indicated by different letters.

5.3.2. Overall glucosinolate profiles

There were no significant differences between profiles when investigating growth stage or the interaction between growth stage and cultivar (data not shown). There was a significant difference between profiles when cultivar was the single factor (Pr<0.001; Figure 5.2). There was a clear separation of Nemat from the other four cultivars along CV 1 which explained 48.27% of the variation; Bento was also significantly different from all other cultivars along CV 1. Ida Gold was discriminated on CV 2, where 30.62% variation was explained. Nemat and Bento grouped together on CV 2 but were significantly different to the other cultivars. The GSL profiles of the *B. juncea* cultivars, ISCI 99 and Scala, were very similar across both CV 1 and CV 2.

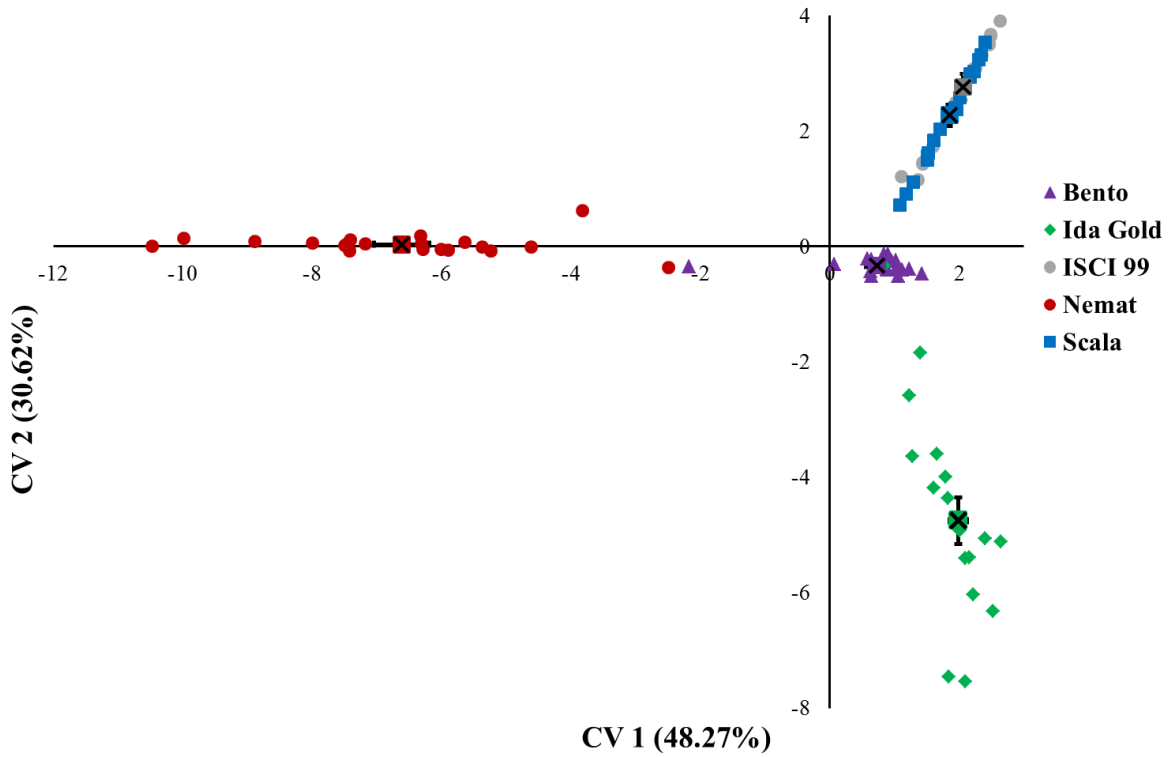


Figure 5.2. Scatterplot of the first and second CVs from CV analysis of the GSL profiles of cultivars. Percentage variation in the data explained by each CV is indicated within brackets. The average of each treatment is indicated by the cross-containing squares and error bars represent the standard error for each CV.

5.4. Discussion

This study provides detailed information on the GSL profiles of five potential biofumigant cultivars at a number of developmental growth stages. There were several differences in GSL concentration within and between cultivars over developmental growth stages and the overall GSL profiles differed between cultivars. This contributes useful knowledge to advance the application of biofumigation in the field, with a focus on PCN control.

5.4.1. Total glucosinolate concentration

When studying the total GSL concentration of samples there were no overall differences between cultivars but there were differences between growth stages. Previous studies have suggested that GSL production and accumulation increases then decreases as the plant develops (Booth, Walker and Griffiths, 1991; Bellostas, Sørensen and Sørensen, 2007; Velasco *et al.*, 2007) with a decline in GSL content as leaves age (Porter *et al.*, 1991; Lambdon *et al.*, 2003). This is consistent with results from this study. In general, the highest GSL concentration was produced between initial flowering and 70% flowering, independent of cultivar, which agrees with the current agricultural practice of incorporating biofumigant material at 50-70% flowering for maximised GSL production. The effectiveness of these

cultivars at incorporation depends more on which GSLs are being produced than total GSL concentration.

5.4.2. Overall cultivar glucosinolate profiles

Overall GSL profiles differed between species included in the study, independent of growth stage. The Nemat GSL profile was the most distinct from the other cultivars and also displayed the highest diversity and greatest number of GSLs. ISCI 99 and Scala overall GSL profiles did not vary between each other which is to be expected as they are both *B. juncea* cultivars. The separation of samples by species and the variations identified in overall GSL profiles is consistent with data compiled in previous reviews (Daxenbichler *et al.*, 1991; Fahey, Zalcmann and Talalay, 2001); this highlights the need to screen Brassicaceae cultivars for their GSL content before use against specific pests.

The primary role of GSL production in plants is not well understood. Previous studies have suggested their main purpose is related to plant defence (Halkier and Gershenzon, 2006; Redovnikovic *et al.*, 2008; Del Carmen Martínez-Ballesta, Moreno and Carvajal, 2013), which could explain the variation in GSL profiles between species depending on their plant-insect interactions. One study noted that *Brassica oleraceae* populations differed in their butenyl GSL levels depending on herbivore pressures present at each population site (Mithen, Raybould and Giamoustaris, 1995). This suggests a role in *B. oleraceae* defence for butenyl GSL but also that the GSLs were only produced when required, leading to variations in *B. oleraceae* GSL profiles. *Brassica napus* cultivars with different GSL profiles have been shown to have varying effects on specialist and generalist herbivores with plants containing shorter side chain GSLs attracting higher specialist herbivore feeding (Giamoustaris and Mithen, 1995). These studies provide evidence to support that the GSL profiles of Brassicaceae species can differ, partly, due to which GSLs are required in each species in response to specialist and generalist herbivores.

In addition to acting as attractants or repellents to herbivores, several studies have indicated that GSLs may play a role in plant defence systems by responding to signal molecules which mediate a plant response and activate defence genes. These studies have been discussed extensively (Redovnikovic *et al.*, 2008) where it has been noted on several occasions that indole GSLs are involved in this system with other GSL types, such as aliphatic, less so. This research suggests that plant species which utilise GSLs in their signal molecule recognition system will potentially have a different GSL profile to species which use a different defence gene activation system.

In addition to playing a role in plant defence, it has been suggested that certain GSL types play a part in auxin (Indole-3-acetic acid; IAA) regulation. Auxin is a key regulator of many plant growth and development processes therefore the regulation of its production is of great importance to plant growth. In relation to the role of GSLs, indole GSLs are believed to be precursors for the plant hormone (Halkier and Gershenzon, 2006; Redovnikovic *et al.*, 2008). In one study, an *Arabidopsis* mutant which was deficient in indole GSLs had only partly reduced levels of auxins compared to a wild-type *Arabidopsis* plant (Zhao *et al.*, 2002) suggesting the presence of a second auxin production pathway. This implies that certain GSLs may be present in species which utilise indole GSLs in an auxin production pathway, and that the same GSLs may be absent from other plant species which make use of a separate pathway.

The combination of varying plant defence systems and auxin production pathways between Brassicaceae species could account, in part, for variations between species GSL profiles. The specifics of these systems are not fully understood and further work is required to understand why different GSLs are produced in various species and the functions that they may have.

5.4.3. Brassica juncea *cultivar differences*

Within species variation was low for the two *B. juncea* investigated as they showed similar overall GSL profiles to each other. Nonetheless there were a few differences between cultivars which have been noted in this study when considering the individual GSLs. Two GSLs, gluconapin and gluconasturtiin, were detected in ISCI 99 in significantly higher concentrations than Scala at the first leaf and plant browning growth stages, respectively. Variation in gluconapin content within *B. juncea* has been shown to occur in previous studies where GSL concentration differed significantly between a number of cultivars (Sexton, Kirkegaard and Howlett, 1999; Sodhi *et al.*, 2002). During this study, 4-methoxyglucobrassicin was identified in ISCI 99 samples but absent from Scala; the concentration was low and inconsistently present between experimental replicates therefore there was not a significant difference between the cultivars implying that this is a minor GSL and is unlikely to influence the overall GSL profile or play a role in biofumigation.

The differences between cultivars in this study were considered to be minor, especially as the GSLs in question were found at relatively low concentrations and the differences were identified at the early and late stage of growth, when the plant is unlikely to be incorporated as a biofumigant due to low biomass. As considered in further discussion, several previous studies have identified differences in the GSL profiles of *E. sativa* cultivars (D'Antuono, Elementi and Neri, 2008; Pasini *et al.*, 2012; Bell, Oruna-Concha and Wagstaff, 2015)

suggesting that, in contrast to this study, within species variation can be high, potentially dependent upon the species in question and the environmental conditions during growth.

In this study only two cultivars were compared from *B. juncea*. This is too small a subset to draw conclusions about within species variation on a larger scale. Nevertheless it can be concluded that these cultivars have the same overall GSL profiles and their use as biofumigants should produce similar results. In saying this, there are other factors to consider, such as the level of myrosinase activity (Dosz *et al.*, 2014) and the relative abundance of ITCs produced compared to other degradation products in each species. The measurement of GSL content is a useful indicator for ITC production although relative GSL abundances are not necessarily reflective of the ITC concentrations that will be released (Morra and Kirkegaard, 2002).

5.4.4. Major glucosinolates identified

There was generally one dominating GSL in each of the cultivars that differed between species. The exception to this was Nemat which contained two dominating GSLs whose concentrations changed depending on growth stage. Differences in the major GSL would impact on the cultivar's effectiveness as a biofumigant against PCN, though the presence of a dominant GSL which produces an ITC toxic to other soil-borne pests would be advantageous to identify.

Sinigrin dominated the ISCI 99 and Scala samples which is consistent with a number of previous studies reviewing the GSL content of *B. juncea* (Morra and Kirkegaard, 2002; Gimsing and Kirkegaard, 2006; Bellostas, Sørensen and Sørensen, 2007). Sinigrin in Brassicaceae has been studied extensively in pest control and biofumigation (Zasada, Meyer and Morra, 2009; Lord *et al.*, 2011; Brolsma *et al.*, 2014; Vervoort *et al.*, 2014; Ngala, Woods and Back, 2015a; Zuluaga *et al.*, 2015) and cultivars which contain high concentrations are of considerable interest. In relation to this study, sinigrin is the parent GSL of AITC, which has been shown to be effective against encysted *G. pallida* in Chapter 4; therefore ISCI 99 and Scala are potentially suitable biofumigant cultivars for the control of PCN.

Ida Gold GSL profiles contained a high concentration of glucosinalbin which is consistent with previous studies where glucosinalbin was identified in Ida Gold (Zasada, Meyer and Morra, 2009), and five other *S. alba* cultivars (Kirkegaard and Sarwar, 1998). Concentration varied over plant development with levels highest at 70% flowering which is consistent with the current practice of incorporating at this time. Glucosinalbin breakdown products have been shown to suppress plant-parasitic nematodes *in vitro* (Avato *et al.*, 2013) and when released from *S. alba* seed meal (Zasada, Meyer and Morra, 2009). In addition, *G. pallida*

movement was inhibited after *S. alba* green manures addition *in vitro* (Lord *et al.*, 2011). In comparison, the GSL breakdown product had no effect on *G. rostochiensis* mortality *in vitro* (Buskov *et al.*, 2002) and *S. alba* green manures did not reduce *G. rostochiensis* hatch (Valdes *et al.*, 2011). Glucosinalbin-containing Brassicaceae, specifically *S. alba* cultivars, could be effective biofumigants against free-living nematodes. Further work is still required to determine if *S. alba* and its breakdown products are effective against the encysted nematodes, PCN.

Glucoraphenin was the major GSL in Bento. This is consistent with one previous study (Zhang *et al.*, 2010) but not with a number of others. In one such study glucoraphenin was detected in high concentrations in *R. sativus* with glucoraphasatin being the major GSL (Lord *et al.*, 2011). A second study also identified glucoraphasatin as the major GSL in seven different *R. sativus* cultivars (Neubauer, Heitmann and Müller, 2014). In a separate study, glucoraphanin was identified as the predominant GSL in Bento tissue (Ngala *et al.*, 2014; Ngala, Woods and Back, 2015a). This inconsistency may be due to influencing environmental conditions. Variations between cultivars in the dominant GSL identified imply that within species variation can occur and provides further evidence towards the importance of screening potential biofumigants for GSLs of interest.

Glucoraphenin does not appear to play a major role in biofumigation as its breakdown products were ineffective against *G. rostochiensis* juveniles *in vitro* (Buskov *et al.*, 2002) and a high concentration of the released ITC was required to control the soil-borne fungus, *Verticillium dahliae, in vitro* (Neubauer, Heitmann and Müller, 2014). The contradictions between studies with respect to the major GSL of *R. sativus* cultivars means that a different cultivar to Bento, containing an alternate GSL, may be more effective as a biofumigant.

Nemat contained two dominant GSLs, glucoraphanin and DMB, both of which have been noted in previous *E. sativa* cultivar studies as the major GSLs (Bennett *et al.*, 2007; D'Antuono, Elementi and Neri, 2008; Pasini *et al.*, 2012; Bell, Oruna-Concha and Wagstaff, 2015). In one study, Nemat leaves were found to contain a high concentration of DMB and low concentration of glucoraphanin when sampled as plants began to flower (Lord *et al.*, 2011). This is consistent with the profile obtained at a similar growth stage in the current study. In the same study, Nemat was shown to reduce *G. pallida* viability in soil in a glasshouse trial; as the GSL profile was dominated by DMB it may be advantageous for PCN biofumigants to contain this GSL.

In contrast to these results, a field study by Ngala *et al.* (2014) did not identify glucoraphanin or DMB in Nemat samples, with the profiles dominated by glucobrassicanapin. This GSL was

not detected in Nemat in the current study. The identification of different GSLs is most likely due to changing GSL profiles in response to the influence of environmental conditions and stresses on GSL content.

In the current study, the concentration of both dominant GSLs differed significantly between Nemat growth stages. Differences in growth stages for optimal production of the GSLs and variations in *E. sativa* dominant GSLs between studies further demonstrates the importance of researching the GSL content of potential biofumigant cultivars if a specific GSL is required for pest control. In addition, inconsistencies in identified GSLs implies that GSL content can differ between studies and that external factors can influence GSL production therefore it is important to confirm that the GSL of interest can be formed in field-grown material.

5.4.5. Commonly identified glucosinolates

Two GSLs were identified in all of the cultivars: glucobrassicin and neoglucobrassicin. Although identified in all of the cultivars, neither were present at every growth stage and significant differences in concentration between growth stages were noted for multiple cultivars. In general the concentration was highest between initial flowering and seed development; this was not true for Nemat where the concentration increased with plant age. Previous studies are not consistent in their identification of these GSLs in cultivars of the same species. Both GSLs have been identified in *B. juncea* samples (Fahey, Zalcmann and Talalay, 2001; Bellostas, Sørensen and Sørensen, 2007) and *S. alba* samples (Fahey, Zalcmann and Talalay, 2001). In Bento tissue one or the other was identified in samples from different growing seasons although they were not identified together (Ngala *et al.*, 2014; Ngala, Woods and Back, 2015b). At least one of the GSLs were detected in a number of *E. sativa* cultivars at low concentrations (Kirkegaard and Sarwar, 1998; D'Antuono, Elementi and Neri, 2008; Pasini *et al.*, 2012), including Nemat (Ngala *et al.*, 2014). A separate study found neither GSL in a subset of the same *E. sativa* cultivars (Bell, Oruna-Concha and Wagstaff, 2015). These GSLs have been identified in several Brassicaceae cultivars during a *G. pallida* biofumigation trial, but they were not the major GSLs and any suppressive effect of the cultivars on encysted *G. pallida* was attributed to the breakdown products of more dominant GSLs (Ngala *et al.*, 2014). No other studies have been performed to determine if they have a toxic effect on soil pests. Although common, the GSLs in question were found at low concentrations in this study, as well as previous studies, suggesting that any potential biofumigant effect would be limited.

5.4.6. Glucosinolates identified of importance to this study

Six of the GSLs identified in this study breakdown into ITCs tested in Chapters 3 and 4; sinigrin (AITC), glucotropaeolin (BITC), gluconasturtiin (PEITC), glucoraphanin (sulforaphane; SUL), glucocapparin (MITC) and glucolepiidin (EITC). None of the cultivars contained all of the six GSLs further highlighting the need to screen potential biofumigant cultivars for the GSLs of interest before use. The parent GSLs of four of the ITCs screened against *G. pallida* juveniles in 3.3.1 (propyl – PITC, glucoputranjivin – IITC, phenyl – PHITC and *n*-butyl – BUITC) were not identified in the cultivars from this study. These were the least effective ITCs against *G. pallida* so the lack of presence of their parent GSLs in these cultivars is not a concern when considering suitability for *G. pallida* control.

When studying the GSL profiles of potential biofumigant cultivars it is not only important to determine which GSLs are produced but also to determine if GSL concentration is sufficient to result in the ITC concentration required for pest suppression. As discussed in 1.5.2, measuring GSL content does not accurately predict ITC concentration when released into soil (Gimsing and Kirkegaard, 2009). In one study, only 1% of the GSL was converted to ITC in soil 24hrs after incorporation (Morra and Kirkegaard, 2002). In order to determine the lowest GSL concentration required for a minimum of 50ppm ITC release, a 1% conversion was assumed. 1mg g^{-1} DW GSL would release 10ppm ITC; therefore in order for a minimum concentration of 50ppm ITC to be released, 5mg g^{-1} DW GSL is required.

With respect to this study, sinigrin is the main GSL of interest as it releases AITC which has been shown to suppress *G. pallida* in soil in Chapter 4. Only ISCI 99 and Scala, both *B. juncea*, produced this GSL with growth stage having no effect on concentration. Sinigrin was present between concentrations of 19.98-45.40mg g^{-1} DW which is consistent with previous studies (Bellostas, Sørensen and Sørensen, 2007; Ngala, Woods and Back, 2015a). Assuming a 1% conversion, GSL levels in this study would release minimum AITC concentrations between 200-454ppm. Data from Chapter 4 showed that 500ppm AITC in soil was required for hatch suppression explained entirely by juvenile mortality. Lower concentrations of 250ppm were also effective at significantly reducing *G. pallida* populations and increasing juvenile mortality *in vitro*. Assuming a 1% GSL to ITC conversion, sinigrin concentrations from this study do not quite reach levels required for 500ppm AITC production, although both cultivars exceed the levels required to produce 250ppm AITC at certain growth stages. In addition, higher ITC concentrations are possible if the GSL to ITC conversion efficiency is increased by providing adequate moisture, a neutral soil pH and ensuring maximum GSL

hydrolysis through increased contact with myrosinase by efficiently chopping the plant material (Morra and Kirkegaard, 2002).

The highest sinigrin concentration was present at seed development and initial flowering of ISCI 99 and Scala, respectively. These results are not consistent with the current practice of incorporating biofumigant cultivars when the plant reaches 50-70% flowering but concentrations in both cultivars are sufficient to produce over 250ppm AITC if incorporated at this stage. For both cultivars, a higher average concentration of sinigrin was produced at initial flowering proposing that the incorporation of these cultivars as biofumigant material should occur earlier in the plant's life cycle for optimised GSL content and ITC release.

Glucoraphanin was identified in Bento and Nemat at every growth stage. The GSL concentration in Bento has the potential to release over 50ppm SUL with samples at first leaf able to release 93.28ppm SUL; this is consistent with an earlier study where *R. sativus* leaf material contained high concentrations of glucoraphanin when the plant had flowered fully (Ngala, Woods and Back, 2015a). Nemat contained high concentrations of glucoraphanin with the maximum concentration at seed development potentially releasing 201.38ppm SUL and the lowest concentration at first leaf able to release just under 50ppm SUL; this is inconsistent with an earlier study which identified glucoraphanin in *E. sativa* cultivars, including Nemat, at concentrations <1.5mg g^{-1} DW with the potential minimum release of 15ppm SUL (Lord *et al.*, 2011).

In the current study, differences in GSL concentration over Nemat growth stages were significant and further emphasises that GSL profiles change with plant development and that the optimal incorporation stage will differ depending on cultivar and desired GSL. In this case Nemat at seed development released the highest SUL concentrations. Experiments in Chapter 3 showed that 50ppm SUL was able to increase *G. pallida* mortality but did not affect encysted juveniles; as glucoraphanin concentrations in these cultivars could hypothetically produce higher ITC concentrations than previously tested there is the potential that they could be used as *G. pallida* biofumigants. In a previous study, Bento leaf material containing 11.11mg g^{-1} DW glucoraphanin had little effect on *G. pallida* suppression *in vitro,* except at high concentrations (50-100% w/v; Ngala, Woods and Back, 2015a). An earlier field study found that Bento, containing 10-22mg g^{-1} DW glucoraphanin, was able to reduce *G. pallida* viability in soil (Ngala *et al.*, 2014). Differences between studies are most likely due to the added stem and organ tissue in the field experiment contributing to the biofumigant effect. Inconsistencies between results illustrate that further research is required to determine how

useful glucoraphanin would be as a *G. pallida* biofumigant and at what concentration for high SUL release.

Glucotropaeolin and gluconasturtiin were identified in a number of cultivars. Although there were significant differences in GSL concentration between cultivar and growth stage, concentration did not exceed 5mg g^{-1} DW. Gluconasturtiin and glucotropaeolin concentrations in above-ground material from this study are consistent with results from previous studies. Both GSLs have been identified in *S. alba* (Kirkegaard and Sarwar, 1998; Fahey, Zalcmann and Talalay, 2001) and *E. sativa* cultivars (D'Antuono, Elementi and Neri, 2008), with *B. juncea* cultivars containing gluconasturtiin but not glucotropaeolin (Kirkegaard and Sarwar, 1998; Gimsing and Kirkegaard, 2006; Bellostas, Sørensen and Sørensen, 2007). The identification of these GSLs does depends on the cultivar as several studies have not detected the GSLs in the species investigated (Fahey, Zalcmann and Talalay, 2001; Pasini *et al.*, 2012; Bell, Oruna-Concha and Wagstaff, 2015). GSL concentrations identified in this study would produce less than 50ppm of the corresponding ITC (BITC and PEITC for glucotropaeolin and gluconasturtiin, respectively); results from Chapters 3 and 4 show that 50ppm BITC and PEITC are unable to suppress *G. pallida* hatch *in vitro* therefore cultivars which contain these GSLs and produce these ITCs are unlikely to be effective *G. pallida* biofumigants.

The GSLs which breakdown into MITC and EITC (glucocapparin and glucolepiidin, respectively) were only produced at one growth stage of one cultivar and not consistently within technical replicates indicating that they are not commonly produced GSLs. In addition, the GSL concentrations were so low that less than 1ppm ITC would be produced. A similar concentration of glucolepidiin has been noted in several *E. sativa* cultivars (D'Antuono, Elementi and Neri, 2008; Bell, Oruna-Concha and Wagstaff, 2015) and other studies have not detected it (Fahey, Zalcmann and Talalay, 2001; Pasini *et al.*, 2012). As neither MITC nor EITC were able to reduce hatch at 50ppm in Chapter 3 then it is unlikely that either parent GSL will be present at a high enough concentration in a *G. pallida* biofumigant cultivar to be considered an important GSL. Furthermore, 12.5-25ppm MITC enhanced hatch in Chapter 4; the potential release of MITC from glucocapparin in Ida Gold is so low (<1ppm MITC) that incorporating Ida Gold as a biofumigant should not increase *G. pallida* populations in soil. Other *S. alba* cultivars have not been shown to contain glucocapparin (Fahey, Zalcmann and Talalay, 2001) so using another cultivar as a biofumigant is unlikely to pose the same risk with respect to MITC and enhanced hatch. Due to these results it is unlikely that EITC or MITC will be released from these cultivars at levels able to suppress *G. pallida.*

5.4.7. Concluding remarks

This study provides information on the GSL profiles of five potential biofumigant cultivars. Total GSL production was influenced by plant growth stage with the lowest GSL concentrations produced at the start of the plant's life cycle and the highest between initial flowering and 70% flowering. The number and type of GSLs produced in each cultivar differed depending on the plant species with each displaying significantly different GSL profiles; this high variation exhibits the need to screen potential biofumigant cultivars for effective GSLs to targeted pests.

With respect to *G. pallida* control using sinigrin-containing biofumigants, only two cultivars appear to be suitable: ISCI 99 and Scala, both *B. juncea* cultivars. Although sinigrin concentration did not significantly vary between growth stage and cultivar, optimal incorporation time appears to be at initial flowering for Scala and seed development for ISCI 99. These differences in concentration between growth stages shows a need to determine GSL profiles of cultivars over various stages in the plant's life cycle in order to establish the optimal incorporation stage for maximum ITC release.

Differences in GSL concentration between growth stage and cultivar were noted with other GSLs implying that the optimal stage of incorporation will differ between biofumigant cultivars depending on the GSL of interest.

Although Ida Gold, Bento and Nemat did not produce sinigrin, they did contain GSLs in high concentrations whose breakdown ITCs have not been assessed in previous chapters: glucosinalbin, glucoraphenin and DMB, respectively. In addition, Nemat produced the parent GSL of SUL with the potential to release four-times the highest concentration tested in Chapter 3. The effect of these breakdown products on encysted *G. pallida* has not been greatly researched therefore further work is required to evaluate the cultivars suitability as effective *G. pallida* biofumigants. This has been explored in Chapter 6.

Chapter 6. Assessing the Effect of Biofumigation on Encysted *G. pallida* in Pot and Field Trials

6.1. Introduction

Previous chapters have investigated the effect of pure isothiocyanates (ITCs) on *Globodera pallida* where several were effective at increasing juvenile (J2) mortality *in vitro* with one, AITC, capable of suppressing encysted *G. pallida in vitro* and in soil. In Chapter 5, two biofumigant cultivars contained high concentrations of the AITC parent glucosinolate (GSL), sinigrin. The three other cultivars assessed contained different dominant GSLs whose breakdown ITC products have not been evaluated against encysted *G. pallida* in previous chapters. Further experimentation is required in order to determine if these cultivars and GSLs would be effective as *G. pallida* biofumigants.

Although positive results have been collected from *in vitro* experiments in Chapters 3 and 4 it is known that these effects may not be as pronounced when moving into biofumigant glasshouse and field trials (Brolsma *et al.*, 2014). This is due to several factors: GSL content is highly variable between and within cultivars, ITC conversion and release from plant material is not consistent, introducing soil as a factor reduces the direct contact of released ITCs to the encysted *G. pallida* J2s, and environmental conditions can affect the growth of plants and the resulting biomass of material at time of incorporation; this is discussed in detail in Chapter 1.5. As a result of these added variables when moving from *in vitro* ITC studies to biofumigant trials it is important to assess the robustness of observed effects on *G. pallida* under controlled glasshouse conditions and in field trials before implementing as a potato cyst nematode (PCN) control strategy.

Previous studies have not produced consistent results when investigating the effect of biofumigant green manures on PCN in soil. One study found that incorporating *Sinapis alba*, *Brassica napus* and *Raphanus sativus* cultivars as green manure had no effect on *Globodera rostochiensis* hatch under glasshouse conditions (Valdes *et al.*, 2011). Similarly, *S. alba* had no effect on *G. rostochiensis* hatch in a field trial (Valdes, Viaene and Moens, 2012). In contrast, a more recent study noted a significant effect of *S. alba*, *B. napus* and *R. sativus* green manures on *G. rostochiensis* viability and multiplication under glasshouse conditions (Fatemy and Sepideh, 2016). Differences between studies in *G. pallida* suppression have also been seen: Brolsma *et al.* (2014) did not observe an effect of *B. juncea* green manure on *G. pallida* hatch under glasshouse conditions whereas Ngala *et al.* (2014) observed a decrease in the formation of new *G. pallida* cysts and viability of eggs post-potato harvest after exposure to *B. juncea* and *R. sativus* green manures in a field trial. Inconsistencies between studies

highlight how variation between different cultivars and experimental parameters can greatly impact the efficiency of biofumigation for PCN control.

In the current study, the effect of five potential biofumigant cultivars on encysted *G. pallida* was investigated in pot and field trials. Cultivars were chosen due to their inclusion in previous studies and because they are sold as commercial biofumigants. In the first pot trial, Brassicaceae material was grown and incorporated into the pots that they were grown in and compared to three controls: a Fallow (no plant) control, a low-GSL *Brassicaceae* cultivar, and a nematicide which kills hatched J2. Due to differences in biomass at incorporation resulting in high variation between repeats, a second pot trial was completed where Brassicaceae material was grown and combined before being split equally between pots at incorporation to address and reduce this variability. In both pot trials the effect of Brassicaceae material on *G. pallida* hatch during growth and after incorporation as well as subsequent *G. pallida* multiplication on potatoes was investigated.

Two field trials were completed, at locations which contained natural PCN populations, in order to determine the effectiveness of biofumigation on a larger scale and under field conditions. In the first trial, all cultivars were included. The effect of Brassicaceae material on *G. pallida* hatch during growth and after incorporation as well as *G. pallida* multiplication on potatoes was investigated. Before potato crop planting, a nematicide was applied to one half of each plot in order to determine if the combination of biofumigation and nematicide application affected *G. pallida* multiplication. In the second field trial, two cultivars, Nemat and Scala, were excluded due to poor results in the previous trial. As no potato crop was planted in the second field trial following biofumigation, only the effect of Brassicaceae material on *G. pallida* hatch during growth and after incorporation was investigated.

GSL profiles of Brassicaceae material at time of incorporation were analysed using liquid chromatography-mass spectrometry (LC-MS) to provide insight into results collected relating to GSL content and potential ITC release. The GSL profiles of all cultivars were determined for the second pot trial and first field trial. The GSL profiles of the *B. juncea* cultivars alone were examined in the first pot trial and no samples were collected from the second field trial due to a lack of positive results, time constraints and access to resources.

The main aims of this study were to:

- Assess the ability of growing and incorporating Brassicaceae material to suppress *G. pallida* hatch under glasshouse and field conditions

- Investigate the result of incorporating biofumigants on encysted *G. pallida* viability under controlled conditions
- Determine the effect of biofumigation on *G. pallida* multiplication under glasshouse and field conditions
- Define and compare the GSL profiles of Brassicaceae cultivars at time of incorporation under glasshouse and field conditions to identify key GSLs and concentrations

6.2. Materials and Methods

6.2.1. Plant cultivars

Brassicaceae seeds were sown during field trials at the recommended seed rates and during pot trials at the equivalent seed rates scaled down to the area of the pots used (Table 6.1). Pot trial seed rates were calculated from the seed rates used in the first field trial except Temple, which was scaled down from 20kg ha^{-1}, and Bristle Oats, which was calculated from the second field trial seed rates.

		Seed Rate			
Species	**Cultivar**	**Pot Trial 1 (mg 2 L pot^{-1})**	**Pot Trial 2 (mg 5 L pot^{-1})**	**Field Trial 1 (kg ha^{-1})**	**Field Trial 2 (kg ha^{-1})**
Raphanus sativus	**Bento**	45.40	83.10	20	15
Sinapis alba	**Ida Gold**	15.89	29.10	7	8
Brassica juncea	**ISCI 99**	20.40	37.40	9	8
Eruca sativa	**Nemat**	13.62	24.90	6	-
Brassica juncea	**Scala**	20.40	37.40	9	-
Brassica napus	**Temple**	45.40	83.10	-	-
Avena strigosa	**Bristle Oats**	-	332.40	-	80

Table 6.1. Plant treatments and seed rates applied in each trial. 2 L pots have a 227cm^2 surface area and 5 L pots have a 415.5cm^2 surface area.

6.2.2. Pot trials

Two pot trials investigating the effect of incorporating green manures on *G. pallida* viability and multiplication were set up under glasshouse conditions in pots three-quarter filled with John Innes No. 2 soil.

In the first pot trial, three muslin bags containing thirteen cysts each were placed in 2 L pots. Thirteen cysts were chosen as two viability assays were completed; five cysts were included

in a hatching assay and eight cysts were included in a trehalose viability assay (van den Elsen *et al.*, 2012). Results from the second viability assay were inconsistent and unreliable (data not shown) so it was decided to exclude the analysis from this study. Seeds of five potential biofumigant cultivars were then sown at the indicated seed rate in Table 6.1 and left to grow for eight weeks (Figure 6.1). Additionally, a Fallow negative and low-GSL (Temple) green manure control was included. Six pots were prepared and left empty until two days before potato planting for the nematicide positive control. Six replicates of each treatment were included and pots were arranged in a randomised block design layout. After eight weeks one cyst bag was removed and subsets of five cysts were subjected to a hatching assay as described in 2.3.2 in order to determine if the growth of GSL-producing cultivars suppressed hatch. ISCI 99 and Scala plant samples were collected from four of the six replicates, as in 2.4.5, freeze-dried and stored in preparation for LC-MS analysis.

All above-ground material from each pot was roughly chopped, weighed (data not shown) and blended with 200 mL H_2O before being incorporated back into the same pot and sealed for three weeks (Figure 6.2). 200 mL H_2O was the smallest volume required to efficiently blend the largest biomass of plant material. Pots were then unsealed, one cyst bag was removed and subsets of five cysts were subjected to a hatching assay (2.3.2). At this time, 125mg Vydate® (DuPont Ltd, UK; scaled down from the recommended 55kg ha^{-1} field rate) was incorporated to the prepared pots as a positive nematicide control. Two days later Desiree potato tubers were planted in each pot containing the remaining cyst bag and PCN multiplication proceeded as 2.4.3. Newly formed cysts were collected as described in 2.6.1. The number of new cysts was counted for each sample and Meldola's Blue dye (MB) stain (2.3.3) was applied to a subsample of ten cysts to determine total number of new eggs; when less than ten new cysts were counted the entire sample was analysed.

Figure 6.1. Cultivar growth at time of incorporation in the first pot trial.

Figure 6.2. Sealed pots after the incorporation of plant material in the first pot trial.

In the second pot trial, a muslin bag containing ten cysts was placed in 5 L pots. Seeds of five potential biofumigant cultivars were sown at the indicated seed rate in Table 6.1 and left to grow for eight weeks. In addition to the five cultivars of interest, a low-GSL (Temple) green manure control and non-GSL (Bristle Oats) catch crop control was incorporated. Four

replicates of each treatment were included and pots were arranged in a randomised block design layout. After eight weeks the cyst bag was removed and the ten cysts were subjected to a hatching assay as described in 2.3.2. Plant samples from all cultivars were collected (2.4.5) and freeze-dried in preparation for LC-MS analysis.

To determine the biomass of material that would be incorporated for each plant treatment the above-ground plant material from the four replicates of each cultivar were combined and the total fresh weight was recorded. This was divided by the number of replicates for each treatment to determine the maximum fresh weight (g) of material that would be incorporated into 2 L pots (Table 6.2).

In addition to controlling the biomass of plant material incorporated between treatment replicates, the moisture content of the soil at incorporation was standardised as each cultivar contained different H_2O contents. A known weight of plant material for each cultivar was dried out completely at 37°C for three days and the difference in weight, attributed to H_2O loss, was recorded. This was used to determine the H_2O content of the total fresh weight of material incorporated in each pot. Six 2 L pots of dry soil were weighed and averaged and the volume of H_2O required to provide 40% soil moisture content was calculated from the soil weights. The H_2O content of the cultivar treatments was then subtracted from the total volume of H_2O necessary for 40% soil moisture content, leaving the amount of added H_2O required for each cultivar replicate (Table 6.2). 40% soil moisture content was chosen as this was the recommended water content for completing the bacterial analysis in Chapter 7.

Two muslin bags containing ten cysts each were placed in 2 L pots. The biofumigant material was blended with H_2O (Table 6.2) before being incorporated into pots and sealed for four weeks. In addition to the seven plant treatments, a Fallow negative control was included where 342 mL H_2O was mixed into soil. Six pots were prepared and left empty until two days before potato planting for the nematicide positive control. Six replicates of each treatment were included and pots were arranged in a randomised block design layout. After four weeks: pots were unsealed, one cyst bag was removed, and the cysts were subjected to a hatching assay and MB stain as described in 2.3.2 and 2.3.3. At this point, 125mg Vydate® was incorporated to the prepared pots as a positive nematicide control. Two days later, Desiree potato tubers were planted in each pot containing the remaining cyst bag and PCN multiplication proceeded as described in 2.4.3 (Figure 6.3). Newly formed cysts were collected from the soil as described in 2.6.1. The number of new cysts was counted for each sample and a hatching assay followed by MB stain (2.3.2 and 2.3.3) was applied to a

subsample of ten cysts to determine total number of new eggs and their viability; when less than ten new cysts were counted the entire sample was analysed.

Treatment	Fresh material (g 2 L pot^{-1})	Fresh material (t ha^{-1})	Added H_2O (mL 2 L pot^{-1})
Bento	120.00	52.86	233.67
Ida Gold	146.67	64.61	216.80
ISCI 99	100.00	44.05	252.02
Nemat	26.67	11.75	322.99
Scala	106.67	46.99	246.77
Temple	115.00	50.66	243.48
Bristle Oats	40.00	17.62	310.62
Fallow	-	-	342.40
Vydate®	-	-	342.40

Table 6.2. Biomass incorporated, comparative biomass if in the field and H_2O added to achieve 40% soil moisture content in the second biofumigant trial. 2 L pots have a 227cm^2 surface area.

Figure 6.3. Potato growth during PCN multiplication in the second pot trial. Flowers were removed from growing plants as they appeared in order to make potato growth last longer and to ensure the efficient production of new cysts.

6.2.3. Field trials

Two field trials were completed with soil sampling and plant incorporation occurring as described in 2.5. The first field trial was carried out in a sandy silt loam soil type with a pH of 7.4 in Lincolnshire, UK (53°00'03.5"N 0°17'22.3"W; Appendix E). Plots were marked out 3m

x 8m with six plots per row in five columns; there was a 0.5m gap between columns and 1m gap between rows. Plots were arranged in a randomised complete block design layout as shown in Appendix F. PCN soil samples were collected before seed addition on 31/7/14 where cysts were extracted (2.6.1) and a hatching assay was completed on subsets of five cysts (2.3.2). Seeds of five potential biofumigant cultivars were sown at the indicated seed rate in Table 6.1 and left to grow for ten weeks. A Fallow negative control was included in the trial. Five replicates of each treatment were performed. PCN soil samples were collected during plant growth, prior to incorporation, on 14/10/14 where cysts were extracted (2.6.1) and a hatching assay was run on subsets of five cysts (2.3.2). Leaf material was collected at time of incorporation (14/10/14) from four of the five replicates of each cultivar and freeze-dried in preparation for LC-MS analysis. Green manure plant material was then incorporated (Figure 6.4). Post-incorporation PCN soil samples were collected four months post-incorporation on 1/2/15 where cysts were extracted (2.6.1) and a hatching assay was run on subsets of five cysts (2.3.2). At potato planting, the nematicide Nemathorin® 10G (Syngenta, UK) was applied to half of each plot in a split-plot design (Appendix F) at the manufacturers recommended rate of 30kg ha^{-1}. A Maris Piper potato crop was sown on 20/4/15. Post-potato harvest PCN soil samples were collected on 30/9/15 from both the nematicide and non-nematicide treated halves of the plots. Cysts were extracted (2.6.1) and the number of new cysts was counted for each sample; an MB stain (2.3.3) was applied to subsamples of five cysts to determine total number of new eggs. Due to differences in total soil per sample, soil was weighed and results were displayed per g soil.

Figure 6.4. Plots at the time of incorporation in the first field trial. This is taken standing at the top right of plot 303 facing north-east. In order of appearance from the first full plot starting top left clockwise: Ida Gold, Bento, Fallow, Scala, Fallow and Bento.

The second field trial was carried out in a sandy silt loam soil type with a pH of 8.0 in Lincolnshire, UK (52°50'55.8"N 0°02'04.6"E; Appendix E). Plots were marked out 7m x 2m with four plots per row in seven columns; there was a 1m gap between columns and 1.5m gap between rows. Plots were arranged in a randomised complete block design layout as shown in Appendix F. PCN soil samples were collected before sowing on 4/9/15 where cysts were extracted (2.6.1) and a hatching assay (2.3.2) was run on subsets of five cysts. Seeds of three potential biofumigant cultivars and one negative plant control were sown at the indicated seed rate in Table 6.1 and left to grow for eight weeks (Figure 6.5). A Fallow negative control was also included in the trial. Five replicates of each treatment were performed. PCN soil samples were collected before plant incorporation on 2/11/15 where cysts were extracted from a known volume of soil (2.6.1) and a hatching assay (2.3.2) was run on subsets of five cysts. Green manure plant material was then incorporated. Post-incorporation PCN soil samples were collected twenty weeks later on 23/3/16 where cysts were extracted (2.6.1) and a hatching assay (2.3.2) was run on subsets of ten cysts from each plot; in order to compare hatch to the previous sampling points, the final total hatch was halved.

Figure 6.5. Plots during cultivar growth in the second field trial. This is taken standing at the top right of plot 401 facing south-west.

6.2.4. LC-MS analysis of Brassicaceae species

Plant material from the *B. juncea* cultivars in the first pot trial, all cultivars in the second pot trial and all cultivars in the first field trial were collected and freeze-dried. Four replicates of each cultivar were analysed. Collected plant material was prepared (2.8.1), GSLs extracted (2.8.2) and LC-MS analysis run (2.8.3) on two technical replicates of each sample by Dr Luke Bell, Department of Food & Nutritional Sciences, University of Reading.

6.2.5. Analysis of LC-MS results

Compounds were identified and quantified as described in 2.8.4. See Appendix D for a list of GSLs identified, retention times, primary ions and relative response factors. GSL concentrations (mg g^{-1} dry weight (DW)) were determined from samples and used to analyse differences in GSL profiles between cultivars and trials at time of incorporation.

6.2.6. Statistical data analysis

Data was analysed as in 2.10.1. One-way ANOVA analysis was performed with Treatment as a factor in the 6.3.1 viability and multiplication analysis. Cultivar was the factor in the 6.3.3 GSL ANOVA analysis. Two-way ANOVA analysis was performed with Treatment x Sampling Point as factors in the 6.3.1 and 6.3.2 hatching assays and Cultivar x Trial in the 6.3.3 sinigrin concentration analysis. Treatment was the factor investigated in the 6.3.2 multiplication analysis, with Nematicide Application being included as a sub-plot factor when considering the split plot design. Significant ANOVA P-values (P<0.05) were further investigated using the means comparison test, Tukey's HSD test.

6.3. Results

6.3.1. Biofumigation and encysted G. pallida *viability and multiplication under glasshouse conditions*

There was a significant treatment and treatment x sampling point effect on hatch in the first pot trial in a hatching assay (Table 6.3). During plant growth there were no differences in hatch. Post-incorporation there was a significant difference in hatch between Scala and the Ida Gold, Bento, ISCI 99 and Temple treated soils as well as between the Ida Gold and Nemat treatments. There were no differences between the Fallow control and biofumigant treatments post-incorporation or between individual treatment samples pre- and post-incorporation.

	Total *G. pallida* hatch	
Treatment	**During Growth**	**Post-incorporation**
Fallow	112.33 (±31.53)^{abc}	33.50 (±9.08)^{abc}
Bento	69.50 (±40.03)^{abc}	13.50 (±6.66)^{ab}
Ida Gold	57.00 (±25.95)^{abc}	11.00 (±5.33)^{a}
ISCI 99	63.83 (±11.11)^{abc}	142.33 (±92.64)^{ab}
Nemat	93.17 (±15.84)^{abc}	283.50 (±126.86)^{bc}
Scala	43.50 (±18.34)^{abc}	247.17 (±56.66)^{c}
Temple	111.33 (±27.58)^{abc}	17.67 (±4.96)^{ab}
	ANOVA P-values	
Treatment	**0.014**	
Sampling Point	0.101	
T x SP	**0.004**	

Table 6.3. Hatch of *G. pallida* J2s in the first pot trial and associated ANOVA P-values. The standard errors are indicated within brackets. For all data, means followed by the same letter are not significantly different (P<0.05).

PCN multiplication was unaffected by the incorporation of Brassicaceae green manures (Table 6.4). The Vydate® nematicide treatment significantly reduced the number of newly formed cysts 2 L pot^{-1} and eggs 2 L pot^{-1} compared to the Fallow control; the number of eggs cyst^{-1} was unaffected.

Treatment	**Cysts 2 L pot^{-1}**	**Eggs 2 L pot^{-1}**	**Eggs Cyst^{-1}**
Fallow	48.00 (±13.27)	3802.67 (±1576.75)	71.00 (±8.68)
Bento	21.25 (±11.50)	2383.00 (±1443.78)	75.00 (±18.60)
Ida Gold	47.60 (±21.58)	4014.40 (±1719.98)	83.00 (±7.17)
ISCI 99	18.83 (±10.10)	2364.00 (±1405.69)	76.67 (±22.71)
Nemat	48.83 (±21.60)	5921.67 (±3173.25)	95.00 (±12.77)
Scala	64.00 (±25.02)	5567.50 (±3054.11)	51.00 (±19.05)
Temple	45.33 (±20.35)	3465.67 (±1471.61)	60.00 (±19.13)
Vydate®	**4.00 (±1.73)**	**128.67 (±72.26)**	38.00 (±17.22)

Table 6.4. The number of new cysts 2 L pot^{-1}, eggs 2 L pot^{-1} and eggs cyst^{-1} post-multiplication in the first pot trial. Standard errors are indicated within brackets. Significant differences (P<0.05) compared to the Fallow control are in bold based on a two-sample t-test.

In the second pot trial, there was an effect of treatment, sampling point and the interaction between the two on *G. pallida* hatch (Table 6.5). During plant growth there were no significant differences between biofumigant cultivars and plant controls. Post-incorporation, ISCI 99 and Scala treatments significantly reduced hatch compared to all other treatments during growth and post-incorporation. None of the other treatments had an effect on hatch compared to the controls and, with the exception of the *B. juncea* treatments, there were no differences before and after plant incorporation for each individual treatment.

	Total *G. pallida* hatch	
Treatment	**During Growth**	**Post-incorporation**
Fallow	-	826.33 (±166.58)[b]
Bento	786.00 (±304.53)[b]	870.00 (±167.46)[b]
Ida Gold	1034.00 (±94.04)[b]	846.00 (±103.27)[b]
ISCI 99	858.00 (±193.81)[b]	**97.00 (±44.66)[a]**
Nemat	755.75 (±293.33)[b]	1013.67 (±139.31)[b]
Scala	1149.50 (±168.36)[b]	**39.33 (±19.44)[a]**
Temple	955.00 (±124.01)[b]	888.67 (±45.68)[b]
Bristle Oats	539.00 (±118.92)[b]	1143.00 (±138.08)[b]
	ANOVA P-values	
Treatment	**<0.001**	
Sampling Point	**0.003**	
T x SP	**<0.001**	

Table 6.5. Hatch of *G. pallida* J2s in the second pot trial and associated ANOVA P-values. Standard errors are indicated within brackets. For all data, means followed by the same letter are not significantly different ($P<0.05$). Treatments in bold are significantly different to the Fallow control within sampling point.

Post-incorporation, the percentage of dead and hatched J2 were significantly affected by treatment ($P<0.001$ for both; Figure 6.6). Both ISCI 99 and Scala increased mortality with a corresponding decrease in hatched J2 compared to the other treatments. Although there was not an overall effect of treatment on unhatched viable J2 ($P=0.096$), ISCI 99 and the Fallow control differed in the percentage of unhatched viable J2 in a means comparison test.

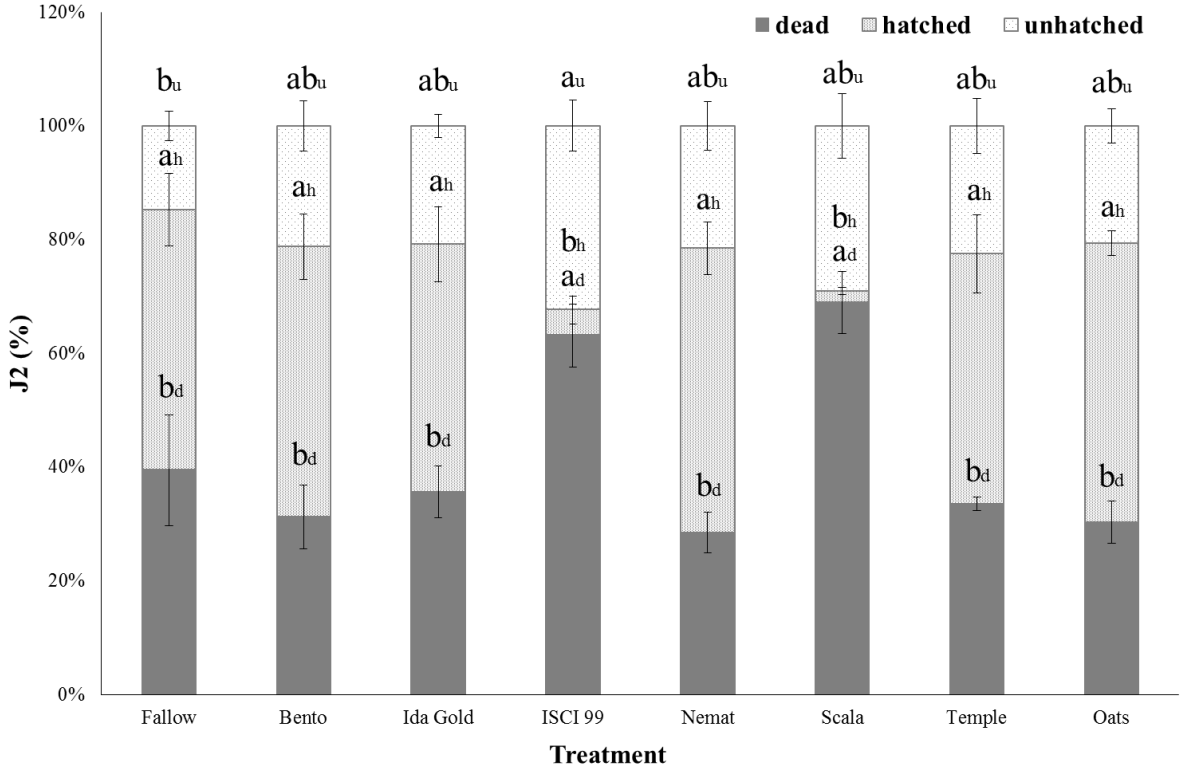


Figure 6.6. Percentage of *G. pallida* J2 that were: dead, hatched and unhatched viable four weeks post-incorporation in the second pot trial. Error bars represent the standard errors. Significant differences (P<0.05) are shown by different letters; the categories to which these relate are indicated by one of the subscript letters: d (dead) h (hatched) u (unhatched viable).

Post-multiplication on a potato crop, the number of newly formed cysts 5 L pot^{-1} and eggs 5 L pot^{-1} were significantly affected by treatments but the number of eggs cyst^{-1} was unaffected (Table 6.6).

The number of new cysts was significantly lower after treatment with ISCI 99 compared to the: Bristle Oats control, Fallow control, and Bento and Nemat treatments. Similarly, Scala treatments significantly reduced the formation of new cysts compared to: Fallow, Bento and Nemat; cyst number was not lower than the plant or nematicide controls. Nemat incorporation led to an increased number of new cysts compared to Ida Gold and the Temple and Vydate® controls. None of the other treatments affected the formation of new cysts compared to the controls.

The number of eggs 5 L pot^{-1} was significantly lower in ISCI 99 and Scala treated pots compared to the Fallow and Nemat treated pots. ISCI 99 also significantly lowered egg number compared to Bristle Oats and Bento. There were no other effects on egg numbers between treatments. ISCI 99 and Scala significantly reduced the number of viable eggs 5 L pot^{-1} compared to Fallow and Nemat treatments. In addition, ISCI 99 incorporation reduced egg viability compared to Bento incorporation. When studying the egg content of individual cysts, there were no differences with respect to eggs cyst^{-1} or viable eggs cyst^{-1}.

Treatment	Cysts 5 L pot^{-1}	Eggs 5 L pot^{-1}	Viable Eggs 5 L pot^{-1}	Eggs Cyst^{-1}	Viable Eggs Cyst^{-1}
Fallow	60.67 (±13.28)^{cd}	11561.65 (±2364.98)^{c}	5955.65 (±1772.64)^{c}	199.47 (±34.89)	101.47 (±25.36)
Bento	39.50 (±7.04)^{cd}	6891.02 (±1666.30)^{bc}	3885.68 (±1194.12)^{bc}	167.15 (±11.35)	91.15 (±12.96)
Ida Gold	18.17 (±9.09)^{abc}	4785.55 (±2517.21)^{abc}	2462.22 (±1390.31)^{abc}	262.54 (±50.78)	148.54 (±39.86)
ISCI 99	**4.50 (±3.04)^{a}**	**843.58 (±570.54)^{a}**	**578.25 (±413.65)^{a}**	135.72 (±61.05)	84.72 (±33.15)
Nemat	93.17 (±19.07)^{d}	18341.30 (±3829.57)^{c}	10476.30 (±2249.30)^{c}	200.63 (±25.21)	115.30 (±17.99)
Scala	**5.33 (±2.35)^{ab}**	**886.18 (±518.41)^{ab}**	**584.85 (±372.41)^{ab}**	154.43 (±43.78)	99.77 (±30.70)
Temple	25.83 (±8.75)^{abc}	6308.78 (±2511.98)^{abc}	3932.12 (±1686.01)^{abc}	181.28 (±50.61)	108.62 (±34.75)
Bristle Oats	22.00 (±4.57)^{bcd}	5195.32 (±1620.00)^{bc}	2973.98 (±1124.06)^{abc}	216.71 (±27.45)	119.05 (±26.23)
Vydate®	20.67 (±9.77)^{abc}	4696.07 (±2592.54)^{abc}	2711.40 (±1571.87)^{abc}	159.28 (±42.64)	94.28 (±28.76)
			ANOVA P-values		
Treatment	**<0.001**	**<0.001**	**<0.001**	0.307	0.684

Table 6.6. The number of new cysts 5 L pot^{-1}, eggs 5 L pot^{-1}, viable eggs 5 L pot^{-1}, eggs cyst^{-1} and viable eggs cyst^{-1} post-multiplication in the second pot trial and associated ANOVA P-values. The standard errors are indicated within brackets. Within columns, means followed by the same letter are not significantly different (P<0.05). Treatments in bold are significantly different to the Fallow control.

6.3.2. Biofumigation and encysted G. pallida *hatch and multiplication under field conditions*

Growing and incorporating Brassicaceae material in the first field trial had no effect on *G. pallida* hatch (Table 6.7).

		Total *G. pallida* hatch	
Treatment	**Before Planting**	**During Growth**	**Post-incorporation**
Fallow	305.40 (±106.76)	583.00 (±217.17)	322.40 (±74.44)
Bento	345.80 (±143.76)	314.60 (±108.48)	428.60 (±123.35)
Ida Gold	617.20 (±61.29)	540.60 (135.79)	306.00 (±79.76)
ISCI 99	571.20 (±143.76)	293.20 (±112.57)	175.80 (±23.81)
Nemat	435.00 (±168.78)	402.40 (±163.68)	377.20 (±97.97)
Scala	538.80 (±73.28)	376.00 (±231.26)	398.80 (±210.50)

Table 6.7. Hatch of *G. pallida* J2s in the first field trial. The standard errors are indicated within brackets.

There was a significant effect of nematicide application on the number of newly formed cysts g soil^{-1} and eggs g soil^{-1} as well as an overall significant effect of treatment on eggs cyst^{-1} five months after potato planting (Table 6.8). Nematicide application led to an average of 0.30 (±0.02) cysts g soil^{-1} and 21.70 (±2.85) eggs g soil^{-1} compared to plots in the absence of nematicide application where there was an average of 0.56 (±0.04) cysts g soil^{-1} and 44.18 (±6.37) eggs g soil^{-1}. The number of cysts g soil^{-1} was significantly reduced after nematicide addition in the Fallow and Nemat plots compared to the non-nematicide Fallow and Nemat plots. There were no differences between treatments and the control in cyst number independent of nematicide application. There was an overall significant effect of treatment on eggs cyst^{-1} but no differences between treatments in a means comparison test.

Nematicide Application	Treatment	Cysts g soil^{-1}	Eggs g soil^{-1}	Eggs Cyst^{-1}
Nemathorin®	**Fallow**	0.26 (±0.04)^{a}	21.09 (±6.08)	80.00 (±15.85)
	Bento	0.37 (±0.08)^{abcd}	17.24 (±5.32)	45.60 (±6.91)
	Ida Gold	0.29 (±0.03)^{abc}	27.48 (±10.10)	87.20 (±22.99)
	ISCI 99	0.27 (±0.03)^{ab}	17.32 (±5.96)	58.00 (±16.25)
	Nemat	0.29 (±0.03)^{abc}	31.13 (±9.92)	101.60 (±23.85)
	Scala	0.30 (±0.07)^{abc}	36.57 (±6.50)	56.80 (±5.78)
None	**Fallow**	0.50 (±0.04)^{bcd}	39.09 (±3.85)	78.40 (±8.52)
	Bento	0.68 (±0.14)^{d}	58.29 (±30.40)	68.80 (±21.66)
	Ida Gold	0.48 (±0.08)^{abcd}	46.66 (±8.35)	98.00 (±12.51)
	ISCI 99	0.52 (±0.05)^{bcd}	26.26 (±6.67)	49.20 (±11.29)
	Nemat	0.64 (±0.11)^{d}	61.75 (±25.67)	92.50 (±25.94)
	Scala	0.57 (±0.09)^{cd}	36.57 (±6.50)	64.40 (±6.76)
		ANOVA P-values		
Nematicide Application		**<0.001**	**<0.001**	0.634
Treatment		0.389	0.181	**0.041**
NA x T		0.954	0.989	0.919

Table 6.8. The number of new cysts g soil^{-1}, eggs g soil^{-1} and eggs cyst^{-1} post-multiplication in the first field trial and associated ANOVA P-values. The standard errors are indicated within brackets. Within columns, means followed by the same letter are not significantly different (P<0.05).

In the second field trial, there was an overall significant effect of sampling point on *G. pallida* hatch but no effect of treatment or the sampling point x treatment interaction (Table 6.9). Although there was an overall significant effect of sampling point on hatch there were no differences between time points in a means comparison test.

Treatment	Before Planting	Total *G. pallida* hatch During Growth	Post-incorporation
Fallow	315.20 (±46.93)	304.00 (±158.76)	308.40 (±91.47)
Bento	421.20 (±83.64)	157.20 (±59.90)	259.50 (±47.69)
Ida Gold	301.60 (±68.77)	338.80 (±164.95)	445.80 (±42.23)
ISCI 99	330.40 (±139.67)	212.40 (±103.35)	430.40 (±101.27)
Bristle Oats	397.20 (±204.27)	205.60 (±40.41)	290.00 (±47.69)
		ANOVA P-values	
Treatment		0.890	
Sampling Point		**0.049**	
T x SP		0.839	

Table 6.9. Hatch of *G. pallida* J2s in the second field trial and associated ANOVA P-values. The standard errors are indicated within brackets.

6.3.3. Cultivar glucosinolate profiles at incorporation

In the first pot trial, ten GSLs were identified in the *B. juncea* cultivars at time of incorporation with no significant effect of cultivar on concentration (Table 6.10). Sinigrin was the major GSL present in both ISCI 99 and Scala making up 88.48% and 89.01% of the total GSL concentration for each cultivar respectively. Eight of the ten GSLs were present in both cultivars. Glucoalyssin was present in ISCI 99 alone and progoitrin was present in Scala alone at low concentrations.

Glucosinolate	ISCI 99	Scala
Epi/Progoitrin	ND	0.04 (±0.03)
Sinigrin	24.73 (±7.68)	20.32 (±4.17)
Glucoalyssin	0.01 (±0.01)	ND
Gluconapin	0.35 (±0.19)	0.11 (±0.03)
4-hydroxyglucobrassicin	0.01 (±0.01)	0.06 (±0.04)
Glucotropaeolin	1.87 (±1.26)	0.92 (±0.61)
Glucobrassicin	0.10 (±0.03)	0.10 (±0.02)
Gluconasturtiin	0.72 (±0.14)	1.16 (±0.21)
4-methoxyglucobrassicin	0.01 (±0.01)	0.06 (±0.05)
Neoglucobrassicin	0.14 (±0.05)	0.07 (±0.03)
Total	27.95 (±8.34)	22.83 (±4.70)

Table 6.10. GSL concentrations (mg g^{-1} DW) identified in the *B. juncea* cultivars in the first pot trial. ND= not detected; values reported as ND were treated as equal to 0 during analysis. The standard errors are stated within brackets.

In the second pot trial, twenty-three GSLs were identified in the six incorporated Brassicaceae cultivars (Table 6.11). GSL content differed between cultivars with a significant effect of cultivar on the concentration of fifteen individual GSLs and total GSL concentration. LC-MS analysis of the Bristle Oats cultivar was also completed and no GSLs were detected. The highest overall GSL concentration was in Ida Gold and the lowest in the low-GSL control

Temple. Nemat and Bento had similar total GSL levels to the control cultivar. GSL profiles differed between species, but not cultivars, with each species containing one dominant GSL; the exception to this was Temple which contained low concentrations of nine GSLs, none of which dominated the GSL profile. The major GSL in ISCI 99 and Scala was sinigrin, Ida Gold had a high level of glucosinalbin, dimeric glucosativin (DMB) was the major GSL in Nemat and glucoraphenin dominated the Bento profile.

Glucosinolate	Bento	Ida Gold	ISCI 99	Nemat	Scala	Temple	Cultivar
Epi/Progoitrin	0.02 (±0.02)^{a}	4.78 (±0.77)^{b}	ND^{a}	0.18 (±0.07)^{a}	0.22 (±0.13)^{a}	0.33 (±0.19)^{a}	**<0.001**
Sinigrin	ND^{a}	ND^{a}	32.33 (±9.25)^{b}	0.12 (±0.08)^{a}	41.23 (±4.14)^{b}	ND^{a}	**<0.001**
Glucoalyssin	ND	0.01 (±0.01)	ND	0.03 (±0.03)	ND	ND	0.529
Gluconapin	ND^{a}	0.43 (±0.10)^{b}	0.31 (±0.08)^{b}	ND^{a}	0.26 (±0.05)^{ab}	0.26 (±0.09)^{ab}	**<0.001**
4-hydroxy-glucobrassicin	ND	0.01 (±0.01)	0.05 (±0.04)	0.01 (±0.01)	0.08 (±0.04)	ND	0.157
Glucotropaeolin	ND^{a}	7.79 (±0.73)^{b}	0.25 (±0.21)^{a}	ND^{a}	0.40 (±0.33)^{a}	0.01 (±0.01)^{a}	**<0.001**
Glucobrassicin	0.01 (±0.01)^{a}	5.54 (±1.07)^{b}	0.02 (±0.01)^{a}	0.01 (±0.01)^{a}	0.12 (±0.02)^{a}	0.01 (±0.005)^{a}	**<0.001**
Gluconasturtiin	0.76 (±0.70)	0.82 (±0.26)	0.55 (±0.08)	0.26 (±0.17)	1.32 (±0.14)	0.45 (±0.24)	0.336
4-methoxy-glucobrassicin	0.50 (±0.30)	0.09 (±0.07)	0.01 (±0.01)	0.15 (±0.07)	ND	0.04 (±0.02)	0.110
Glucosinalbin	ND^{a}	40.88 (±8.23)^{b}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	**<0.001**
Glucoerucin	0.27 (±0.27)^{ab}	ND^{a}	ND^{a}	1.64 (±0.70)^{b}	ND^{a}	ND^{a}	**0.007**
DMB	ND^{a}	ND^{a}	ND^{a}	8.41 (±2.56)^{b}	ND^{a}	ND^{a}	**<0.001**
Glucoraphanin	ND^{a}	0.76 (±0.46)^{a}	ND^{a}	2.47 (±0.52)^{b}	ND^{a}	ND^{a}	**<0.001**
Digluco-thiobeinin	ND^{a}	ND^{a}	ND^{a}	0.39 (±0.12)^{b}	ND^{a}	ND^{a}	**<0.001**
Gluco-brassicanapin	ND	ND	ND	ND	0.10 (±0.10)	0.16 (±0.09)	0.176
Gluco-napoleiferin	0.02 (±0.02)^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.13 (±0.05)^{b}	**<0.001**
Glucocapparin	ND^{a}	0.12 (±0.06)^{b}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	**0.022**
Methylpentyl-GSL	ND	ND	ND	0.11 (±0.11)	ND	ND	0.446
Hexyl-GSL	ND	ND	ND	0.08 (±0.08)	ND	ND	0.446
Glucosativin	ND^{a}	ND^{a}	ND^{a}	2.02 (±0.96)^{b}	ND^{a}	ND^{a}	**0.008**
Glucoiberin	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.07 (±0.01)^{b}	**<0.001**
Glucoraphenin	1.74 (±0.35)^{b}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	**<0.001**
Gluco-raphasatin	0.01 (±0.01)	ND	ND	ND	ND	ND	0.446
Total	3.32 (±1.10)^{a}	61.24 (±9.55)^{d}	33.52 (±9.33)^{bc}	15.90 (±4.13)^{ab}	43.71 (±4.55)^{cd}	1.46 (±0.44)^{a}	**<0.001**

Table 6.11. GSL concentrations (mg g^{-1} DW) identified in the second pot trial and associated ANOVA P-values. ND= not detected; values reported as ND were treated as equal to 0 during analysis. The standard errors are stated within brackets. Within rows, means followed by the same letter are not significantly different (P<0.05).

In the first field trial, nineteen GSLs were identified at time of incorporation (Table 6.12). GSL content differed between cultivars with a significant effect of cultivar on the concentration of sixteen GSLs. Total GSL concentration did not differ between cultivars. GSL profiles differed between species, but not cultivars, with each containing one dominant GSL. The major GSL in ISCI 99 and Scala was sinigrin, Ida Gold had a high level of glucosinalbin, glucosativin was the major GSL in Nemat and glucoraphenin dominated the Bento profile.

Glucosinolate	Bento	Ida Gold	ISCI 99	Nemat	Scala	Cultivar
Epi/Progoitrin	ND^{a}	1.03 (±0.20)^{b}	ND^{a}	ND^{a}	ND^{a}	**<0.001**
Sinigrin	ND^{a}	1.12 (±0.48)^{a}	23.52 (±8.78)^{b}	ND^{a}	24.48 (±1.77)^{b}	**<0.001**
Glucoalyssin	ND^{a}	ND^{a}	ND^{a}	0.04 (±0.03)^{a}	ND^{a}	**0.040**
Gluconapin	0.08 (±0.06)	0.04 (±0.01)	0.61 (±0.53)	ND	0.17 (±0.03)	0.452
4-hydroxy-glucobrassicin	ND	ND	0.003 (±0.003)	ND	0.02 (±0.01)	0.356
Glucotropaeolin	0.55 (±0.34)^{ab}	5.98 (±0.12)^{d}	3.04 (±1.09)^{bc}	ND^{a}	3.32 (±0.58)^{c}	**<0.001**
Glucobrassicin	3.25 (±0.28)^{c}	ND^{a}	1.30 (±0.77)^{ab}	ND^{a}	2.92 (±0.23)^{bc}	**<0.001**
Gluconasturtiin	0.58 (±0.37)^{a}	5.07 (±0.33)^{b}	2.61 (±1.53)^{ab}	ND^{a}	6.04 (±0.67)^{b}	**<0.001**
4-methoxy-glucobrassicin	1.64 (±0.33)^{b}	0.98 (±0.20)^{ab}	0.32 (±0.19)^{a}	0.47 (±0.12)^{a}	0.45 (±0.04)^{a}	**0.003**
Neogluco-brassicin	ND^{a}	2.13 (±0.14)^{b}	0.79 (±0.38)^{a}	0.49 (±0.06)^{a}	0.34 (±0.03)^{a}	**<0.001**
Glucosinalbin	ND^{a}	15.90 (±1.13)^{b}	ND^{a}	ND^{a}	ND^{a}	**<0.001**
Glucoerucin	1.34 (±0.31)^{a}	ND^{a}	ND^{a}	5.26 (±1.61)^{b}	ND^{a}	**<0.001**
DMB	ND^{a}	ND^{a}	ND^{a}	6.88 (±1.41)^{b}	ND^{a}	**<0.001**
Glucoraphanin	3.00 (±0.95)^{b}	ND^{a}	ND^{a}	3.74 (±0.64)^{b}	ND^{a}	**<0.001**
Digluco-thiobeinin	ND^{a}	ND^{a}	ND^{a}	0.23 (±0.03)^{b}	ND^{a}	**<0.001**
Gluco-brassicanapin	0.07 (±0.04)	ND	ND	ND	ND	0.071
Glucosativin	ND^{a}	ND^{a}	ND^{a}	31.93 (±3.71)^{b}	ND^{a}	**<0.001**
Glucoraphenin	21.81 (±4.04)^{b}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	**<0.001**
Glucoraphasatin	3.34 (±1.16)^{b}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	**0.002**
Total	35.65 (±2.98)^{b}	32.24 (±1.46)	32.19 (±10.77)	49.04 (±4.74)	37.73 (±2.12)	0.325

Table 6.12. GSL concentrations (mg g^{-1} DW) identified in the first field trial and associated ANOVA P-values. ND= not detected; values reported as ND were treated as equal to 0 during analysis. The standard errors are stated within brackets. Within rows, means followed by the same letter are not significantly different (P<0.05).

Sinigrin concentrations in ISCI 99 and Scala at time of incorporation from the two pot trials and the first field trial were compared. Sinigrin concentration did not differ between trials, cultivars or the interaction between the two; in both ISCI 99 and Scala the highest sinigrin levels were produced in the second pot trial.

6.4. Discussion

In general, the biofumigant cultivars had little effect on *G. pallida* hatch and multiplication under controlled and field conditions with the exception of ISCI 99 and Scala in one pot trial where potentially influencing factors were standardised to reduce variation.

6.4.1. The effect of growing plants on G. pallida *hatch*

In all biofumigant trials, *G. pallida* hatch was unaffected by the growth of biofumigant cultivars compared to the plant controls. Previous studies have noted that biofumigant cultivars can increase encysted *G. pallida* mortality before incorporation (Ngala *et al.*, 2014; Ngala, Woods and Back, 2015b) which would suggest the release of toxic compounds during growth from the hydrolysis of root GSLs (McCully *et al.*, 2008) by myrosinase-producing soil microorganisms (Borek, Morra and McCaffrey, 1996; Sakorn *et al.*, 1999; Rakariyatham *et al.*, 2005). Ngala *et al.* (2014) noted the greatest effect on *G. pallida* mortality during the growth of *R. sativus* cv. Bento in a field trial, where the roots contained a high concentration of gluconasturtiin. Root material was not analysed in this study therefore a direct comparison cannot be made. As the same seed rate was used, the lack of effect is most likely due to a lower GSL content in roots in the current trials. Current results suggest that the cultivars used in this study are unlikely to release a high enough ITC content from roots to be effective at reducing PCN populations during plant growth.

6.4.2. Variability in G. pallida *suppression relating to GSL content and ITC release*

From the four trials only one noted a biofumigant effect relating to GSL hydrolysis and ITC release. In the first pot trial a significant treatment effect was detected. The differences were between cultivars and there was not an effect compared to the control. In contrast, when the biomass of incorporated material and moisture content of soil was standardised within treatments in the second pot trial, encysted *G. pallida* was effectively controlled after *B. juncea* incorporation.

Where there were significant differences between biofumigant cultivars and the Temple control in the first pot trial, the sinigrin concentrations reported after analysis of the *B. juncea* GSL profiles would suggest that shifts are unlikely to be related to GSL content and ITC release; ISCI 99 and Scala had similar GSL profiles but did not affect *G. pallida* hatch

similarly. Since seed rate and GSL profiles were the same between the two *B. juncea* cultivars, the most likely reason for differences in hatch is inconsistencies in biomass incorporation and moisture content between replicates leading to high variation between replicates. In addition, hatch and multiplication from several replicates was extremely low, potentially due to the inadvertent drowning of encysted J2 in high moisture pots and the low number of cysts per sample. The resulting significance tests may not be reliable and further experimentation should be undertaken to confirm if there is a true difference between treatments or not.

Due to this high variation between replicates, biomass and water content was standardised between replicates in the second trial. The *B. juncea* (ISCI 99 and Scala) green manures effectively suppressed *G. pallida* populations. A reduction in hatch was noted with a corresponding increase in encysted J2 mortality providing evidence to support that these cultivars release nematotoxic compounds. These results are consistent with a soil microcosm study where three sinigrin-containing *B. juncea* green manures applied to *G. pallida* cysts in soil caused over 95% mortality of encysted J2 (Lord *et al.*, 2011). In contrast, a previous study found no effect of *B. juncea* incorporation on *G. pallida* hatch in a pot trial (Brolsma *et al.*, 2014). This is most likely due to lower sinigrin concentrations where, assuming a 1% GSL to ITC conversion (Morra and Kirkegaard, 2002), the plants released <50ppm AITC which is too low to control *G. pallida* in soil (Chapter 4).

In the current study, the ISCI 99 and Scala cultivars reduced the number of newly formed cysts compared to a Fallow control but had no effect on the egg content of cysts. This suggests that the released ITCs are toxic to the encysted J2 present but have no effect on the ability of the *G. pallida* J2 to reproduce once hatched or on the viability of the second generation of encysted J2.

Encysted *G. pallida* suppression by ISCI 99 and Scala compared to other treatments cannot be attributed to differing soil moisture content, due to standardisation, or to the amount of fresh material incorporated, as high and low biomass treatments affected encysted *G. pallida* similarly, rather there appears to be a clear biofumigant effect of the *B. juncea* cultivars. This is most likely due to the major GSL found in ISCI 99 and Scala, sinigrin. Sinigrin concentration was high in both cultivars at time of incorporation and able to release a minimum of 323ppm and 412ppm AITC for ISCI 99 and Scala, respectively, if assuming a minimum 1% GSL to ITC conversion during hydrolysis. This provides evidence that cultivars containing high concentrations of sinigrin (above 30mg g^{-1} DW) can be effective at suppressing *G. pallida* in soil.

In contrast to the *B. juncea* cultivars, none of the other treatments had a suppressive effect on encysted *G. pallida* in the second pot trial. Ida Gold was incorporated at a high biomass with a high GSL content so the lack of suppression would suggest that this *S. alba* cultivar is not an effective PCN biofumigant. Bento and Nemat cannot be as easily ruled out as PCN suppressants due to issues with either growth or GSL content in this pot trial. Although Bento was incorporated at a high biomass, GSL content was very low whereas Nemat growth and GSL content was low leading to a lower biomass at incorporation in conjunction with a low GSL content. A potential reason for this has been discussed in 6.4.6.

A lack of effect with *S. alba* and *R. sativus* incorporation on *G. rostochiensis* hatch has been shown in a previous glasshouse trial (Valdes *et al.*, 2011). In a later study *S. alba* and *R. sativus* green manures reduced *G. rostochiensis* multiplication on potato in a pot trial (Fatemy and Sepideh, 2016). Fatemy and Sepideh, (2016) did not analyse the GSL content of incorporated material and therefore it is not known if inconsistencies are due to the type and concentration of ITCs released; experimental conditions did differ from the present study as they used a different soil type (40% sandy loam) and did not track the moisture content of soil during biofumigation. Differences between biofumigant cultivars highlight the importance of the major type and concentration of GSL produced in each cultivar with respect to targeted pest control. The differences between cultivars on *G. pallida* suppression under controlled conditions demonstrates the necessity to study the effect of potential biofumigants on pathogens prior to use, as different biofumigants can exert a variety of effects depending on their GSL content and biomass.

The lack of effect of biofumigation in the field trials implies that using these cultivars as biofumigants in the field would have no impact on *G. pallida* suppression. These results are consistent with previous studies where *S. alba* had no effect on *G. rostochiensis* hatch (Valdes, Viaene and Moens, 2012) and *E. sativa* did not reduce *G. pallida* populations (Ngala *et al.*, 2014) under field conditions, but not with results from the same study where the incorporation of sinigrin-containing *B. juncea* and glucoraphanin-containing *R. sativus* green manure reduced PCN populations (Ngala *et al.*, 2014). Differences in effectiveness between the current field trials and those by Ngala *et al.* (2014) are most likely due to the major GSLs identified and their concentration at time of incorporation. Although sinigrin was the major GSL identified in *B. juncea* in both studies, field grown cultivars in the earlier study contained higher levels (90μmol g^{-1} DW), implying that sinigrin concentrations in the current field trials were too low to release an effective level of AITC. In contrast, the major GSL in the *R. sativus* plant material at time of incorporation differed between trials and this could

account for the lack of consistency in *R. sativus* related biofumigation and *G. pallida* control. The lack of biofumigant effect in the current field trials could be due to a number of factors, including GSL content and the influence of environmental factors, which will be discussed in detail later.

6.4.3. A contributory green manure effect on G. pallida *suppression*

In addition to a direct effect of AITC-release from biofumigants in the second pot trial, there was an intermediary effect of green manure incorporation on the number of newly formed cysts and eggs after potato harvest. Green manure incorporation can contribute towards pest suppression by: breaking disease and pest cycles (Snapp *et al.*, 2005), releasing sulphur-containing toxins during decomposition (such as methyl sulphide, dimethyl sulphide and carbon disulphide) that are toxic to soil pathogens (Lewis and Papavizas, 1970; Abawi and Widmer, 2000; Wang *et al.*, 2009), and altering the soil environment which can increase the activity of soil microorganisms (Bernard *et al.*, 2012; Hueso, García and Hernández, 2012; Mocali *et al.*, 2015) potentially negatively influencing soil pathogen populations.

In the second pot trial the low-GSL and non-GSL green manure controls reduced the formation of new cysts compared to the Fallow control and there was a lack of significant difference between the successful biofumigant cultivars, ISCI 99 and Scala, and the green manure control. This provides evidence for a contributory green manure effect when utilising a biofumigation method, independent of GSL content and ITC-release. A green manure effect could also account for the significant difference between Nemat and Temple treatments in the second pot trial, with the incorporation of a high Temple biomass leading to a greater suppressive effect compared to the lower biomass incorporation of Nemat. Variations in experimental and environmental conditions need to be considered and minimised for maximum *G. pallida* suppression as a similar green manure effect was not noted with all treatments or in the other pot and field trials.

6.4.4. Nematicide application and G. pallida *suppression*

Nematicide application was effective at reducing the number of newly formed cysts post-potato harvest under glasshouse and field conditions. Both Vydate® and Nemathorin® are granular nematicides who act directly on emerging PCN J2 at the beginning of potato planting by killing them prior to root invasion. Due to this, their effectiveness can be measured by a reduction in the formation of new cysts post-potato harvest with no effect on reproduction or the second generation, represented by the egg content and viability of new cysts.

Under controlled conditions, Vydate® performed as expected but the reduction in cyst number was not significant in the second pot trial. It is known that timing and distribution prior to

potato planting are critical during nematicide application when targeting naturally hatching J2, therefore there is the possibility that the nematicide was applied too early or that the nematicide had broken down before late emerging *G. pallida* were affected (Whitehead, 1992; Ryan *et al.*, 2000).

In the field trial, Nemathorin® effectively reduced the formation of new populations, independent of biofumigation. There was no additional benefit of combining biofumigation and nematicide application in the same year on *G. pallida* control. The incorporation of biofumigant material was not effective on its own in this trial therefore there is the potential that a greater suppressive effect would result when integrating the two control methods after an effective biofumigant approach has been implemented.

6.4.5. The influence of external and experimental conditions on the effectiveness of biofumigation on G. pallida *suppression*

Variations in the experimental set up such as: moisture content, plant material biomass, differences in incorporation, and the influence of environmental conditions could account for the large variability in biofumigation effectiveness between trials.

Differences between pot trials are likely due to differences in moisture content and biomass of fresh material at incorporation. In the first trial, cultivars were planted, grown and incorporated into the same pot throughout, where not all planted seeds sprouted; this would have led to inconsistencies in biomass between replicates potentially masking an effect. In addition, water availability can influence the rate of plant material decomposition with higher moisture content leading to a faster rate of decomposition (Singh and Gupta, 1977; Omirou *et al.*, 2013). Variations in moisture content between replicates could have influenced the persistence of the green manures effects. Due to this, a lack of consistency between replicates led to more variability between samples and a lack of *G. pallida* control in the first glasshouse experiment.

In a separate issue, a lower GSL content in the *B. juncea* cultivars was noted in the first pot trial compared to the second, therefore the potential ITC release from cultivars may have been too low to control *G. pallida* populations. Potential AITC release averaged at 247ppm and 203ppm AITC in the first pot trial and 323ppm and 412ppm AITC in the second pot trial for ISCI 99 and Scala respectively, assuming a 1% GSL to ITC conversion.

Differences between the pot and field trials are most likely due to environmental factors and differences in experimental set up. Environmental conditions in the glasshouse studies were controlled with consistent temperature and moisture. In comparison, the field trials would have been affected by varying environmental conditions including changes in temperature

(Davidson, Belk and Boone, 1998), pH (Lauber *et al.*, 2009) and rainfall (Hueso, García and Hernández, 2012; Hagemann *et al.*, 2016). This would potentially influence the GSL content of cultivars (Charron, Saxton and Sams, 2005; Velasco *et al.*, 2007) as well as affect the formation of the breakdown products with the possibility of nitrile over ITC formation. The GSL analysis shows that the sinigrin content of *B. juncea* cultivars was lower in the first field trial compared to the glasshouse grown cultivars. This in turn could have led to lower ITC release and hence reduced impact on soil *G. pallida* populations. In addition, inconsistent soil environments could have led to variability in the growth and biomass of biofumigant cultivars over the field sites leading to less consistency between plot replicates compared to pot replicates.

Experimental methodology between glasshouse and field trials needs to be considered as an impacting factor; although seed rate and biomass were standardised across trials, certain incorporation approaches were not consistent when transferring the process to a larger scale. ITC release was maximised in the pot trials through plant material blending and sealing of pots which increased exposure of cysts to the volatiles. The field trial would not have achieved the same tissue disruption and sealing from flail mowing, rotovating and rolling leading to less efficient GSL hydrolysis and quicker volatisation into the atmosphere; the biofumigation process would have had less of an impact on encysted *G. pallida*. Chopping plant material over blending could have led to slower GSL hydrolysis and extended ITC release compared to the experiments completed under controlled conditions. In this case, the initial concentration required for suppression would have been reduced and instead a consistent release of ITCs at too low concentrations to have an effect might occur. An increased surface area in the field plots could have limited the initial concentration of released ITCs potentially reducing the impact of released compounds on encysted *G. pallida* in the first instance.

The lack of effect in the field trials demonstrates that a number of factors can influence the effectiveness of biofumigation in practice. Further work is required to optimise the experimental methodology and maximise GSL content so that environmental conditions have less of an impact.

6.4.6. Glucosinolate profiles of glasshouse- and field-grown Brassicaceae cultivars

In general, there were little differences between glasshouse- and field-grown cultivars although overall GSL concentration did vary between trials and GSL diversity was generally reduced in field-grown cultivars.

The *B. juncea* profiles (ISCI 99 and Scala) were similar between the two glasshouse trials. Sinigrin concentrations were higher in the second trial compared to the first. This demonstrates how the GSL content of cultivars can differ between trials under standard conditions. Differences may be due to: natural variations in GSL content between individual plants, slight differences between the trials such as length of exposure to light (Engelen-Eigles *et al.*, 2005; Huseby *et al.*, 2013) or differences in plant growth due to the pot sizes and root competition for soil water and nutrients (Booth, Walker and Griffiths, 1991; Zhao *et al.*, 1994; Zhang *et al.*, 2008). Similarly, the GSL profiles of glasshouse- and field-grown *B. juncea* cultivars were alike although the total GSL and sinigrin concentrations were higher in the second pot trial compared to the field trial. As previously discussed, this is likely due to the influence of environmental factors which negatively impact plant growth and GSL production in material. The lowered GSL content in field-grown material and lack of suppressive effect on *G. pallida* highlights the need to further improve biofumigation practice in the field by either investigating cultivars with a higher potential sinigrin concentration or increasing the biomass of sinigrin-containing material being grown and incorporated.

GSL profiles of the other three cultivars differed slightly between trials. There were differences in the presence and concentration of several minor GSLs identified in Bento between the glasshouse and field trials. The major GSL was the same in both, glucoraphenin. Similarly, Ida Gold profiles differed slightly with lower diversity in the field material compared to the glasshouse material; glucosinalbin was identified as the major GSL under both growth conditions. The number of GSLs in Nemat was reduced in field-grown material and the major GSL identified differed between trials. In the glasshouse trial DMB was the major GSL whilst in the field trial glucosativin dominated. These are the dimeric and monomeric forms of the same GSL, implying that the major GSL can shift between the two forms depending on growth conditions. Differences between trials with respect to cultivar GSL profiles are to be expected as changing field conditions are likely to impact on the production and accumulation of different GSLs during plant growth when compared to controlled glasshouse conditions. In spite of this, the major GSL in each cultivar was generally consistent between trials and agree with results in Chapter 5.

In the second pot trial, overall GSL content differed between species which contrasts from results in Chapter 5 where glasshouse-grown cultivars contained similar total GSL concentrations. As all other glasshouse conditions remained the same, this suggests that increasing the pot size during plant growth did not benefit all cultivars with respect to GSL content. Bento and Nemat had significantly lower GSL levels at incorporation compared to

the other cultivars. This is likely due to the species of these cultivars and the way that they grow. *R. sativus* (Bento) and *E. sativa* (Nemat) are both compact leafy species with an extensive root system but very little shoot system. In comparison, *S. alba* (Ida Gold) and *B. juncea* (ISCI 99 and Scala) are tall mustard species with a number of shoots and a smaller root system. Due to the randomised design layout of the pots, the mustards would have grown tall and blocked the light for the non-mustard species, reducing their rate of growth and GSL content. The lack of light access combined with the larger root system, increasing nutrient competition within pots, can account for the differences between the species. In contrast, field-grown cultivars contained similar overall GSL concentrations. This implies that the issues with light and soil nutrient competition were not present which is to be expected due to the larger plot sizes and increased soil depth for the Bento and Nemat root systems to expand.

6.4.7. Concluding remarks

Results from this chapter provide information on the ability of biofumigation to be utilised to suppress *G. pallida* populations. Biofumigation had little effect on *G. pallida* hatch and multiplication under controlled and field conditions with the exception of ISCI 99 and Scala in a pot trial where potentially influencing factors were standardised to reduce variation.

Sinigrin-containing cultivars were the most effective against encysted *G. pallida*. GSL concentration and external factors can greatly influence biofumigation efficiency. *Brassica juncea* cultivars able to release a minimum of 200-247ppm AITC under controlled and field conditions were ineffective at controlling *G. pallida*. In contrast, cultivars able to release a minimum of 323-412ppm AITC had a significant effect on populations and were able to decrease hatch, increase mortality and reduce the formation of new cysts. This is consistent with results from Chapter 4 and highlights that a high concentration is required for control. Although ISCI 99 and Scala suppressed encysted *G. pallida*, there was little effect on the egg content of the cysts implying that incorporating biofumigants affects the J2 directly but has no effect on the reproductive abilities of the J2 which do hatch. Whilst sinigrin-containing *B. juncea* cultivars have been shown to be effective *G. pallida* biofumigants in this study, the lack of effect in several of these trials highlights that external conditions need to be controlled where possible to maximise ITC release and the suppressive effect on *G. pallida* populations. None of the other cultivars suppressed PCN. The major GSLs identified in glasshouse- and field-grown Ida Gold (glucosinalbin) and field-grown Nemat (glucosativin) were not effective PCN biofumigants. Further research is needed to determine the effectiveness of Bento and Nemat (containing glucoraphenin and DMB) against PCN considering the issues with biomass and GSL content in the presented trials. In addition to a direct ITC-related

biofumigant effect, biofumigation provides other suppressive benefits through the incorporation of green manures. Green manure incorporation can improve the soil environment and assist with soil pathogen suppression. Cultivars which do not contain sinigrin may still provide some use with respect to the suppression of soil-borne pathogens, although this is not necessarily a cultivar-independent effect.

Although effective biofumigant cultivars and sinigrin concentrations were identified, cultivar incorporation was ineffective in the field trials. Further work is required in order to maximise GSL content and ITC release and reduce the impact of environmental factors under field conditions if biofumigation is to become an effective alternative to nematicides for *G. pallida* control.

Chapter 7. Assessing the Effects of Isothiocyanates and Biofumigation on Soil Microbial Communities

7.1. Introduction

The study of soil microbial diversity is important in determining the influence of various practices and environmental conditions on key soil functions, including C and N cycles. Soil microorganisms play a role in many important soil nutrient pathways including the cycling of organic compounds (Kirk *et al.*, 2004). In addition they can influence above-ground ecosystems and mitigate various stresses through their roles in these pathways (Kirk *et al.*, 2004; Boyle *et al.*, 2008; Rincon-Florez, Carvalhais and Schenk, 2013).

Soil diversity can be easily affected by stresses from external sources such as loss of organic matter, soil erosion, pollution, temperature fluctuations and pH alterations. Although several of these factors can change naturally over time, agricultural practices are known to lead to sudden physical, chemical and biological changes in the soil resulting in adverse effects on the soil community and hence plant health and soil functions as discussed in 1.7.1.

Studies into the effects of biofumigation on soil microbial diversity have noted that isothiocyanates (ITCs) and the incorporation of green manure can alter soil microbial community structure and function. ITCs have been shown to reduce nitrifying bacteria populations and inhibit bacterial growth *in vitro* (Bending and Lincoln, 2000). In a later study, fungal populations decreased after AITC exposure but bacterial populations were not as impacted with only a transient increase in *Firmicutes* populations (Hu *et al.*, 2015). Differences are most likely due to discrepancies in ITC concentration and methodology: Bending and Lincoln (2000) cultured bacteria on plates whereas Hu *et al.* (2015) used community quantitative polymerase chain reaction (qPCR) assays, which allows the analysis of culture-independent bacterial species. In a microcosm study, *Brassica oleracea* residues increased microbial activity in the presence of myrosinase (Omirou *et al.*, 2011). Later studies found that *B. juncea* and *R. sativus* growth and incorporation increased microbial activity (Ngala, Woods and Back, 2015b) and altered the carbon substrate utilization of communities under controlled conditions with the greatest effect occurring within fourteen days of incorporation (Fouché, Maboeta and Claassens, 2016). An earlier study found that incorporation of the same cultivars had no effect on soil communities when terminal restriction fragment length polymorphism (T-RFLP) was utilised to detect changes (Taylor, 2013). *Brassica napus* and *Brassica carinata* green manures have also been shown to alter fungal and bacterial communities (Bernard *et al.*, 2012; Wang *et al.*, 2014; Mocali *et al.*, 2015).

Previous studies have shown that ITCs and biofumigation can affect soil microorganisms but there are several significant gaps in the research. There are few studies on the effects of pure ITCs on soil microbial populations, and inconsistencies between results with most studies investigating high concentrations. When investigating the effect of biofumigation on microbial communities, results are more consistent. The incorporation of biofumigant material has a transient positive effect on microbial activity although the persistence of shifts in the microbial community over time and short term changes in soil diversity requires further research. Several of the cultivars considered in Chapters 5 and 6 have not been included in previous biofumigation and soil microbial studies. The research presented here addresses these issues and investigates the effect of ITCs and different biofumigant cultivars on soil microorganisms over time in order to determine if biofumigation has an effect on soil microbial respiration and community profiles which could impact key soil functions.

A wide range of methods have been developed for studying the diversity and activity of microorganisms with advantages and disadvantages associated with each (Kirk *et al.*, 2004; Leckie, 2005; Rincon-Florez, Carvalhais and Schenk, 2013). The method used in this study was MicroResp™ (Campbell *et al.*, 2003), a community-level physiological profile (CLPP) method able to detect shifts in the microbial community function based on the soil's ability to utilise different carbon sources and the subsequent detection of respired carbon dioxide (CO_2) using a colorimetric reaction. MicroResp™ has a number of advantages: it is a 'whole soil' technique, has a short incubation time, does not require extraction or culturing of organisms, uses a small sample volume, and is sensitive. In addition to functional diversity analysis, a qPCR protocol was developed to detect changes in specific bacterial groups involved in nutrient cycling which are sensitive to environmental shifts - namely nitrogen fixing bacteria which contain the *nifH* gene.

The main aims of this study were to:

1. Assess the effect of ITCs on soil microbial activity (basal respiration rate) and CLPPs
2. Determine the effect of biofumigation on soil microbial activity (basal respiration rate) and CLPPs
3. Evaluate the persistence of changes in basal respiration and shifts in microbial community profiles after biofumigation
4. Develop an assay to investigate the potential impact of biofumigation on *nifH*-containing soil nitrogen fixing bacteria

7.2. Materials and Methods

7.2.1. Isothiocyanate pot trial

A pot trial investigating the effect of AITC, BITC and PEITC on soil microbial diversity was set up under glasshouse conditions. The experimental design and set up was as previously described in 4.2.4. ITC treatments (Table 7.1) were incorporated in combinations at high (100ppm) and low (5ppm) initial concentrations in 2 L pots of John Innes No. 2 soil which were subsequently sealed for four weeks. Six replicates of each treatment were included and pots were set up in a randomised design layout. After four weeks, Desiree potato tubers were planted in each pot and left to grow for sixteen weeks. Soil samples were collected from three of the six replicates: prior to ITC addition, one day post-treatment, one week post-treatment and one month post-treatment as well as at potato harvest. Three soil samples were collected from each pot and combined to form one composite soil sample per replicate as described in 2.6.2. MicroResp™ was performed as described in 2.9 and Appendix A. The absorbance wavelength used was 405nm and the corresponding calibration curve was utilised to determine CO2 rate.

Identifier	Treatment
C	Water
A	100ppm AITC
B	100ppm BITC
PE	100ppm PEITC
Abpe	100ppm AITC + 5ppm BITC + 5ppm PEITC
aBpe	5ppm AITC + 100ppm BITC + 5ppm PEITC
abPE	5ppm AITC + 5ppm BITC + 100ppm PEITC

Table 7.1. ITC treatments applied in the pot trial.

7.2.2. Biofumigation pot trials

Two pot trials investigating the effect of incorporating green manures on soil microbial diversity were performed under glasshouse conditions. The experimental design and set up was as previously described in 6.2.2. In the first pot trial biofumigant material was grown and incorporated into the same pots. In the second pot trial, biofumigant material was grown and combined prior to incorporation, after which time the material was split between pots and incorporated in equivalent amounts to reduce variability. In addition, the moisture content of soil was standardised to 40%. The variations in the second pot trial were completed in order to reduce inconsistencies between replicates and also to determine how important consistent moisture and biomass is on the impact of biofumigation on soil microorganisms.

In the first pot trial, seeds of five cultivars were sown in 2 L pots, at the indicated seed rate in Table 7.2, and left to grow for eight weeks. Six replicates of each treatment were included and set up in a randomised block design layout. After eight weeks all above-ground material from each pot was cut and blended with 200 mL H_2O before being incorporated back into the same pots and sealed for three weeks. Soil samples were collected from three of the six treatment replicates: prior to seed planting, during cultivar plant growth, one day post-incorporation, one week post-incorporation, one month post-incorporation and at potato harvest. Three soil samples were collected from each pot and combined to form one composite soil sample per replicate as described in 2.6.2. MicroResp™ was performed as described in 2.9 and Appendix A. The absorbance wavelength used was 405nm and the corresponding calibration curve was utilised to determine CO_2 rate.

In the second pot trial, seeds of five cultivars were sown in 5 L pots, at the seed rate in Table 7.2, and left to grow for eight weeks. Four replicates of each treatment were prepared in a randomised design layout. After eight weeks the above-ground plant material from the replicates of each cultivar were combined, weighed and blended with H_2O before being incorporated into 2 L pots of soil at the rates indicated in Table 7.2 and sealed for four weeks. Six replicates of each treatment were included in a randomised block design layout. Soil samples were collected from three of the four or six replicates: prior to seed planting, during cultivar growth, one day, one week and one month post-incorporation. Samples from all replicates were collected from the Fallow control in order to obtain a more accurate untreated CLPP. Three soil samples were collected from each pot and combined to form one composite soil sample per replicate as described in 2.6.2. MicroResp™ was performed as described in 2.9 and Appendix A. The absorbance wavelength used was 570nm and the corresponding calibration curve was utilised to determine CO_2 rate.

Species	Cultivar	Seed Rate		Pot Trial 2	
		Pot Trial 1 (mg 2 L pot^{-1})	Pot Trial 2 (mg 5 L pot^{-1})	Fresh Material (g 2 L pot^{-1})	Added H_2O (mL 2 L pot^{-1})
Raphanus sativus	**Bento**	45.40	83.10	120.00	233.67
Sinapis alba	**Ida Gold**	15.89	29.10	146.67	216.80
Brassica juncea	**ISCI 99**	20.40	37.40	100.00	252.02
Eruca sativa	**Nemat**	13.62	24.90	26.67	322.99
Brassica juncea	**Scala**	20.40	37.40	106.67	246.77
Brassica napus	**Temple**	45.40	83.10	115.00	243.48
Avena strigosa	**Bristle Oats**	-	332.40	40.00	310.62

Table 7.2. Plant treatments and seed rates applied in each pot trial and biomass and water incorporation in the second pot trial. 2 L pots have a 227cm^2 surface area and 5 L pots have a 415.5cm^2 surface area.

7.2.3. Biofumigation field trial

A field trial investigating the effect of incorporating green manures on soil microbial diversity was designed and performed as previously described in 2.5 and 6.2.3. The field trial was initiated on 4/9/15 where seeds of three potential biofumigant cultivars and one negative (Bristle Oats) green manure control were sown at the seed rates in Table 7.3 and left to grow for eight weeks. Five replicates of each treatment were included in a randomised block design layout. Plants were incorporated on 2/11/15. Soil samples were collected from three of the five replicates: prior to seed planting, during cultivar plant growth, one day post-incorporation, one week post-incorporation and four months post-incorporation. Thirty cheese-cored samples per plot were randomly taken and combined to form one composite soil sample per plot as described in 2.6.2. MicroResp™ was performed as described in 2.9 and Appendix A. The absorbance wavelength used was 570nm and the corresponding calibration curve was utilised to determine CO_2 rate.

Species	Cultivar	Seed Rate (kg ha^{-1})
Raphanus sativus	**Bento**	15
Sinapis alba	**Ida Gold**	8
Brassica juncea	**ISCI 99**	8
Avena strigosa	**Bristle Oats**	80

Table 7.3. Plant treatments and seed rates applied in the field trial.

7.2.4. NifH *qPCR assays*

Several qPCR assays were developed to detect and quantify changes in nitrogen-fixing bacterial populations which contain the *nifH* gene. Species from the genera *Rhizobium, Azospirillum, Azotobacter* and *Pseudomonas*, as well as nitrogen-fixing species of cyanobacteria, were selected after being identified in literature as important soil nitrogen-fixers (Marusina *et al.*, 2001; Levy-Booth and Winder, 2010; Orr *et al.*, 2011). Primers and probes were designed to detect these bacteria as described in 2.7.2. From this, three *NifH* qPCR primer sets were designed to detect the nitrogen-fixing bacteria of interest.

The genomic DNA of four bacterial species were purchased and standards were prepared as described in 2.7.3. *Rhizobium etli* ATCC 51251 DSM-11541, *Nitrosomonas europaea* ATCC 25978 DSM-28437 and *Azotobacter chroococcum* ATCC 9043 DSM-2286 were supplied by DSMZ, Germany and *Anabaena variabilis* ATCC 29413 was supplied by ATCC, UK. *R. etli*, *A. chroococcum* and *A. variabilis* were the positive nitrogen-fixing standards (one for each assay) and *N. europaea* was included as a negative ammonia-oxidising bacteria control. Once standards were prepared, the *NifH* assays were run and amplification occurred as described in 2.7.4.

Microbial DNA was extracted from the second pot trial soil samples as described in 2.7.1. The three qPCR assays were tested on the extracted samples as described in 2.7.4. An H_2O control and the appropriate four standard dilutions were included in each run to produce a standard curve for quantification and analysis of DNA in the soil samples. Data was expressed as ng *nifH* DNA μg^{-1} total DNA.

7.2.5. Data analysis

Changes in the basal respiration of soil samples were analysed by two-way ANOVA as described in 2.10.1 with Treatment x Sampling Point as factors in all experiments. Significant ANOVA P-values ($P<0.05$) were further investigated with Tukey's HSD test. One-way ANOVA analysis followed by Tukey's HSD test was performed with Treatment as the factor in the qPCR assays.

Changes in the CLPPs of soil samples were analysed by multivariate analysis as described in 2.10.2. Canonical variate (CV) analysis was performed on data sets from each sampling point with Treatment as the single factor. CV analysis was also completed with all data grouped by Sampling Point. All seven carbon sources were used as variates during the analysis. The significance of distances were investigated using Analysis of Distance with the treatment structure reflecting the factor used in the CV analysis. Where this produced a significant P-value ($Pr<0.05$), scatter plots of the first and second CV ordinates were formed with data sets grouped by the factor displaying significance. Tukey's HSD test was performed on the first and second CV ordinates to identify differences between groups.

7.3. Results

7.3.1. Isothiocyanates and soil microorganisms under glasshouse conditions

Basal respiration was unaffected by treatment or the interaction between ITC treatment and sampling point (data not shown). CO_2 rate did change over time independent of treatment ($P<0.001$; Figure 7.1). Basal respiration was significantly reduced one day post-treatment compared to all other sampling points. One week post-treatment, basal respiration recovered and was significantly higher than at all other time points. One month post-treatment, basal respiration was lower than pre-treatment and one week post-treatment. At potato plant growth, basal respiration had recovered and was at a similar level as the soil samples pre-treatment.

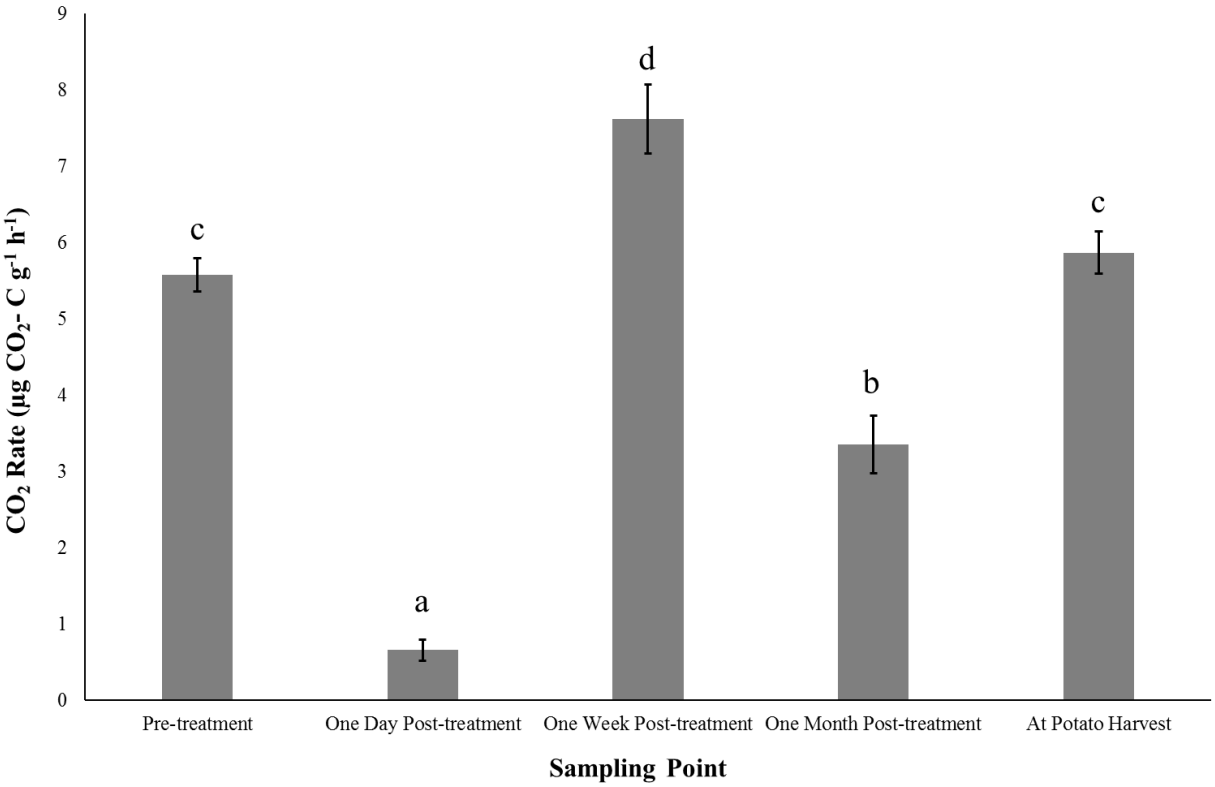


Figure 7.1. Soil basal respiration (µg CO_2- C g^{-1} h^{-1}) over five sampling points in the ITC pot trial. Error bars represent the standard error. Significant differences (P<0.05) are indicated by different letters.

CLPP was unaffected by ITC treatment at each sampling point (data not shown). CLPPs did differ significantly over time (Pr<0.001; Figure 7.2). All sampling points were significantly different from each other along CV 1 (73.89% variation) with the exception of the pre-treatment and one month post-treatment samples which clustered together. CV 2 (13.76% of variation) discriminated between potato harvest samples and all other time points.

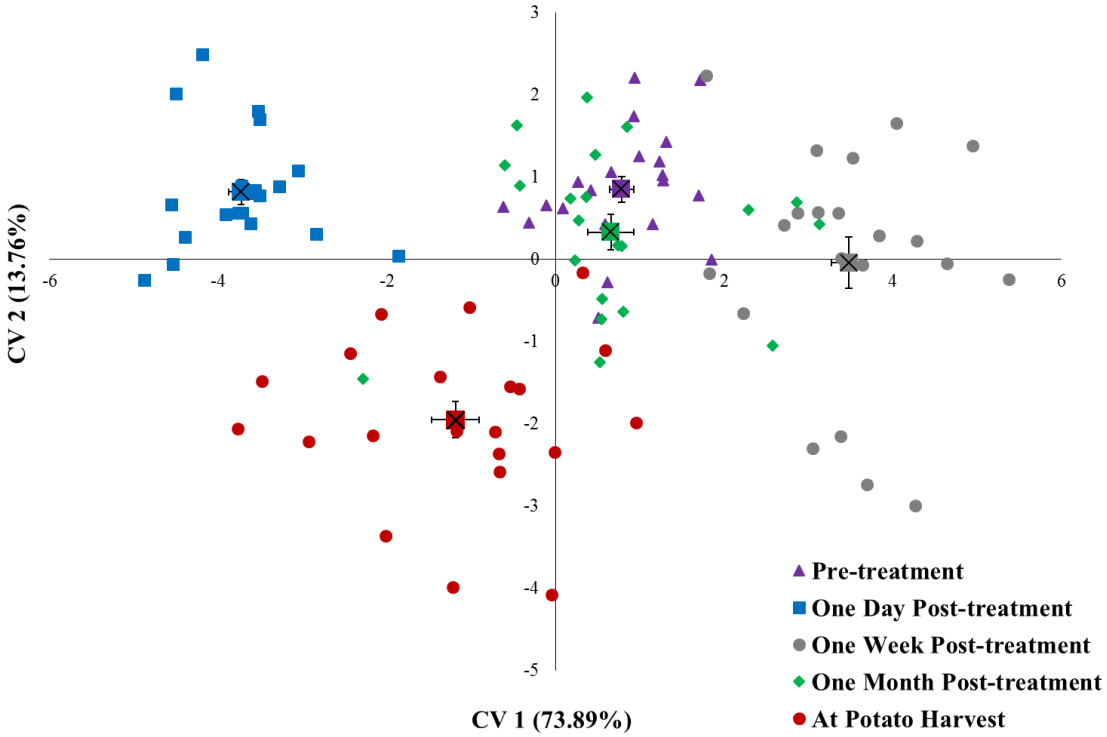


Figure 7.2. Scatter plot of the first and second CVs of CLPPs at five sampling points in the ITC pot trial. The average of each sampling point is indicated by the cross-containing squares. Error bars represent the standard error for each CV.

7.3.2. Biofumigation and soil microorganisms under glasshouse conditions

In the first biofumigation pot trial, basal respiration was unaffected by treatment or the interaction between treatment and sampling point (data not shown). CO_2 rate did change over time independent of treatment (P<0.001; Figure 7.3). CO_2 rate was at its lowest before seed planting and one month post-incorporation. Respiration increased significantly during cultivar plant growth, one day post-incorporation and one week post-incorporation. Basal respiration increased further after potato plant growth.

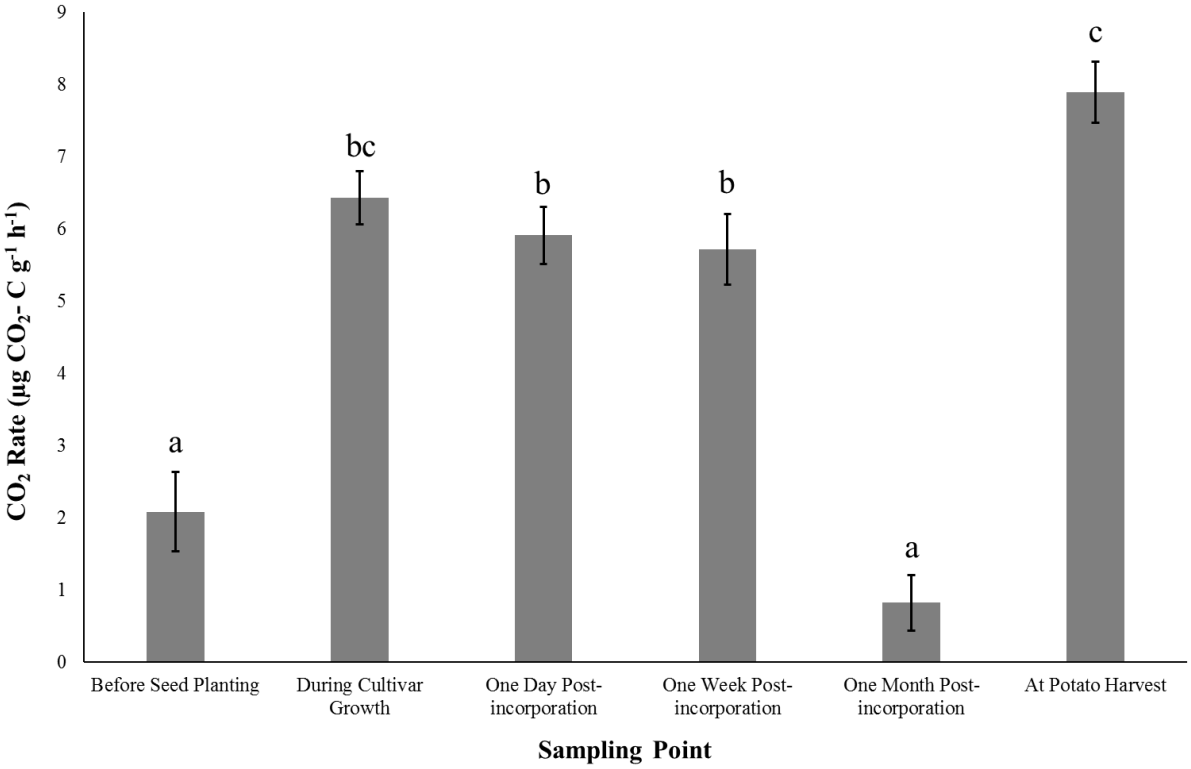


Figure 7.3. Soil basal respiration (µg CO_2- C g^{-1} h^{-1}) over six sampling points in the first biofumigation pot trial. Error bars represent the standard error. Significant differences (P<0.05) are indicated by different letters.

Microbial community function was significantly affected by treatments one day post-incorporation and there was an overall sampling point effect (Table 7.4).

One day post-incorporation, there was a clear separation of the Fallow samples from the six plant treatments along CV 1 which explained 84.64% of the variation (Figure 7.4). ISCI 99 samples had a different physiological profile from the Ida Gold and Scala samples. Along CV 2 (8.28% variation), Ida Gold profiles were significantly different from Nemat and Temple profiles. ISCI 99 and Nemat CLPPs also differed on CV 2.

When comparing profiles over time, all groups were significantly different from each other along CV 1 (80.52% variation) with the exception of before seed planting and one day post-incorporation samples (Figure 7.5). The before seed planting profile was significantly different to all other sampling points along CV 2, which explained 11.71% variation in the data. The one month post-incorporation and potato harvest CLPPs were significantly different from all earlier sampling points.

Data Set	Factor	% Variation	Pr
Before Seed Planting	Treatment	83.99	0.758
During Cultivar Growth	Treatment	76.54	0.988
One Day Post-incorporation	Treatment	92.92	**0.001**
One Week Post-incorporation	Treatment	93.75	0.244
One Month Post-incorporation	Treatment	80.26	0.075
At Potato Harvest	Treatment	84.01	0.198
All Data	Sampling Point	92.23	**0.001**

Table 7.4. Variation explained by the first 2 CVs and significance of each factor based on analysis of distance of CLPPs in the first biofumigation pot trial. Significant results are in bold.

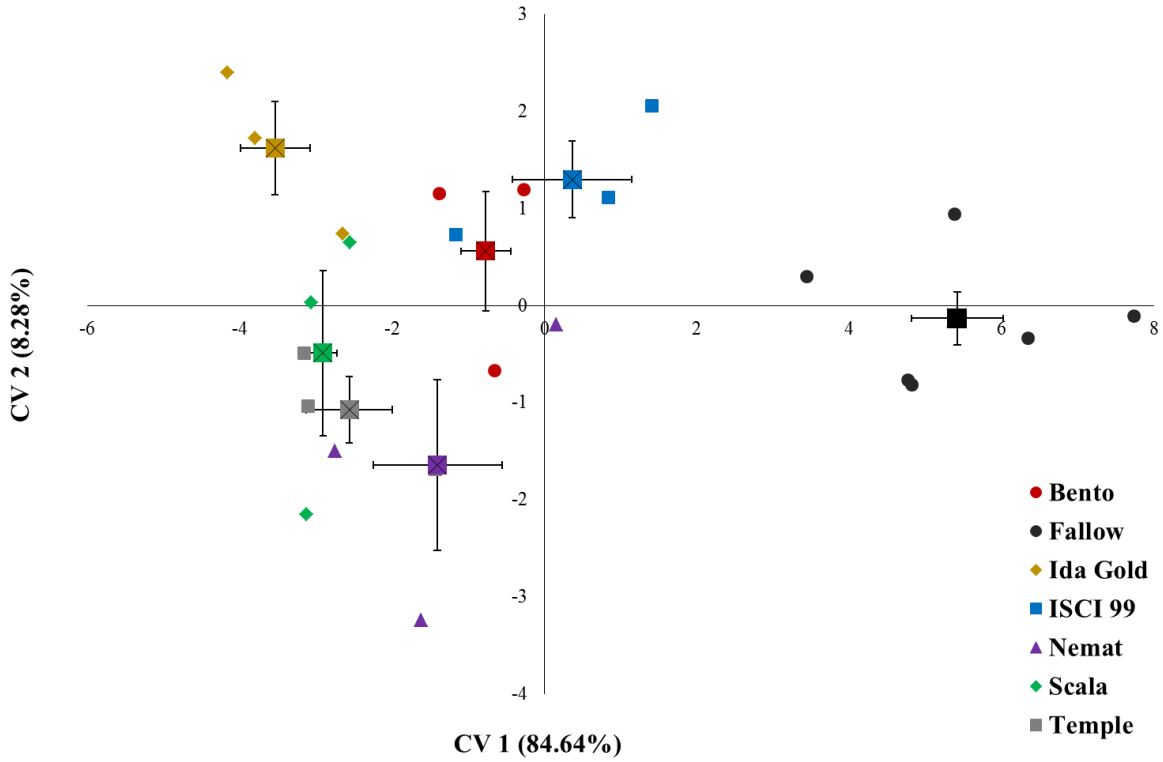


Figure 7.4. Scatter plot of the first and second CVs of CLPPs one day post-incorporation in the first biofumigation pot trial. Three replicates taken from empty positive control pots were included as Fallow samples to give a total of six control replicates. The averages of each treatment are indicated by the cross-containing squares. Error bars represent the standard error for each CV.

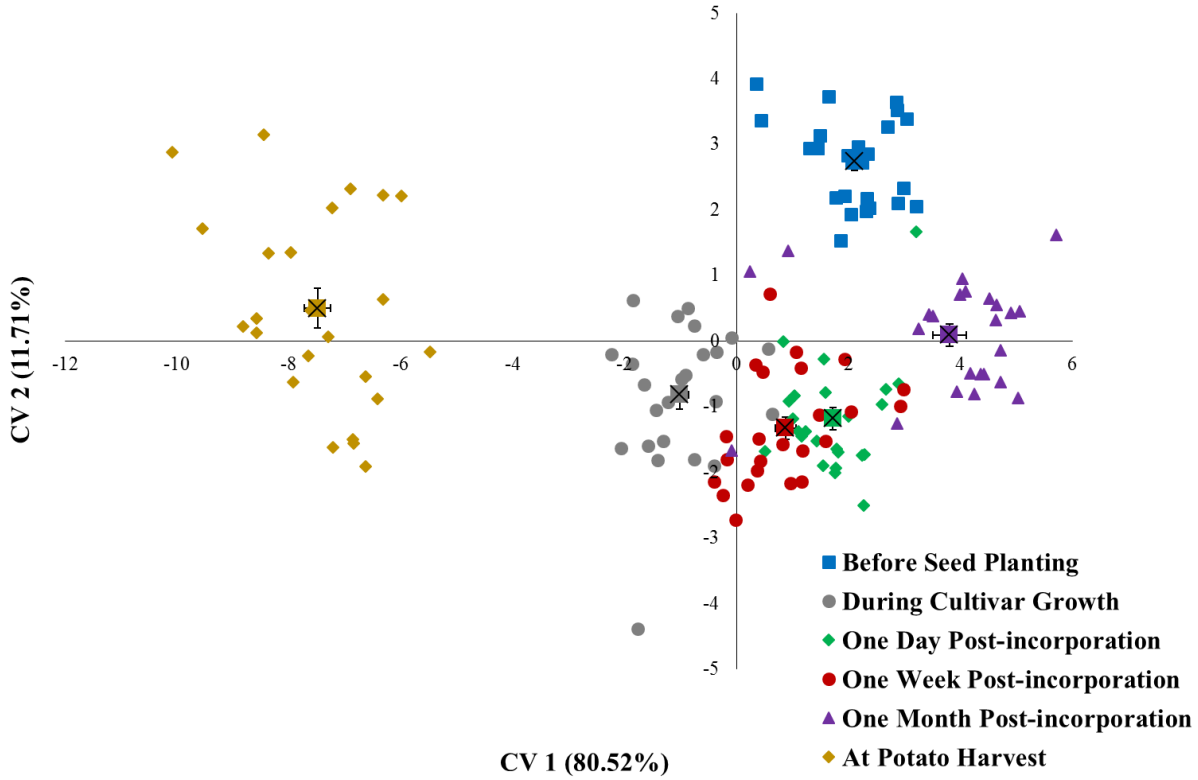


Figure 7.5. Scatter plot of the first and second CVs of CLPPs at six sampling points in the first biofumigation pot trial. The averages of each sampling point are indicated by the cross-containing squares. Error bars represent the standard error for each CV.

In the second pot trial, basal respiration was affected by sampling point and treatment as well as the interaction between the two (Table 7.5). Basal respiration was significantly lower at seed planting compared to the following three sampling points. CO_2 rate was lowest in the Fallow treatments, independent of time, and significantly different to the CO_2 rates in the Ida Gold, ISCI 99 and Scala treated samples. When the interaction between Sampling Point x Treatment was investigated, there was an overall significant effect due to differences in basal respiration between treatments one day and one week post-incorporation. One day post-incorporation, CO_2 rate in the Fallow control was significantly lower than in the Ida Gold, Scala and ISCI 99 treatments. In addition, the non-GSL Oats control had a lower CO_2 rate compared to the ISCI 99 treatment. One week post-incorporation, CO_2 rate in the Fallow and Oat controls was significantly lower than in the Ida Gold, Scala and ISCI 99 treatments.

Treatment	Sampling Point					All Sampling Points
	Before Seed Planting	During Cultivar Growth	One Day Post-incorporation	One Week Post-incorporation	One Month Post-incorporation	
Bento	3.65 (±0.55)	5.14 (±0.53)	6.79 (±2.17)^{abc}	5.68 (±0.32)^{ab}	5.09 (±0.23)	5.27 (±0.48)^{ab}
Fallow	-	-	2.88 (±0.45)^{a}	4.05 (±0.23)^{a}	4.14 (±0.23)	3.69 (±0.22)^{a}
Ida Gold	3.88 (±0.78)	4.92 (±0.52)	**7.58 (±1.56)^{bc}**	**6.56 (±0.47)^{b}**	5.49 (±0.67)	**5.69 (±0.48)^{b}**
ISCI 99	3.75 (±0.24)	5.41 (±0.54)	**8.60 (±1.36)^{c}**	**6.18 (±0.35)^{b}**	4.60 (±0.14)	**5.71 (±0.51)^{b}**
Nemat	3.46 (±0.47)	5.45 (±0.60)	6.56 (±1.37)^{abc}	5.45 (±0.26)^{ab}	4.62 (±0.54)	5.11 (±0.39)^{ab}
Oats	3.30 (±0.32)	5.22 (±0.05)	3.72 (±0.20)^{ab}	4.16 (±0.24)^{a}	4.50 (±0.15)	4.18 (±0.19)^{ab}
Scala	3.96 (±0.75)	5.04 (±0.50)	**8.09 (±0.67)^{bc}**	**6.50 (±0.75)^{b}**	5.33 (±0.81)	**5.78 (±0.46)^{b}**
Temple	3.57 (±0.77)	4.74 (±0.23)	3.84 (±0.10)^{abc}	4.80 (±0.30)^{ab}	5.16 (±0.53)	4.42 (±0.24)^{ab}
All Treatments	3.65 (±0.19)^{a}	5.13 (±0.16)^{b}	5.66 (±0.54)^{b}	5.27 (±0.22)^{b}	4.78 (±0.16)^{ab}	
			ANOVA P-values			
Sampling Point			**<0.001**			
Treatment			**<0.001**			
SP x T			**0.007**			

Table 7.5. Soil basal respiration (μg CO_2- C g^{-1} h^{-1}) over five sampling points in the second biofumigation pot trial and associated ANOVA P-values. The standard errors are indicated within brackets. For sampling points (within columns) and for all treatments (bottom row), means followed by the same letter are not significantly different (P<0.05) according to Tukey's HSD test. Significant differences compared to the Fallow control within sampling points are in bold.

In the second pot trial, treatment significantly affected microbial communities post-incorporation, but had no effect pre-incorporation; CLPPs also varied over time (Table 7.6).

One day post-incorporation, the Fallow microbial profile was significantly different to all other treatments along CV 1 (89.12% variation; Figure 7.6). The Oats and Temple control profiles were similar to each other but distinct from all other treatments. Bento samples were significantly different from the Nemat and Ida Gold samples. Along CV 2, which explained 7.04% of the data variation, the Temple, Oats and Ida Gold CLPPs were significantly different to the Fallow, ISCI 99 and Bento CLPPs. One week post-incorporation, differences between CLPPs were less pronounced (Figure 7.7). Along CV 1, which explained 70.71% of the variation, Fallow samples were significantly different from all other treatments except Oats. The Oats profile was distinct from all biofumigant cultivars. The Temple profiles were significantly different to all other cultivar profiles except Nemat. ISCI 99 CLPPs were significantly different from Nemat, Temple and Bento profiles along CV 2 (13.09% variation). Nemat and Temple profiles were distinct from Fallow and Scala profiles. One month post-incorporation, Fallow and ISCI 99 profiles were significantly different to Ida Gold, Bento and Temple profiles along CV 1 where 56.03% variation was explained (Figure 7.8). Ida Gold microbial communities were also different to the Nemat and Scala microbial communities. CV 2 explained 27.85% of the variation with significant differences in profiles noted between Scala and the Fallow, Oats and Temple samples.

When studying the effect of time on CLPPs, before seed planting and during cultivar growth samples were significantly different from all later sampling points along CV 1 (68.33% variation; Figure 7.9). The one day post-incorporation profile was distinct from all other sampling points on this axis. CV 2 explained 22% of the data variation with the one day post-incorporation samples displaying a significantly different CLPP to all other sampling points.

Data Set	Factor	% Variation	Pr
Before Seed Planting	Treatment	89.96	0.992
During Cultivar Growth	Treatment	85.37	0.768
One Day Post-incorporation	Treatment	96.16	**0.001**
One Week Post-incorporation	Treatment	83.80	**0.001**
One Month Post-incorporation	Treatment	83.88	**0.032**
All Data	Sampling Point	90.33	**0.001**

Table 7.6. Variation explained by the first 2 CVs and significance of each factor based on analysis of distance of CLPPs in the second biofumigation pot trial. Significant results are indicated in bold.

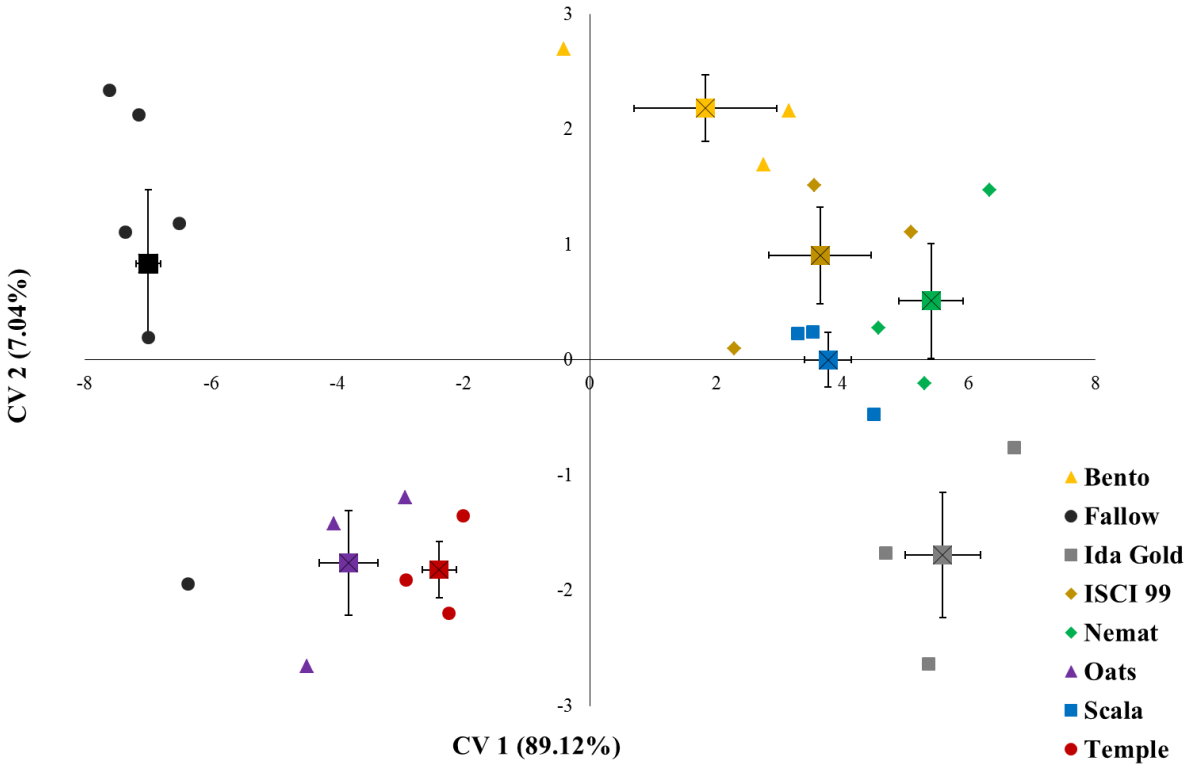


Figure 7.6. Scatter plot of the first and second CVs of CLPPs one day post-incorporation in the second biofumigation pot trial. The averages of each treatment are indicated by the cross-containing squares. Error bars represent the standard error for each CV.

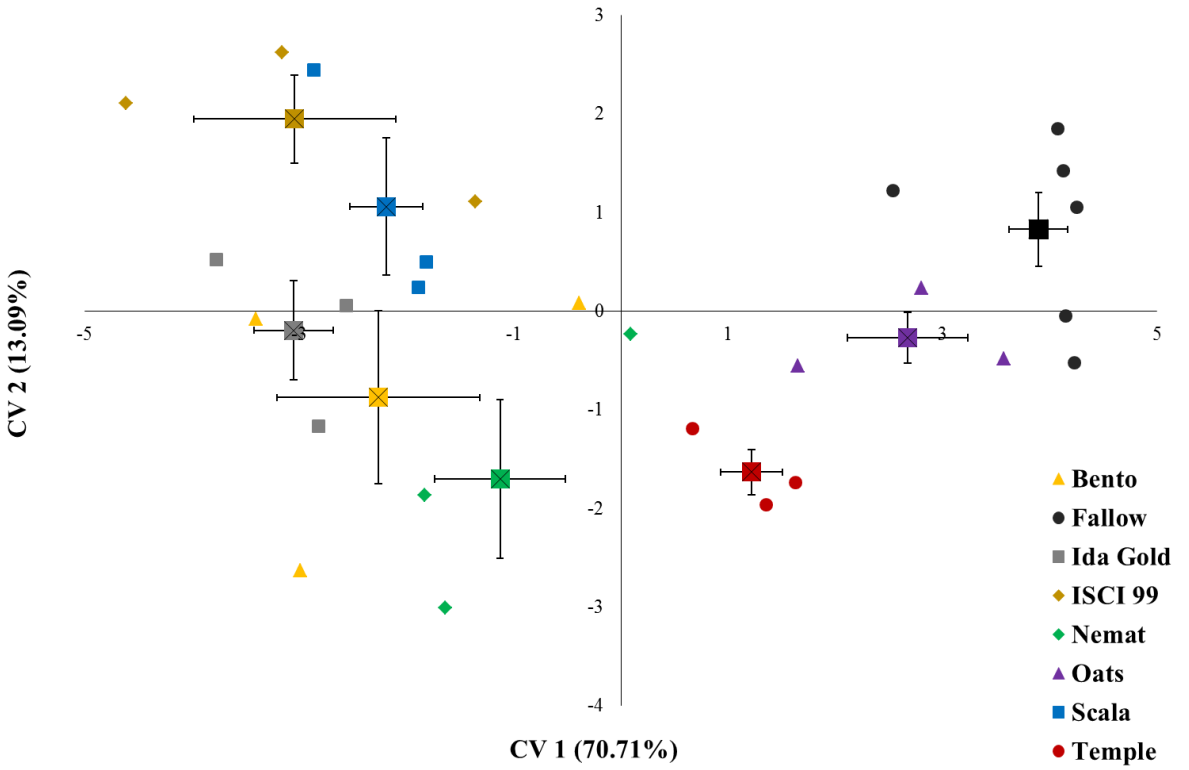


Figure 7.7. Scatter plot of the first and second CVs of CLPPs one week post-incorporation in the second biofumigation pot trial. The averages of each treatment are indicated by the cross-containing squares. Error bars represent the standard error for each CV.

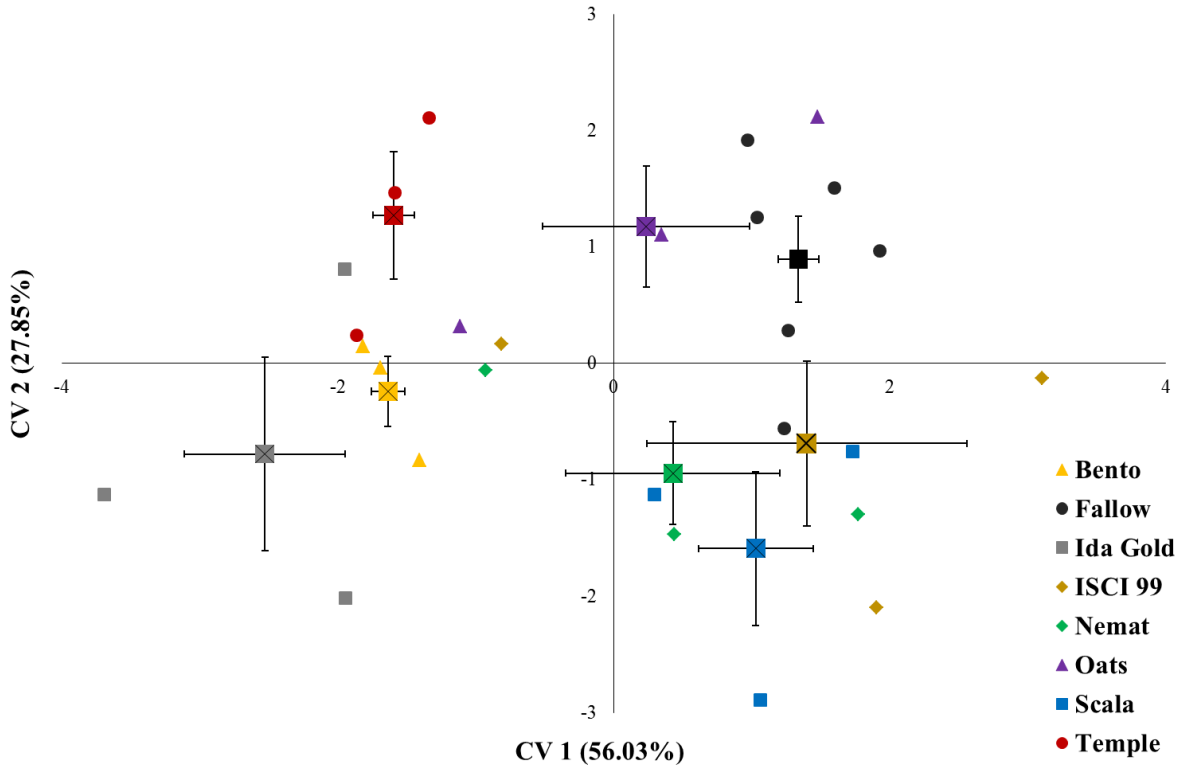


Figure 7.8. Scatter plot of the first and second CVs of CLPPs one month post-incorporation in the second biofumigation pot trial. The averages of each treatment are indicated by the cross-containing squares. Error bars represent the standard error for each CV.

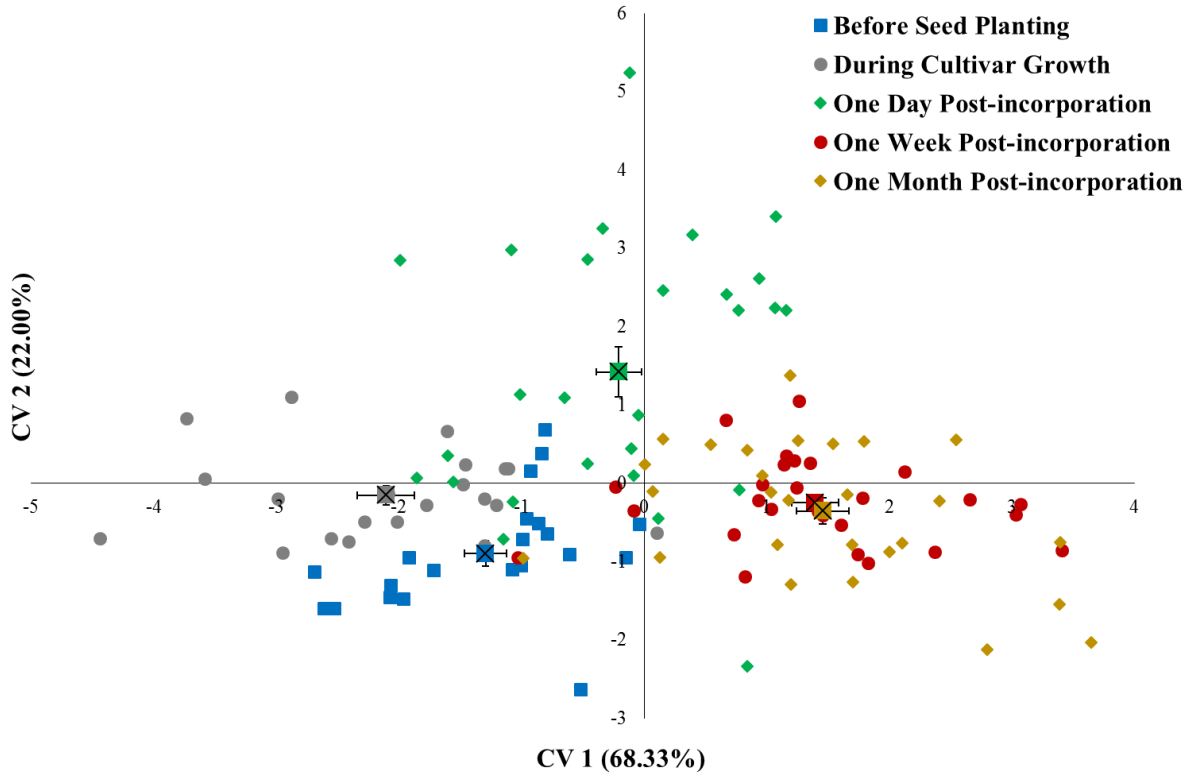


Figure 7.9. Scatter plot of the first and second CVs of CLPPs over five sampling points in the second biofumigation pot trial. The averages of each sampling point are indicated by the cross-containing squares. Error bars represent the standard error for each CV.

7.3.3. Biofumigation and soil microorganisms under field conditions

In the field trial, basal respiration was unaffected by treatment or the interaction between treatment and sampling point (data not shown). Respiration was affected significantly by sampling point (P<0.001; Figure 7.10). Basal respiration was similar over the first three sampling points (<0.35 µg CO_2-C g^{-1} h^{-1}) after which CO_2 rate increased significantly to 1.87 µg CO_2-C g^{-1} h^{-1} one week post-incorporation, before declining to 0.90 µg CO_2-C g^{-1} h^{-1} four months post-incorporation.

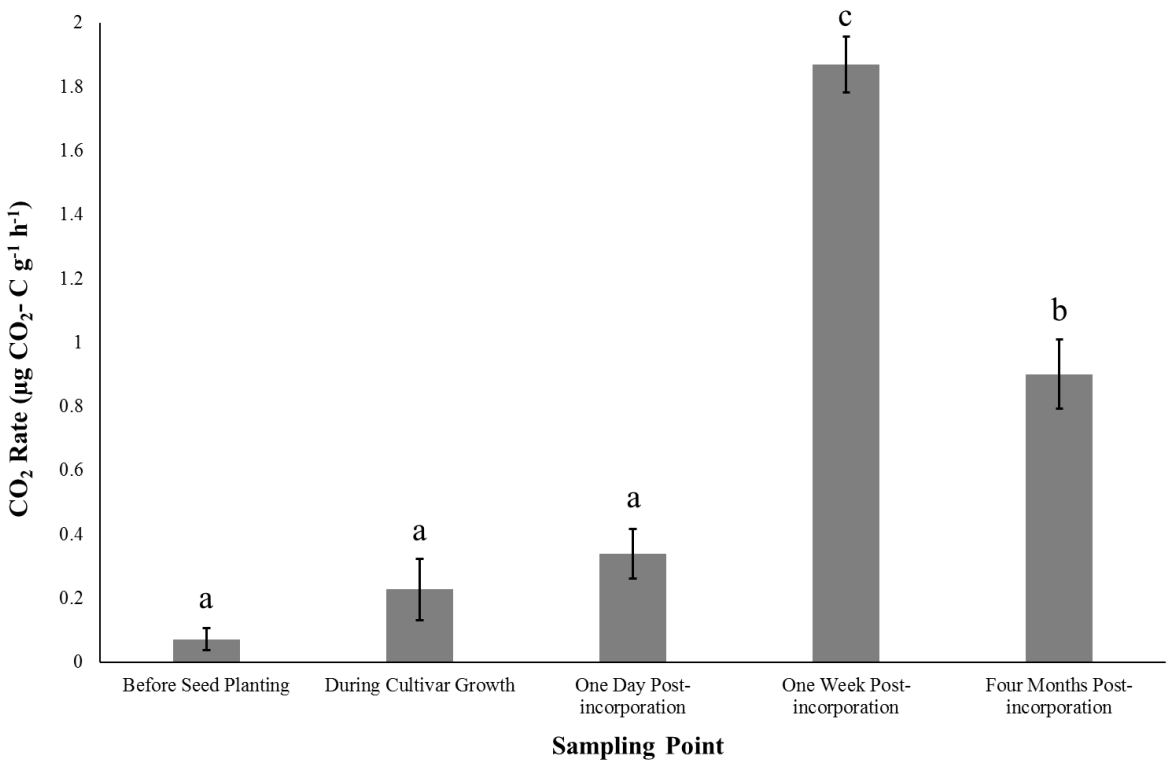


Figure 7.10. Soil basal respiration (µg CO_2- C g^{-1} h^{-1}) over five sampling points in the field trial. Error bars represent the standard error. Significant differences (P<0.05) are indicated by different letters.

Microbial community function was unaffected by treatment at any sampling point (data not shown) although there were shifts in communities over time (Pr<0.001; Figure 7.11). One week post-incorporation samples differed significantly from all other sampling points along CV 1 (56.95% variation). The before seed planting profile was significantly different from the one day and four month post-incorporation profiles. In addition, the during plant growth CLPP was distinct from the one day post-incorporation CLPP. All CLPPs were significantly different from each other along CV 2, which explained 26.35% variation, with the exception of the during cultivar growth and one week post-incorporation profiles which clustered.

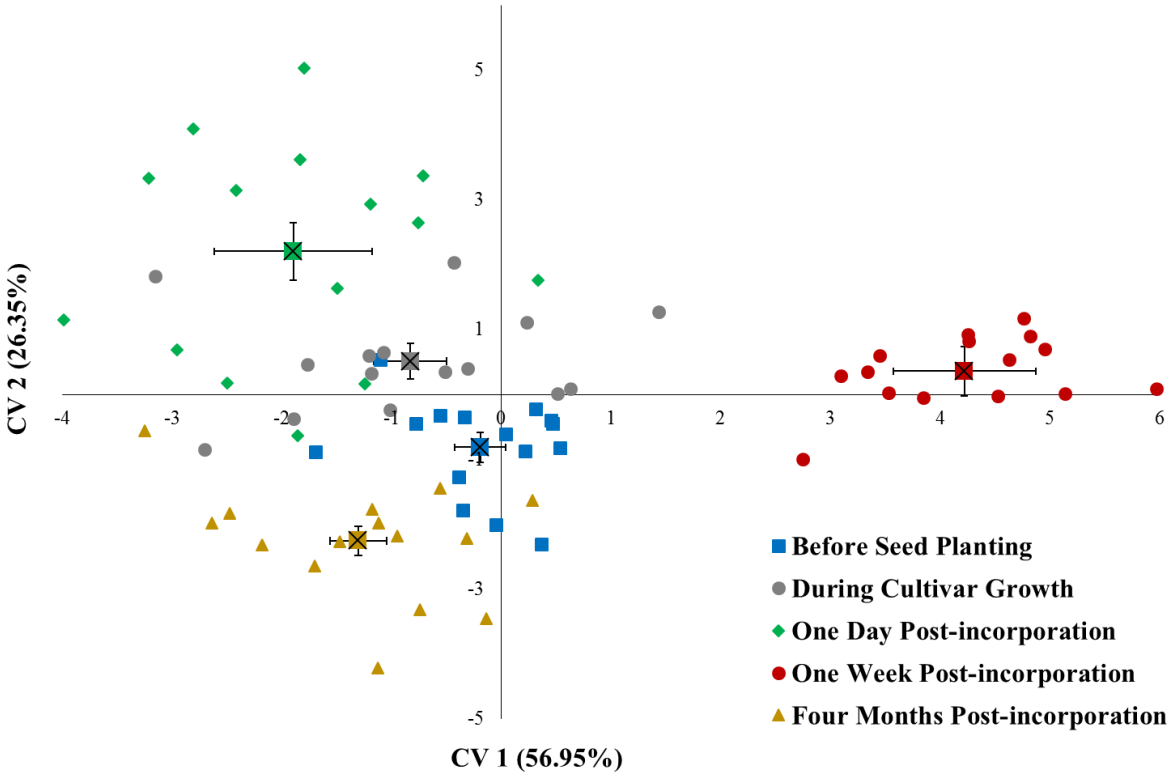


Figure 7.11. Scatter plot of the first and second CVs of CLPPs over five sampling points in the field trial. The averages of each sampling point are indicated by the cross-containing squares and error bars represent the standard error for each CV.

7.3.4. Preliminary qPCR assay results

Three qPCR primer sets were designed to detect *nifH*-containing nitrogen-fixing bacteria (Table 7.7). Testing these assays with four different bacterial standards showed that the primers and probes were specific to the species being targeted and that the individual sets did not amplify DNA standards from the other *nifH*-containing species or the negative ammonia-oxidising bacterial control (data not shown). A standard curve was obtained for each of the assays from serial dilutions of the standards (Table 7.8).

Target Organisms	Primers and Probes	Sequence (5'-3')
Cyanobacteria	CW_NifH_1F	TCCTACGACGTATTAGGTGA
	CW_NifH_1P	GGTGGTTTCGCTATGCCTAT
	CW_NifH_1R	TTCTTGTGCTTTACCTTCAC
***Azotobacter* sp. & *Pseudomonas* sp.**	CW_NifH_2F	CGATCAACTTCCTGGAAGA
	CW_NifH_2P	CTTCGCCATGCCCATCC
	CW_NifH_2R	ATTTCCTGAGCCTTGTTTTC
***Azospirillum* sp. & *Rhizobium* sp.**	CW_NifH_3F	GCTACAAGGGCATCAAGT
	CW_NifH_3P	GCGGCGTCATCACCTC
	CW_NifH_3R	TCTCTTCCAGGAAGTTGATC

Table 7.7. qPCR target genera, primer & probe names and DNA sequences. Each assay has a forward (F) primer, reverse (R) primer and probe (P).

Assay	DNA Standard	Standard Curve	R^2
NifH_1	*A. variabilis*	Ct = -3.409log(*nifH* DNA) + 23.74	0.998
NifH_2	*A. chroococcum*	Ct = -3.932log(*nifH* DNA) + 21.28	0.996
NifH_3	*R. etli*	Ct = -3.683log(*nifH* DNA) + 28.10	0.983

Table 7.8. qPCR assay, associated DNA standard organism, standard curve equation and the percentage of variance explained by the curve (R^2).

The three qPCR assays were run with microbial DNA extracted from the second biofumigant pot trial 'during cultivar growth' soil samples. In the NifH_1 and NifH_3 assays, DNA amplification of samples was unsuccessful (data not shown). In the NifH_2 assay, DNA amplification occurred in all soil samples and ng *nifH* DNA μg^{-1} total DNA was calculated from the NifH_2 standard curve (Figure 7.12). *NifH* DNA in soil differed significantly between cultivars during growth (P<0.001); the lowest amount of *nifH* DNA was identified in the ISCI 99 and Bento samples and varied from the Nemat and Oats soil samples.

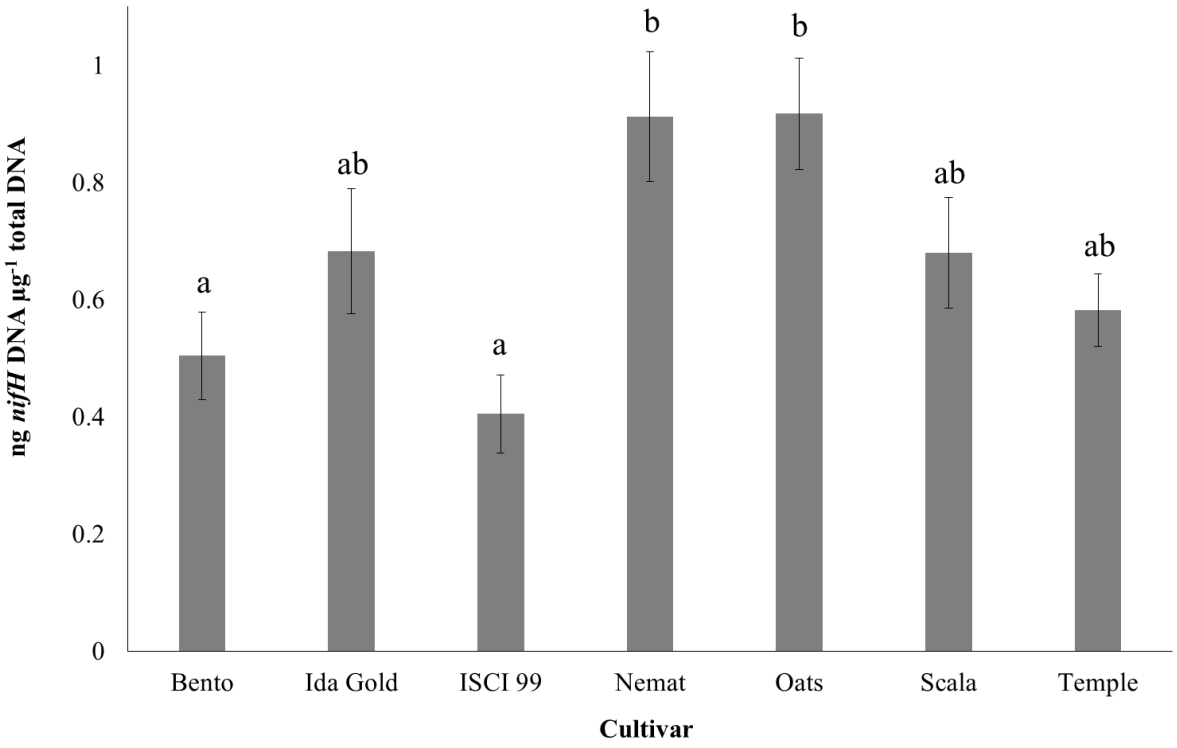


Figure 7.12. ng *nifH* DNA μg^{-1} total DNA in the NifH_2 assay from soil during the growth of cultivars. Error bars represent the standard error. Significant differences (P<0.05) are indicated by different letters.

7.4. Discussion

In all studies, sample point was the dominant factor affecting soil basal respiration and CLPPs with cultivar having no effect. The exception to this was a glasshouse trial completed under

controlled conditions. Differences over time are likely due to changing soil and environmental conditions; a number of contributing factors, as discussed further, can alter microbial communities and this is consistent with previous studies (Larkin and Honeycutt, 2006; Griffiths *et al.*, 2011; Orr *et al.*, 2011, 2012).

7.4.1. The impact of isothiocyanates on soil microorganisms

Incorporating pure ITCs into soil had no effect on microbial activity or the soil microbial community function under these conditions and using this diversity analysis method. This contradicts some previous studies. Incorporating pure AITC and PEITC into soil inhibited the growth of ammonia-oxidising and nitrite-oxidising bacteria, dependent on soil and ITC type, with populations recovered one week post-treatment (Bending and Lincoln, 2000). Similarly a later study showed that incorporating PEITC continuously for five days led to a shift in the microbial community structure of the soil rhizosphere (Rumberger and Marschner, 2003). In contrast, incorporating 200ppm PEITC into soil in a later study had no effect on microbial communities although it did decrease soil respiration for one week temporarily (Omirou *et al.*, 2011). Fungal communities can be influenced by AITC incorporation with high concentrations causing a shift in communities one week and one month post-addition (Hu *et al.*, 2015). In the same study, BITC had no effect on fungal communities but did cause a temporary shift in bacterial communities two days post-addition where communities had recovered by one week post-addition. These studies show that both fungal and bacterial soil communities are influenced by ITC addition although the persistence of shifts in the soil microbial communities were short suggesting that ITC release from biofumigation would not significantly impact soil processes in the long-term. A range of analysis approaches were taken in each study: bacterial plating, community qPCR analysis and denaturing gradient gel electrophoresis (DGGE), which would have contributed to the lack of consistency between studies.

Past experiments have investigated higher initial ITC concentrations (127-2000ppm) than used in the current study. BITC and PEITC are unlikely to be found at concentrations higher than 100ppm in the field (Chapter 5; Gimsing and Kirkegaard, 2006; Ngala *et al.*, 2014), therefore community changes due to their release are improbable with important soil nutrient cycles unlikely to be affected. It is possible for AITC to be released into the soil at higher concentrations (Chapter 5; Chapter 6; Bellostas, Sørensen and Sørensen, 2007; Ngala *et al.*, 2014) so biofumigation with sinigrin-containing cultivars could affect soil microbial communities.

7.4.2. The effect of growing plants on microbial CLPPs and activity

Cultivar growth increased basal respiration and induced a shift in microbial communities under glasshouse conditions, independent of the GSL content of the cultivars. It is known that growing plants release a number of beneficial nutrients from their roots such as amino acids, sugars, carbohydrates, vitamins and organic acids (Dakora and Phillips, 2002; Bertin, Yang and Weston, 2003) and that these can stimulate microbial growth and activity (Rincon-Florez, Carvalhais and Schenk, 2013). A number of the compounds released from plant roots are carbon substrates; all substrates utilised in the CLPP analysis in this study have been identified in root exudates. There was an increase in CO_2 rate of soil samples after exposure to substrates compared to CO_2 rate after exposure to water (Figure 7.13). This provides evidence to support that released carbon substrates from roots can stimulate activity and induce a shift in microbial physiological profiles by attracting the soil microbes which utilise them.

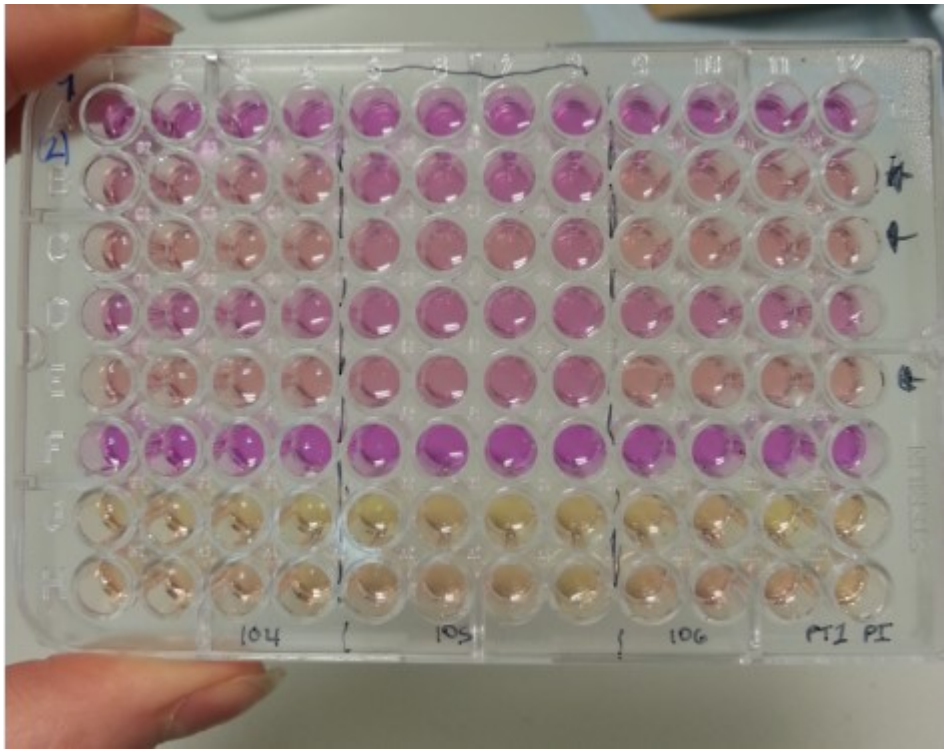


Figure 7.13. Example of a MicroResp CO_2 detection plate after CO_2 production. The gel begins pink and turns yellow with increasing CO_2 concentrations. Rows A-H: water, trehalose, fructose, glucose, lysine, arginine, citric acid and oxalic acid. Columns 1-4, 5-8 and 9-12 are three different soil samples.

Previous studies have noted that biofumigant cultivars can have an effect on target organisms before incorporation (Motisi *et al.*, 2013; Ngala *et al.*, 2014) which would suggest the release of toxic compounds from the hydrolysis of root GSLs (McCully *et al.*, 2008) by myrosinase-producing soil microorganisms (Borek, Morra and McCaffrey, 1996; Sakorn *et al.*, 1999; Rakariyatham *et al.*, 2005). Although there is the potential of ITC release during cultivar

growth, a lack of effect on the microbial community is to be expected when considering the ITC concentration required to impact soil communities in these studies. Because 100ppm ITC and incorporated cultivars containing high GSL concentrations (see results from 6.3.3) did not affect microbial communities it is unlikely that these cultivars would be able to release high enough ITC levels from their roots in the absence of tissue disruption. The lack of effect during cultivar growth would also suggest that the long term release of low doses of ITCs is unlikely to affect soil communities. Biofumigant plant growth should not impact the soil microbial composition compared to non-GSL green manures, as is the case in these studies.

7.4.3. Variability in microbial CLPPs relating to GSL content and ITC release

Only one of three biofumigation trials produced an effect relating to GSL hydrolysis and ITC release.

During the first pot trial, there was a significant effect of treatment one day post-incorporation which was not GSL related. Where there were significant differences between biofumigant cultivars and the Temple control, the sinigrin concentrations of the *B. juncea* cultivars reported in 6.3.3 would suggest that shifts are unlikely to be related to GSL content and ITC release. The two cultivars had similar GSL profiles but resulted in different CLPPs. Since seed rate and GSL profiles were the same between the two, the most likely reason for differences in microbial communities is inconsistencies in biomass incorporation and moisture content between treatments.

Shifts in soil microbial communities occurred one day and one week after biofumigant green manures were incorporated in the second pot trial, where variation in the biomass of incorporated material and moisture content of soil was controlled. This is consistent with a previous study which demonstrated a shift in microbial communities three weeks after treatment due to biofumigation (Wang *et al.*, 2014). In the current study, microbial communities exposed to all biofumigant cultivars clustered separate to the Fallow profiles and the green manure controls one day post-incorporation. Shifts cannot be attributed to differing soil moisture content, due to standardisation, or to the amount of fresh material incorporated, as high and low biomass CLPPs were not separated, rather there appears to be a clear division of biofumigant cultivars from the controls. This provides evidence for a short-term GSL-breakdown induced effect on the soil microbial community during the process of biofumigation.

Shifts in the microbial communities also occurred when comparing between biofumigant cultivars. This implies that variations in total GSL content and the major GSLs in each cultivar can impact the microbial communities differently. The greatest distinction was

between the Ida Gold and Bento microbial profiles; this can be attributed to a significant difference in total GSL content as well as in the major type of GSLs in each cultivar (6.3.3; glucosinalbin and glucoraphenin for Ida Gold and Bento respectively). Nemat CLPPs grouped closely with the Scala and ISCI 99 CLPPs even though their GSL profiles are dissimilar. The released ITCs may have properties which could influence soil physiological profiles in a likewise manner; both are aliphatic ITCs and known to be toxic to either: soil-borne pests (AITC) or human cells (SUL), therefore they may also share attributes which could influence the soil environment equally.

The lack of effect of biofumigation in the field trial implies that using these cultivars as biofumigants in the field would have no impact on the soil microbial community. This is consistent with a *B. carinata* seedmeal experiment (Wei, Passey and Xu, 2016) and a *B. napus* green manure study where incorporation had no effect on microbial activity four weeks after incorporation at two field sites (Bernard *et al.*, 2012). In contrast, a *B. napus* green manure rotation led to a reduction in bacterial populations in field soil four weeks after incorporation under conventional management practices, although the populations recovered after potato harvest, suggesting a lack of long-term effect (Bernard *et al.*, 2012). *Brassica napus* has not been investigated in the current trial so a direct comparison cannot be made. Differences are most likely due to the release of an alternative ITC. *Brassica napus* contains gluconasturtiin, which releases PEITC (Gardiner *et al.*, 1999), which none of the cultivars included in this study produced at high levels (see 6.3.3).

7.4.4. A contributory green manure effect and microbial CLPPs and activity

Although there was little GSL-related effect, green manure incorporation increased basal respiration and induced a shift in microbial communities under glasshouse conditions. Similar benefits on soil microbial growth and activity as growing plant material has been suggested for the incorporation of green manures. Green manure incorporation: increases the amount of organic matter introduced into the soil, improves nutrient availability, provides appropriate moisture and creates a more favourable soil pH which is beneficial for soil microbial activity and communities (Lupwayi, Rice and Clayton, 1998; Tiedje *et al.*, 1999; Bernard *et al.*, 2012; Hueso, García and Hernández, 2012.

In both pot trials all plant cultivars, including the low-GSL green manure controls, caused a short-term but significant shift in microbial profiles compared to the Fallow control. The shift in profiles after incorporation of the low-GSL control provides evidence for a green manure effect instead of an ITC-induced effect. A green manure effect is evident in previous studies where the incorporation of low- or non-GSL treatments has altered soil microbial

communities alongside GSL-containing treatments (Cohen, Yamasaki and Mazzola, 2005; Mocali *et al.*, 2015). This demonstrates that the effect of biofumigation on soil microorganisms is in part due to a secondary green manure effect.

The incorporation of green manure treatments appears to increase microbial activity in the soil. In the first pot trial, basal respiration increased independent of cultivar. In contrast, respiration increased in the second pot trial and the extent of which depended on the cultivar; Ida Gold, ISCI 99 and Scala incorporation increased basal activity the most. *B. oleracea* green manure incorporation has been shown to temporarily stimulate soil respiration and activity for up to one month (Omirou *et al.*, 2011). *Brassica napus* seedmeal was shown to increase bacterial populations for two weeks (Wang *et al.*, 2014) and the incorporation of *B. juncea* and *R. sativus* stimulated microbial activity for six weeks after incorporation (Ngala, Woods and Back, 2015b). Although the *S. alba* (Ida Gold) and *B. juncea* (ISCI 99 and Scala) cultivars increased respiration the most, this was not significantly so compared to the low-GSL Temple control. This was attributed to an intermediary increase in basal respiration after the low-GSL treatment due to a beneficial green manure effect.

Although there was no effect of biofumigation on the CLPPs in the field trial, soil basal respiration did increase one week post-incorporation where there was also a treatment-independent shift in microbial community profiles. This differs from the pot trials where the largest effects were noted one day post-incorporation. There was likely a delayed green manure effect in the field trial due to the experimental protocol when on a larger scale. Less efficient tissue disruption would have occurred due to plant material being chopped compared blending in the pot trials. This could have led to slower GSL hydrolysis and extended ITC release compared to the experiments completed under controlled conditions. In addition, an increased surface area in the field plots could have reduced the initial concentration of released ITCs; this would limit the impact of released compounds on the soil microorganisms in the first instance.

7.4.5. The persistence of the biofumigation impact on soil microorganisms

Changes in microbial activity and shifts in the microbial physiological profiles due to biofumigation were short-term and occurred one day and one week post-incorporation depending on the trial. The effects of cultivar incorporation persisted longer in the second biofumigant pot trial compared to the first and the differences between the soil microbial profiles became less pronounced as both trials progressed. This shows that the effect of incorporating biofumigant material into soil is not persistent and that soil microorganisms are able to recover within a short time frame under controlled conditions.

The temporary shift in the microbial community can be attributed to efficient ITC release from biofumigants and their subsequent rapid breakdown (Brown *et al.*, 1991; Borek *et al.*, 1995; Gardiner *et al.*, 1999; Petersen *et al.*, 2001; Morra and Kirkegaard, 2002) or the rapid breakdown of the green manure material leading to the re-establishment of the original microbial communities. This transient effect is consistent with previous trials: *B. oleracea* incorporation led to a shift in the microbial community one and two weeks after incorporation but not three months after incorporation (Omirou *et al.*, 2011), and *B. juncea*, *B. oleracea* and *R. sativus* altered soil communities most drastically two weeks after incorporation (Fouché, Maboeta and Claassens, 2016). The lack of persistence of effects confirms that biofumigation is unlikely to lead to the establishment of different microbial populations which could affect the efficiency of important soil processes.

In the second pot trial there were significant differences between treatments one month post-incorporation but there was no longer a distinction between the controls and biofumigant treatments. Therefore the shifts in profiles at this sampling point were unrelated to GSL content or green manure addition indicating an additional factor at play.

7.4.6. The influence of external and experimental conditions on the impact of biofumigation on soil microorganisms

There were several differences in the impact of biofumigation within the pot trials and between the pot and field trials attributed to variations in experimental parameters such as: moisture content, plant material biomass, differences in incorporation and the influence of environmental conditions.

Differences between pot trials are likely due to differences in moisture content and fresh material incorporation. In the first trial, cultivars were planted, grown and incorporated into the same pot throughout; not all planted seeds sprouted, leading to inconsistencies in biomass between replicates potentially masking an effect similar to that in the second trial. In addition, the moisture content of soil was not standardised which could have affected the establishment of different microbial communities in each pot and persistence of the effects. Omirou *et al.* (2013) found that increased soil respiration after *B. oleracea* incorporation persisted longer at a low soil moisture content (20%) compared to a high moisture content (90%). Water availability can influence the rate of plant material decomposition with higher moisture content leading to a faster rate of decomposition (Brandt, King and Milchunas, 2007; Powers *et al.*, 2009), implying that variations in moisture content between replicates would have influenced the persistence of the green manures effects. Due to this, a lack of consistency

between replicates led to more variability between samples and a lack of identifiable shifts in the soil community in the first experiment.

In a separate issue, a lower GSL content in the *B. juncea* cultivars was noted in the first pot trial compared to the second (see 6.3.3); the potential ITC release may have been too low to induce a shift in microbial communities in the first experiment compared to the second.

Differences between the pot and field trials with respect to the impact of biofumigation on soil communities is most likely due to environmental factors and differences in methodology. Environmental conditions in the glasshouse studies were controlled, with consistent temperature and moisture. In comparison, the field trial would have been affected by varying environmental conditions including changes in temperature (Davidson, Belk and Boone, 1998), pH (Lauber *et al.*, 2009) and rainfall (Clark *et al.*, 2009; Hueso, García and Hernández, 2012; Hagemann *et al.*, 2016). This could potentially alter the soil environment, and soil microbial communities, over both location and time leading to less consistency between plot replicates compared to pot replicates. Environmental conditions would also influence the GSL content and growth of potential biofumigant cultivars (Ciska, Martyniak-Przybyszewska and Halina, 2000; Jeffery *et al.*, 2003; Charron, Saxton and Sams, 2005; Velasco *et al.*, 2007). Results from 6.3.3 show that GSL content was lower in field-grown cultivars compared to glasshouse-grown cultivars which in turn would have led to lower ITC release and a lower impact on soil microorganisms.

Experimental methodology between glasshouse and field trials needs to be investigated as an impacting factor. Although seed rate and biomass were standardised across trials, incorporation soil depth in the pot trial was not considered. In addition, ITC release was maximised in the pot trials through plant material blending and sealing of pots which increased exposure of the soil microorganisms to the volatiles. The field trial would not have achieved the same effect from flail mowing, rotovating and rolling leading to less efficient GSL hydrolysis and quicker volatisation into the atmosphere; this would have had less of an impact on the soil microbial community (Mattner *et al.*, 2008). The lack of effect in the field trial is not necessarily a negative result as this study has not determined if the shifts in the microbial community under controlled conditions are advantageous for important soil processes.

7.4.7. Shifts in microbial CLPPs and activity relating to experimental and environmental conditions

The largest effect on soil microbial communities was attributed to changes in the soil environment over time due to variations in external and experimental conditions. CLPP shifts

indicate that changing soil conditions can affect soil microorganisms and should be considered when measuring changes in communities in response to agricultural processes over time.

When treatments consisted of adding liquid, as in the ITC experiment, there was a significant reduction in basal activity and a shift in the community profile of samples, independent of ITC addition, one day post-treatment. This is likely due to the disturbance of soil during treatment incorporation. The disturbance of soil through tillage has been shown to reduce soil diversity compared to soil where tillage did not occur (Lupwayi, Rice and Clayton, 1998). This was attributed to a number of factors relating to soil disturbance such as: mechanical destruction, compaction of soil, altered pore volume and a disruption of access to food sources (Giller, 1996). Giller (1996) suggested that soil changes due to these influences led to the establishment of dominant microorganisms reducing the presence of competing inferior species and causing lower overall diversity. Due to this, disturbance of the soil in the current ITC study would both shift the community profile due to the establishment of different species and reduce basal respiration after the sudden change in the soil environment. One week post-treatment, there was a second shift in CLPPs and an increase in basal respiration which suggests a settling of the microbial communities and increase in activity as they became established. A decrease in activity and return to the original community profile one month into the trial most likely occurred due to the return of the soil environment to its original state in the absence of plant growth or further soil disturbance. At potato harvest, basal respiration was similar to pre-treatment samples although there was a clear shift in the community profiles. This is to be expected as the soil would have remained undisturbed since seed planting and different microbial communities will have established in the new soil environment, most likely due to the influence of potato root exudates on the soil microorganisms (Dakora and Phillips, 2002).

The general shifts in the soil physiological profiles over time in the biofumigant trials can be attributed to the changing soil conditions from bare soil to actively growing plants to green manure incorporation to potato plant growth. This is due to the release of different compounds and nutrients at each stage by the plant roots and incorporated material which affects the soil environment, as discussed previously.

7.4.8. The detection of shifts in nitrogen-fixing bacterial populations

The real-time assays were developed in order to quantify changes in nitrogen-fixing bacterial groups. Nitrogen-fixing bacteria were chosen as they are considered good indicator species which are sensitive to environmental changes and shifts in their populations could negatively

impact the nitrogen cycle which is a key soil process (Orr *et al.*, 2011, 2012). It is important to include information on how biofumigation affects specific bacterial groups to complement the community profile data as the current study does not provide knowledge on if the shifts in microbial communities are advantageous or not. Although primer sets were designed successfully to detect important nitrogen-fixing bacteria, two of the assays were unsuccessful when applied to DNA extracted from soil samples. This would suggest that either the designed assays were unable to detect and amplify the target DNA from a mixed DNA sample or that the targeted species were not present in the soil. Further work is required to optimise these assays.

In contrast, the assay which was designed to detect *Azotobacter* spp. and *Pseudomonas* spp. amplified DNA from all soil samples taken during plant growth. This indicates that nitrogen-fixing members of these species are present in soil during cultivar growth. This is to be expected as *Azotobacter* spp. is commonly identified in soil and associates with the roots of several plants (Martinez Toledo *et al.*, 1988; Tejera *et al.*, 2005; Kumar *et al.*, 2007). Plant species appears to influence the quantity of *nifH*-containing *Azotobacter* spp. and *Pseudomonas* spp. in the soil, independent of GSL content, with the most DNA quantified within soil during the Bristle Oats and Nemat growth compared to ISCI 99 and Bento growth. Nemat has been shown to have a distinct GSL profile from the other Brassicaceae cultivars (Chapter 5) and Bristle Oats is a non-Brassicaceae species therefore there is the potential that these cultivars release root exudates which could affect these bacterial groups differently to the other cultivars. The lack of effect of growing biofumigant plant material in the CLPP studies contrasts to these qPCR results and highlights the need for complementary studies; overall differences, or a lack thereof, in the microbial community profiles may not represent changes in specific bacterial groups involved in important soil processes. Due to a lack of time only soil samples during plant growth were analysed with the three assays. Further work is required to determine the effect of the biofumigation process on nitrogen-fixing bacteria and establish if GSL hydrolysis and ITC release impacts these species persistently.

7.4.9. Concluding remarks

Results from this chapter provide information on how ITCs and biofumigation can affect soil microbial basal activity and induce shifts in the soil microbial community function. ITC addition had no effects on the soil microbial community. Biofumigation also had very little effect on soil microorganisms with only short-term shifts in microbial activity and communities after green manure incorporation under controlled conditions. When taken into a

field setting, biofumigation no longer altered soil microbial profiles providing evidence that the process is unlikely to negatively impact the soil microbial community.

This study shows that although biofumigation has little persistent effect on the soil community, other factors can greatly influence the structure of the soil with: moisture content, mechanical disturbance, nutrient provision and green manure incorporation all leading to significant shifts in the microbial communities, independent of treatment. In general, these changes were beneficial and led to an increase in microbial respiration. It is unknown if the shifts in the soil physiological profiles impacted on important microbial groups involved in soil processes. The absence of any long-term shifts in overall microbial communities as a result of biofumigant crop incorporation is positive, and there were no adverse effects on basal respiration. Further work is required to identify the effect of biofumigants on bacterial groups involved in important soil processes, such as nitrogen cycling.

Chapter 8. Final Discussion and Conclusions

8.1. Study Findings

The main aim of this study was to determine if biofumigation has the potential to be used to control potato cyst nematodes (PCN), namely *Globodera pallida*. In order to do this, a series of *in vitro* and glasshouse trials were completed with the potentially toxic glucosinolate (GSL) hydrolysis products, isothiocyanates (ITCs), followed by glasshouse and field biofumigation trials with commercially sold biofumigant cultivars. AITC was effective at suppressing *G. pallida* hatch and increasing encysted juvenile (J2) mortality at concentrations above 100ppm. *Brassica juncea* cultivars were able to suppress *G. pallida* hatch, increase mortality and reduce the formation of new cysts under controlled conditions.

The GSL profiles of cultivars were determined in order to relate *G. pallida* suppression to potential ITC release from the biofumigants as well as provide information on GSL profile changes over plant growth to ascertain the optimum incorporation time for maximised GSL content. The major GSL in each cultivar varied with *Brassica juncea* cultivars containing high concentrations of sinigrin. GSL content varied over plant growth depending on the species, growth stage and GSL.

Information was collected on the effect of ITCs and biofumigation on soil microbial communities as new processes involving the exposure of soil microorganisms to toxic compounds may impact key soil processes with negative environmental consequences. The biofumigation process had short term effects on soil communities under controlled conditions but not under field conditions.

8.1.1. The effect of isothiocyanates on PCN

Initial studies found that ITCs had differing effects on free-living *G. pallida* and *G. rostochiensis* J2. Several ITCs at 50ppm were able to cause over 90% J2 mortality over a three day period with AITC being the most effective at increasing mortality for both species within the shortest exposure period. Differences in mortality suggested that ITC toxicity depends upon the species being targeted and therefore initial screening of ITCs should be completed when considering biofumigation as a control method for any given soil pest or pathogen.

In the initial hatching assays it became apparent that although able to cause free-living J2 mortality, the effect of ITCs was greatly reduced when J2 were encysted. Hatch suppression did not occur consistently over concentrations or exposure periods leading to the conclusion that any reduction in hatch was either due to: transient suppression, through temporary

eggshell or cyst wall permeability alterations or J2 paralysis, or a false positive effect due to a high variability in egg number between cysts. A number of assays were completed where experimental conditions were altered and higher ITC concentrations were investigated to determine the conditions and minimum ITC dose required for efficient ITC-related *G. pallida* suppression.

In these assays, AITC was the only ITC able to suppress hatch and increase the mortality of encysted *G. pallida* J2. A delay of four weeks between AITC exposure and hatch stimulation increased the suppressive effects of AITC on encysted *G. pallida* which is advantageous as when implementing biofumigation in the field there is an extended period of time between incorporation and potato planting. PCN control by AITC was determined to be the induction of J2 paralysis followed by mortality, dependent on AITC concentration. Concentrations lower than 50ppm AITC delayed hatch but did not lead to permanent suppression. Concentrations above 50ppm permanently suppressed *G. pallida* hatch and increased J2 mortality with 100ppm AITC being sufficient for complete hatch suppression *in vitro*. This was achieved after one day's exposure when there was a four week delay included before hatch stimulation. This shows that AITC is effective after a short exposure period, and extended time between exposure and hatch stimulation is required to allow the process of J2 paralysis followed by J2 death to occur.

In soil microcosm and glasshouse trials, AITC was effective at suppressing encysted *G. pallida* J2 although a higher concentration of 500ppm was required to completely inhibit hatch and increase mortality. This is to be expected as the soil environment would reduce direct contact between the ITCs and the cyst requiring a higher concentration of AITC for suppression than in *in vitro* studies. Temperature and soil composition were found to have little impact on AITC efficiency in soil, but there was higher variability between replicates in sandy silt loam soil than in the other soil types. Therefore, higher AITC concentrations may be required in soil categorised as sandy silt for consistent AITC-related PCN control. These soil experiments are important as they provide evidence that biofumigation to control *G. pallida* in the field would, in theory, be effective provided that cultivars used are able to release a high AITC concentration.

8.1.2. Glucosinolate profiles of cultivars over plant development

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis of Brassicaceae above-ground plant tissue grown under glasshouse conditions indicated that all cultivars contained high GSL concentrations. In general, each cultivar contained one dominant GSL which made up the majority of the total GSL concentration at each growth stage. Nemat was the exception to this

and contained two dominant GSLs which varied over growth stages. The major GSL in each cultivar was species dependent and only ISCI 99 and Scala, the *B. juncea* cultivars, contained high levels of sinigrin which breaks down into the ITC of interest, AITC. Both cultivars contained levels of sinigrin sufficient to ensure that the resulting AITC-release would equal or exceed the levels determined for efficient *G. pallida* control in Chapter 4.

Results from this study conclude that the presence and concentration of GSLs alters throughout plant development meaning that the time of incorporation for maximum pest suppression may differ depending on the GSL of interest and the cultivar which produces it. In general, the highest GSL content occurred between initial flowering and seed development which is consistent with the current practice of incorporating biofumigants when close to 70% flowering. Although incorporating at this stage would release a high concentration of ITCs, this study shows that it would be advantageous to incorporate ISCI 99 and Scala at initial flowering and seed development, respectively, for higher sinigrin concentration and maximum AITC release. The plant growth stage for optimum GSL content is not restricted to one developmental period. This allows more flexibility in the field when considering when to incorporate, especially as different species grow at different rates depending on environmental factors and plant competition (Velasco *et al.*, 2007; Antonious, Bomford and Vincelli, 2009).

8.1.3. Biofumigation and G. pallida *under controlled conditions*

The biofumigation pot trials (Chapter 6) were not as successful as the AITC studies (Chapter 4). Nevertheless, *B. juncea* cultivars did suppress encysted *G. pallida* under certain conditions.

In one trial, none of the incorporated cultivars had an effect on *G. pallida* hatch or multiplication. An absence of suppression was attributed to a lack of consistency between replicates in moisture content and incorporated plant biomass; this would have led to differences in ITC release which could have masked a potential suppressive effect of the incorporated green manure. In addition, the *B. juncea* cultivars at time of incorporation contained lower sinigrin concentrations than identified in Chapter 5, which in turn would have released lower ITC concentrations.

In a second pot trial, ISCI 99 and Scala reduced encysted *G. pallida* hatch, increased encysted J2 mortality, and reduced the formation of new cysts on the subsequently planted potato crop. Incorporated biomass and moisture content was standardised over replicates so variability between pots was reduced. The *B. juncea* cultivars contained a high sinigrin concentration at time of incorporation (over 32mg g^{-1} DW sinigrin). This suggests that either 300ppm AITC is

sufficient for control, assuming a high biomass, or that the GSL-ITC conversion was higher than 1% in this trial.

None of the other cultivars suppressed encysted *G. pallida* under controlled conditions. GSL profiles from the second trial showed that Nemat and Bento GSL concentrations at incorporation were considerably lower than results from Chapter 5; further experiments under controlled conditions should be completed with these cultivars to confirm the absence of control. In contrast, Ida Gold contained a high concentration of glucosinalbin providing evidence that the ITC released from its hydrolysis is not effective at suppressing *G. pallida*. This *S. alba* cultivar should not be included in PCN biofumigation management programs.

It was noted that there was a positive green manure effect in at least one of the trials. This provides evidence that, in addition to ITC release, biofumigation can contribute to pathogen suppression through factors associated with the decomposition of plant material in soil.

8.1.4. Biofumigation and G. pallida *under field conditions*

None of the cultivars suppressed encysted *G. pallida* in the field trials. The *B. juncea* cultivars at time of incorporation were shown to contain lower sinigrin concentrations than identified in Chapter 5 which in turn would have released lower ITC concentrations (235 and 245ppm AITC for ISCI 99 and Scala respectively). Variable environmental conditions such as fluxes in temperature, moisture and soil pH could have affected the final biomass of plant material at incorporation as well as the rate of GSL hydrolysis and ITC volatisation once incorporated. The methods employed in the field for chopping material and sealing soil would not have resulted in the same level of GSL hydrolysis or ITC exposure as in the glasshouse trial.

None of the other cultivars suppressed encysted *G. pallida* under field conditions. In contrast to the glasshouse trial, GSL profiles from the field trial showed that Nemat, Bento and Ida Gold GSL concentrations at incorporation were similar to those in Chapter 5. This shows that the dominant GSLs in these cultivars: glucoraphenin, glucosinalbin and glucosativin, do not release desirable ITCs for *G. pallida* suppression. These *Eruca sativa*, *Raphanus sativus* and *Sinapis alba* cultivars should not be included in PCN biofumigation management programs.

8.1.5. Isothiocyanates, biofumigation and soil microbial communities

Pure ITC addition had no effect on soil microorganisms. This shows that ITC release below 100ppm will be too low to influence non-target microorganisms. Biofumigation under controlled conditions led to short-term shifts in the soil microbial communities and increased basal respiration for up to one week after incorporation. Communities recovered quickly so there should be no long-term adverse effects of biofumigation and ITC release on key soil

processes. In addition, biofumigation and green manure incorporation had no effect on soil microbial communities under field conditions providing further evidence that biofumigation would not negatively affect non-target soil microorganisms.

Factors unrelated to biofumigation and ITC release had an effect on soil microorganisms with large shifts in communities and basal respiration occurring over time. This was attributed to a number of factors including: changes in moisture content, the mechanical disturbance of soil, the incorporation of green manure and the release of nutrients from active plant growth and root exudates.

8.2. Comparison of Glasshouse and Field Studies

This collection of studies highlights the need to complete *in vitro*, glasshouse and field trials when understanding the effects of biofumigation on a soil pest. It is known that *in vitro* results do not always translate efficiently into *in vivo* studies as the introduction of additional factors can negatively influence the biofumigation process. Differing results between glasshouse and field trials have been noted here with a number of the discrepancies attributed to trial conditions.

8.2.1. Encysted G. pallida *suppression*

The ability of biofumigants to control *G. pallida* in soil was dependent upon the trial conditions and the GSL content of the cultivars being incorporated. Out of two glasshouse and two field trials, biofumigation with AITC-releasing *B. juncea* cultivars was only successful when temperature, moisture content and cultivar biomass were controlled.

The influence of environmental factors can have a negative impact on: GSL hydrolysis and ITC release (Charron, Saxton and Sams, 2005). This is a problem with respect to achieving the ITC concentration required. Biofumigation in the field is likely to be unpredictable with pathogen suppression varying between trial sites and seasons, potentially even between different plots of the same site, due to inconsistencies in plant growth and GSL production.

Although the field trials were unsuccessful in this study, previous field trials have had success at implementing biofumigation for the control of *G. pallida* populations. Therefore further field sites and environmental conditions must continue to be assessed to determine the requirements for an effective biofumigation strategy.

8.2.2. Glucosinolate profiles

When comparing the GSL profiles of cultivars between trials at time of incorporation, it is clear that several factors can influence the GSL content of material; the specifics of these have not been fully investigated in this trial.

The GSL concentration of cultivars differed between pot trials. In general, concentration was highest at 70% flowering in the GSL profile pot trial (Chapter 5) compared to cultivars at incorporation in the biofumigation pot trials (Chapter 6). As glasshouse temperature and moisture conditions were the same for each trial, differences in the GSL content of cultivars could be due to the pot sizes used or seed rates applied. The GSL profile trial and first biofumigation pot trial used the same pot sizes during plant growth although seed rate differed with a greater amount of plant material grown in the biofumigation trial. The lower GSL content in the biofumigation trial cultivars may then be due to: increased root competition for water and soil nutrients, namely sulphur, (Kim *et al.*, 2002; Schenk, 2006; Zhang *et al.*, 2008) and decreased exposure to light (Engelen-Eigles *et al.*, 2005; Huseby *et al.*, 2013) due to inadequate spacing between plants. In contrast, the seed rate for Ida Gold, ISCI 99 and Scala had no effect on GSL content in the second biofumigation trial compared to the GSL profile trial; this may be due to the increased pot size which allowed more space for plant roots to develop and leaves to access light, even with the higher seed rate.

The increased pot size did not benefit all cultivars in the second biofumigation trial. Bento and Nemat had significantly lower GSL levels at incorporation compared to the other cultivars and the equivalent GSL profile trial plants. This is most likely due to inadequate glasshouse spacing. *Raphanus sativus* (Bento) and *E. sativa* (Nemat) are both compact leafy species with an extensive root system but very little shoot system. In comparison, *S. alba* (Ida Gold) and *B. juncea* (ISCI 99 and Scala) are tall mustard species with a number of shoots and a smaller root system (Figure 8.1). Since the plants were grown in close proximity, the mustards would have grown tall and blocked the light for the non-mustard species. The lack of light access combined with the larger root system increasing nutrient competition in the soil could account for the differences in GSL content between the species.

Figure 8.1. Examples of each plant cultivar. Clockwise from top left: Ida Gold, ISCI 99, Scala, Bento and Nemat. These photos were taken at the time of incorporation in the second biofumigant pot trial.

Field-grown cultivars contained similar total GSL concentrations to those in the GSL profile trial. This implies that the issues with light and soil nutrient competition were not present when in a field setting which is to be expected due to the larger plot sizes and increased soil depth for the Bento and Nemat root systems to exploit. In spite of this, the concentration of the major GSL in the field-grown cultivars was generally lower than in the glasshouse-grown cultivars and GSL profiles differed slightly. This is likely due to varying environmental conditions, such as temperature and rainfall, influencing the GSL content of cultivars (Ciska, Martyniak-Przybyszewska and Halina, 2000; Jeffery *et al.*, 2003; Velasco *et al.*, 2007). The lowered GSL content in field-grown material and lack of suppressive effect on encysted *G.*

pallida highlights the need to improve biofumigation practice in the field by either investigating cultivars with a higher sinigrin concentration or by increasing the biomass of sinigrin-containing material being grown and incorporated.

8.2.3. Soil microbial communities

Differences between trials were noted in the soil microbial experiments where GSL content and environmental conditions influenced the effect of biofumigation on soil communities. A biofumigant effect was only identified in one trial under controlled conditions; the greatest effect was with the Ida Gold, ISCI 99 and Scala cultivars, which contained the highest GSL content at time of incorporation.

There were differences in moisture, biomass and GSL content between the replicates incorporated in the first glasshouse trial. As the trials were temperature controlled, these were the factors which could explain why biofumigation related shifts were noted in the second trial and not the first. These studies did not explore if the lack of effect was one of, or a mixture of, these factors. Further work is required to separate out which factor had the greatest influence on the ability of biofumigation to affect soil communities.

Variations in the ability of biofumigants to induce a shift in soil communities between glasshouse and field trials were attributed to both environmental conditions and the incorporation method employed. Although biofumigation had no effect on soil microbial communities in the field trial compared to the glasshouse trial, this is considered a positive outcome. Any change in the natural soil microbial community structure leading to differences in respiration or diversity could negatively impact important soil processes. The knowledge that the effect of biofumigation on soil microorganisms is reduced under field conditions shows that incorporating the process into pest management strategies is unlikely to alter the microbial communities present in field soil if no changes are made to increasing ITC release. In the instance that cultivars containing higher GSL levels are incorporated, the impact on soil communities would need to be re-assessed.

8.3. Optimising *G. pallida* Biofumigation in the Field

Although several advances have been made throughout this study, it is evident that further research is required to gather the information required to increase the efficiency of biofumigation in the field. The major factors that appear to impact on biofumigation effectiveness in these trials are environmental conditions and incorporation methodology.

Although environmental conditions, such as temperature and rainfall, cannot be controlled, steps can be taken to increase the probability of success in the field. While sinigrin-containing

cultivars have been identified as effective under controlled conditions in this study, the lower GSL content in the field trial shows that they do not grow as well under variable environmental conditions. Further research is required to identify cultivars able to accumulate a high sinigrin concentration under field conditions as well as research into controllable factors which could assist the cultivars in producing a high GSL content. During these studies, consistent moisture improved encysted G. pallida suppression; therefore, irrigation of biofumigants may be necessary to maximise GSL content. In addition, seed rate may need to be further investigated for the production of a higher plant biomass, as plant growth can be highly variable; this needs to be balanced with cost further emphasising the importance of selecting high-sinigrin containing cultivars. Experiments determining the specifics of this were not completed in this study and further research is required to determine the optimum conditions for each contributing factor which could help maximise GSL content.

In addition to maximising GSL content, optimising ITC release in the field needs to be addressed further. The method of incorporation used in the field trials did not promote ITC production at concentrations high enough for encysted G. pallida suppression. Different incorporation methods on a large scale need to be compared, with the aim of maximising ITC release and reducing ITC volatisation, to increase contact time between the soil pathogen and toxic GSL-hydrolysis products.

Although the field trials were unsuccessful in this study, there is the possibility that the lack of effect was due to specific environmental conditions experienced over these two trials. Further trials under a range of field conditions and sites must be assessed to identify the specific conditions controlling GSL production and ITC release.

8.4. PCN Diapause and Biofumigation

Results from this study suggest that the current practice of incorporating biofumigants in autumn may not be effective against encysted *G. pallida* due to the unique ability of PCN to enter diapause in the absence of potatoes. The *in vitro* assays showed that AITC was more effective at suppressing *G. pallida* hatch when exposed during J2 hatch stimulation compared to before. In addition, viability experiments noted that there were a proportion of unhatched viable eggs in each assay and trial which remained unaffected by any treatment; these were considered dormant J2 which had not broken diapause. This demonstrates that J2 in a diapause state could be resistant to ITC exposure. Different responses of J2 in various states are likely due to altered gene regulation and eggshell permeability during and after diapause (Ellenby and Perry, 1976; Atkinson and Ballantyne, 1977b; Clarke, Perry and Hennessy, 1978; Beane and Perry, 1990; Kovaleva *et al.*, 2004). Once diapause is broken and encysted

J2 enter a state of quiescence, the J2 are likely to be more vulnerable and open to attack compared to J2 in diapause. Although different J2 states in the *in vitro* assays was low, as the majority of diapause was overcome before use, encysted J2 state may hinder biofumigation use in the field.

It is currently assumed that mature cysts which have undergone diapause and entered quiescence do not return to a diapause state in the absence of a host. This assumption has arisen from studies where cysts which have remained in field soil for a number of years have a high percentage of eggs hatching at time of collection (Kroese, Zasada and Ingham, 2011; Ingham, Kroese and Zasada, 2015). It should be noted that the trials which used mature cysts collected them during warm seasons, when diapause would not be present, whether or not it had reoccurred before winter. Although a number of studies have looked at when diapause is overcome in the first year after formation (Hominick, Forrest and Evans, 1985; Hominick, 1986; Muhammad, 1994; Muhammad and Evans, 1997; Ingham, Kroese and Zasada, 2015), little work has been completed on how seasonality affects potential facultative PCN diapause in future years when optimal hatching conditions have not been met.

In spite of the lack of specific studies, previous data would support the possibility of facultative diapause occurring in PCN cysts in response to a seasonality effect. Hominick, Forrest and Evans (1985) found that two year old *G. rostochiensis* cysts displayed varying hatch percentages over time: cysts hatched in October, December and July displayed between 80-95%, 60-75% and 40-70% hatch, respectively, whilst cysts hatched in February, April and August displayed between 25-50%, 25-50% and 40-50% hatch, respectively. Similarly, *G. rostochiensis* cysts hatched over a year showed an increase in hatch in April (33% hatch) and June (60% hatch) after obligate diapause was overcome in December (20% hatch) and February (15% hatch) followed by a decrease in hatch when initiated in October (40% hatch) (Hominick, 1986). Muhammad and Evans (1997) noted that hatch from mature *G. rostochiensis* cysts stored for one year outside then one year at 20°C was lower in the last hatching period of August (75% hatch) compared to the previous hatching periods of October to July (85-100% hatch). As these experiments were discontinued after the described time periods it is unclear if this decreasing trend continues; nevertheless, the results would suggest an influence of season on *G. rostochiensis* hatch readiness with low temperatures and an absence of host crop initiating a facultative diapause state of cysts until optimal hatching conditions return. In the case that diapause can reoccur, a long-term hatching field trial is required to monitor how cysts, in the absence of crops, respond to changing seasons over multiple years.

Facultative diapause would have implications on the effectiveness of biofumigation on mature encysted PCN in the field and a re-thinking of how the process is implemented for PCN control would be required. The incorporation of biofumigants during spring may increase PCN suppression as a high proportion of encysted J2 will be in a vulnerable state. The environmental conditions at this time of year may not be beneficial for maximum GSL content. Experimentation is also required to determine the GSL profiles of spring grown and incorporated cultivars. Furthermore, the practicalities of implementing the process in crop rotations during this season need to be considered.

8.5. Reducing the Impact of Biofumigation on Soil Microbial Communities

There was little effect of biofumigation on soil communities in the field trial, although biofumigation under controlled conditions did lead to short-term shifts up to a week after incorporation. Although not persistent, any change in the soil communities due to agricultural practices should be considered disadvantageous to key soil processes in the absence of information on specific bacterial groups. Due to this, altering the moisture content of soil during the biofumigation process could reduce the effects seen in the glasshouse trial. During biofumigation, lower moisture content can cause slower GSL hydrolysis and ITC release leading to a longer persistence of the compound in soil (Morra and Kirkegaard, 2002; Gimsing *et al.*, 2006, 2007), in turn this would lead to a longer effect on soil microbial communities (Omirou *et al.*, 2013).

In the current study, the soil microbial communities were affected for up to one week after incorporation at 40% moisture content. Increasing the moisture content of soil to above 40% water holding capacity during incorporation could reduce the effect on the soil microbial communities; it may also adversely affect the efficiency of biofumigation on the soil pest. The higher the water content the quicker GSL hydrolysis and ITC volatisation will occur, minimising the contact time of the ITC with the target pathogen. Due to the issues with reduced pathogen suppression which could occur at a higher moisture content, 40% water holding capacity of the soil may be the best compromise when balancing the suppressive effects on encysted *G. pallida* and the effects on soil microbial communities. Results from this study show that biofumigation under controlled conditions is effective at this moisture content and any shifts in the soil communities are not persistent.

8.6. Potential Study Improvements

Although positive results were collected, there was a high level of variability in several of the *in vitro* hatching assays attributed to the known variability in the egg content of cysts. This led to inconsistent results between repeats and a lack of definitive conclusions in a number of

early experiments. Due to a lack of useable cysts prior to the *in vitro* assays, five cysts per replicate were chosen as this was the maximum possible which allowed all desired treatments to be included. It was then decided that the same number of cysts should be used in later *in vitro* studies for consistency purposes. When moving from *in vitro* to glasshouse trials the number of cysts in a batch was doubled to reduce the variability seen in the *in vitro* studies, especially as soil was being introduced as a factor. Although the number of cysts used was consistent with previous PCN research (Byrne, Maher and Jones, 2001; Valdes *et al.*, 2011; Palomares-Rius *et al.*, 2013; Ngala, Woods and Back, 2015a, 2015b), it may have been advantageous to carry out the *in vitro* experiments with a larger batch of cysts to reduce variability between replicates.

When considering the effects of biofumigation on *G. pallida* with respect to ITC release, it may have been more useful to study the ITCs released instead of the GSL profiles of cultivars. Although important information was gathered from the LC-MS analysis of the GSL profiles, it is known that GSL concentration does not relate directly to ITC release (Warton, Matthiessen and Shackleton, 2001; Morra and Kirkegaard, 2002; Gimsing and Kirkegaard, 2006) with trial conditions and environmental factors affecting the ITC levels that can be released and how long they persist in the soil. Due to this, the relation of the biofumigation effects to the ITC *in vitro* effects was based on conclusions drawn from estimated ITC release instead of exact concentrations. Due to limited access to resources, ITC chemical analysis was unavailable in this study although it would be useful to directly relate released ITC concentrations to the *in vitro* studies.

In addition to analysing the GSL profiles of above-ground material, collecting and analysing root tissue of the cultivars would have helped to provide a more complete picture on GSL accumulation throughout plant development. Although root tissue has been shown to contain different GSLs to above-ground material in high concentrations (Kirkegaard and Sarwar, 1998; Dam, Tytgat and Kirkegaard, 2009), the low biomass of some species roots (eg. *B. juncea* and *S. alba*) means that they probably do not contribute greatly to the overall biofumigation process. Other species, such as *R. sativus*, contain a larger root system so a high GSL content could have more of an impact. With respect to the problems associated with root GSL analysis, root collection is not easy due to their structure and the presence of soil requiring extensive cleaning before LC-MS analysis can be completed. Due to this, it was considered an inefficient use of time to include root material in the GSL profile analysis.

8.7. Future Biofumigation Studies

Several outcomes have led to the development of further research questions on optimising biofumigation for *G. pallida* control.

Effective cultivars have been identified which contain the parent GSL of AITC, sinigrin. Now that an ITC which suppresses encysted *G. pallida* has been identified, a variety of other cultivars should be screened to determine which ones will be effective against PCN in the field. In addition, there is the potential that cultivars can contain high concentrations of more than one GSL, therefore the identification and selection of cultivars which can release ITCs able to suppress multiple pests would be advantageous.

As previously discussed, further research is required to optimise the biofumigation process in the field with respect to *G. pallida* suppression. This involves determining cultivars able to accumulate high sinigrin concentrations and release high AITC concentrations under variable growth conditions. The potential for facultative diapause to occur multiple times in PCN when in unfavourable conditions should be investigated with the aim of increasing the number of J2 in a vulnerable state which are not resistant to ITC exposure. Research into the influence of: soil moisture content, seed rate, plant biomass and incorporation methods on GSL content and ITC release should be completed to develop an optimised biofumigation protocol.

Further studies are required to investigate the effect of biofumigation on soil microbial communities. Although this study gained positive insights into the effect on overall communities, work is still needed to determine if biofumigation affects important bacterial groups involved in nitrogen cycling and if the effect is positive or negative.

The results from this study combined with the outcomes from the indicated future studies would: influence how biofumigation practices evolve, refine protocols for optimal pest suppression, and provide information on the impact of biofumigation on the soil environment with the aim of limiting any negative effects on non-target soil microorganisms.

8.8. The Future of PCN Suppression by Biofumigation

Realistically, biofumigation is more likely to be effective against other soil pests than PCN. PCN is difficult to control due to the presence of the cyst wall, and this can be seen through the trials in this study as well as with previous research (Valdes *et al.*, 2011; Valdes, Viaene and Moens, 2012; Brolsma *et al.*, 2014; Ngala, Woods and Back, 2015b). In comparison, free-living nematodes and soil fungal pathogens are more likely to be susceptible to

biofumigation as they do not have the protective features which make PCN so resilient and notoriously difficult to control.

Results from this study show that biofumigation has the potential to be used to control encysted *G. pallida*, although the influence of external conditions when moving into a field situation reduces the ability of biofumigants to consistently suppress PCN. The variability in GSL content of cultivars between trials would suggest that biofumigation would be unlikely to consistently achieve encysted *G. pallida* suppression over different seasons and years if used as the sole control method.

Biofumigation could contribute to PCN suppression if considered as part of an integrated pest management method. Even if ITC release was low, the incorporation of green manure would have a partial suppressive effect on encysted *G. pallida*. An integrated management approach would involve including biofumigation as one of multiple control strategies with the aim of suppressing PCN using sustainable agricultural practices which do not have the same harmful side-effects of chemical application on soil.

An alternative use of biofumigation research for *G. pallida* control could be through the development and use of Brassicaceae-derived biofumigant products. Biofumigant seed meals have been developed successfully for the control of soil pests (Mazzola *et al.*, 2001; Zasada, Meyer and Morra, 2009). These release high concentrations of ITCs due to concentrated GSLs in the absence of seed oil. This may make it more effective at suppressing *G. pallida* in the field due to greater ITC release g of soil^{-1} compared to traditional growth and incorporation of plant green manure. Similarly, dried biofumigant pellets have been created which can be incorporated into the soil (Lazzeri, Leoni and Manici, 2004). These pellets contain dried plant material with intact GSLs and myrosinase. They remain separate until water is added in the form of irrigation, then GSL hydrolysis occurs releasing ITCs. These methods may be more effective at suppressing soil pests than the biofumigation process as they reduce a number of variables associated with the growth and incorporation of fresh plant material, and it can be easier to gain the ITC release required for pathogen control. The disadvantage associated with these methods is that the higher concentration of toxic ITCs is more likely to negatively impact the environment therefore studies into the hazards associated with their application need to be conducted.

In order for biofumigation to be effective in the field either: on its own, as part of an integrated pest management solution or applied as a biopesticide, effective exchange of knowledge between academic and industrial communities is required. The efficient exchange of information with the growers that will be applying the method practically is essential for

the success of the chosen application method. In addition, expectations of pest suppression through biofumigation needs to be managed to avoid a situation where soil pathogen populations are not controlled at all. As part of this it is essential that the grower is aware of: the target pest, the cultivars which will be effective for the given pest, the optimal conditions required and the success rate of biofumigation, so that additional measures can be taken to suppress the pest if required. In return, the effectiveness of biofumigation should be recorded by the growers and communicated back into academic studies in order to address any issues which may currently be unknown to researchers.

8.9. Concluding remarks

The aim of this study was to determine if biofumigation has the potential to be used to control PCN, namely *G. pallida*. From the data produced by these studies it is clear that ITCs and biofumigation have the potential to control *G. pallida* through hatch suppression and encysted J2 mortality. The efficiency and consistency of this control is dependent upon the influence of external conditions on plant growth and GSL content.

This study successfully identified an ITC required for *G. pallida* toxicity as well as the minimum suppressive concentration. In addition, the importance of time between exposure and hatch stimulation was demonstrated. Results have led to an understanding of the ITCs released from biofumigants; the GSL analysis of Brassicaceae spp. indicated that GSL profiles vary between species and throughout plant growth, with *B. juncea* cultivars containing the highest concentration of the GSL of interest, sinigrin. Cultivars which released AITC were able to suppress *G. pallida* under controlled conditions but environmental factors impacted on the effectiveness of biofumigation in the field trials. In conclusion, although the components for achieving *G. pallida* suppression through ITC release from GSL-containing cultivars appear to be present, the difficulty of implementing biofumigation within a field setting seems to be due to the inability to control the GSL content of plant material grown in variable environmental conditions and an inefficient incorporation method which does not maximise ITC release.

In addition to understanding the effect of biofumigation on soil pests, this study aimed to explore the effect of incorporating ITC-releasing material on non-target soil microbial communities. Transient shifts in microbial community function were found in response to biofumigation under controlled conditions although biofumigation field trials showed little effect on soil microbial community function. This suggests that biofumigation is unlikely to adversely affect the soil environment when implemented in the field.

It is anticipated that this study will contribute to furthering the information available on PCN control and biofumigation. Results from this study have practical implications with respect to the development and selection of biofumigants that can successfully release ITCs and cause PCN suppression. The study also highlights the need to control as many factors that influence plant growth and GSL content as possible if wanting to successfully and consistently implement biofumigation as a *G. pallida* control method. It is hoped that this study will further research into alternative control strategies for pathogen control and promote future work into integrated pest management strategies in response to increasingly restrictive legislative controls on synthetic pesticides.

Appendix A: MicroResp™ Protocol

A1. MicroResp™ set up

Soil samples were added to 96-well DeepWell microplates (Fisher Scientific, UK) using the filling device provided and sealed with Parafilm®. Plates were filled so that each sample occupied three columns and eight rows; seven carbon substrates and a dH_2O control could then be added in three technical replicates per sample. The filled plates were incubated in the dark at 25°C in a sealed container with self-indicating soda lime (Fisher Scientific, UK) and a beaker of dH_2O for six days prior to the addition of the carbon sources and analysis. The weight of soil in each well was recorded.

Seven carbon substrates were selected depending on their ecological relevance to soil, their solubility in water and use in previous studies (Campbell, Grayston and Hirst, 1997; Campbell *et al.*, 2003). Carbon substrates consisted of trehalose, fructose, glucose, lysine, arginine, citric acid and oxalic acid. Each carbon source was dissolved in dH_2O at concentrations able to deliver 30mg substrate g^{-1} soil H_2O as per the MicroResp™ protocol. Arginine was delivered at 7.5mg substrate g^{-1} soil H_2O because of its poor solubility in water. The carbon sources were prepared and stored at room temperature within a day before use.

The colorimetric carbon dioxide (CO_2) detection plates were prepared as per the manufacturer's instructions. The agar and indicator solution were readied separately and then combined prior to use. The indicator was prepared at 65°C; 18.75mg Cresol Red, 16.77g Potassium Chloride (KCl) and 0.315g Sodium Bicarbonate ($NaHCO_3$) were dissolved in 1 L dH_2O to form the stock solution which was stored at 4°C until use. A 3% purified agar (Fisher Scientific, UK) was prepared in dH_2O just before use and cooled to 60°C. A 1:2 ratio (agar: indicator) was mixed thoroughly at 60°C. 150 µL was aliquoted to individual wells of 96-well microplates (Fisher Scientific, UK) using an automated repeating pipette (Multipette® E3; Eppendorf, UK) to give a final concentration of 1% Purified Agar, 12.5µg mL^{-1} Cresol Red, 150mM KCl and 2.5mM $NaHCO_3$ per well. Detection plates were sealed with Parafilm® and stored in a sealed container with self-indicating soda lime and a beaker of H_2O in the dark at room temperature for up to two weeks before use.

A2. MicroResp™ analysis

Immediately before experimental assembly, the CO_2 detection plate was read using a Multiskan EX spectrophotometer microplate reader (Fisher Scientific, UK) and the associated Ascent™ Software v2.6 at absorbance wavelength 405nm or 570nm, depending on the experiment. The carbon sources were added to the appropriate wells of the soil DeepWell

plates in 25 µL volumes using an 8-channel multipipette so that there were three technical replicates of each carbon source for each soil sample; in addition, a 25 µL dH_2O control was added in triplicate for each soil in order to analyse the basal respiration of soil samples. After carbon substrate addition, the provided MicroResp™ seal was added to the filled soil plate followed by the CO_2 detection plate inverted on top. The assembled components were firmly secured with the provided MicroResp™ clamp and incubated at 25°C for 6hrs. After incubation the clamp and plates were disassembled and the CO_2 detection plate was read again on the spectrophotometer at the correct absorbance wavelength.

A3. MicroResp™ CO_2 rate conversion

The absorbance after 6hrs (At6) was normalized for any differences recorded before analysis (At0) for each plate using equation (1):

$$At6n = (At6 / At0) \times Mean (At0) \quad (1)$$

At6n was then converted to headspace CO_2 concentration using an equation calculated from a CO_2% calibration curve obtained as per the MicroResp™ protocol.

The CO_2% calibration curve was obtained as follows: a breakable microplate (Fisher Scientific, UK) filled with the CO_2 detector gel was read on the spectrophotometer at 405nm and 570nm. 40 mL Supelco glass vials with screw top Septa caps were prepared with a strip of four wells from the breakable microplate and vials were sealed. The quantity of air equivalent to the quantity of CO_2 for insertion was removed from vials with a gas chromatography syringe. A Tedlar bag was filled with CO_2 gas standard (10% CO_2 in Nitrogen; Calgaz, UK) and the quantity of CO_2 required was transferred into the correct vials from the bag. Each CO_2 concentration (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3 and 3.5%) was duplicated to total eight wells. The vials were then incubated at 25°C for 6hrs, after which time the vials were opened, microstrips were reassembled and read on the spectrophotometer at the correct absorbance wavelength.

Regression analysis was completed (2.11.3) to determine the best fit curves. The theoretical concentration of CO_2 and the normalised absorbance reading (At6n using equation (1)), were used to obtain the calibration curves for 405nm and 570nm (Figure A1 and A2).

The best fit for both curves was:

$$CO_2\% = A + B*(R\text{^}At6n) + C*At6n \quad (2)$$

CO_2 production rate was calculated by converting the CO_2% data to µg CO_2-C g^{-1} h^{-1} using a number of inputs; gas constants, constants for incubation temperature in °C, headspace volume in the well, incubation time and soil sample dry weight. The full equation is described

in detail in Campbell *et al.* (2003) which, when all fixed factors are inputted, can be simplified to equation (3):

CO_2 rate ($\mu g\ CO_2$- $C.g^{-1}.h^{-1}$) = ((463.73*(CO_2%/100))/0.6fwt)/5 (3)

Where CO_2% is from equation (2) and fwt is the average fresh weight of soil $well^{-1}$ recorded when preparing the soil plates.

The CO_2 rate data were averaged over the three technical replicates for each of the carbon sources so that there was one CO_2 rate value $carbon\ source^{-1}$ $soil\ sample^{-1}$. All multivariate statistical analysis was carried out using the CO_2 rate data from equation (3) for each soil sample.

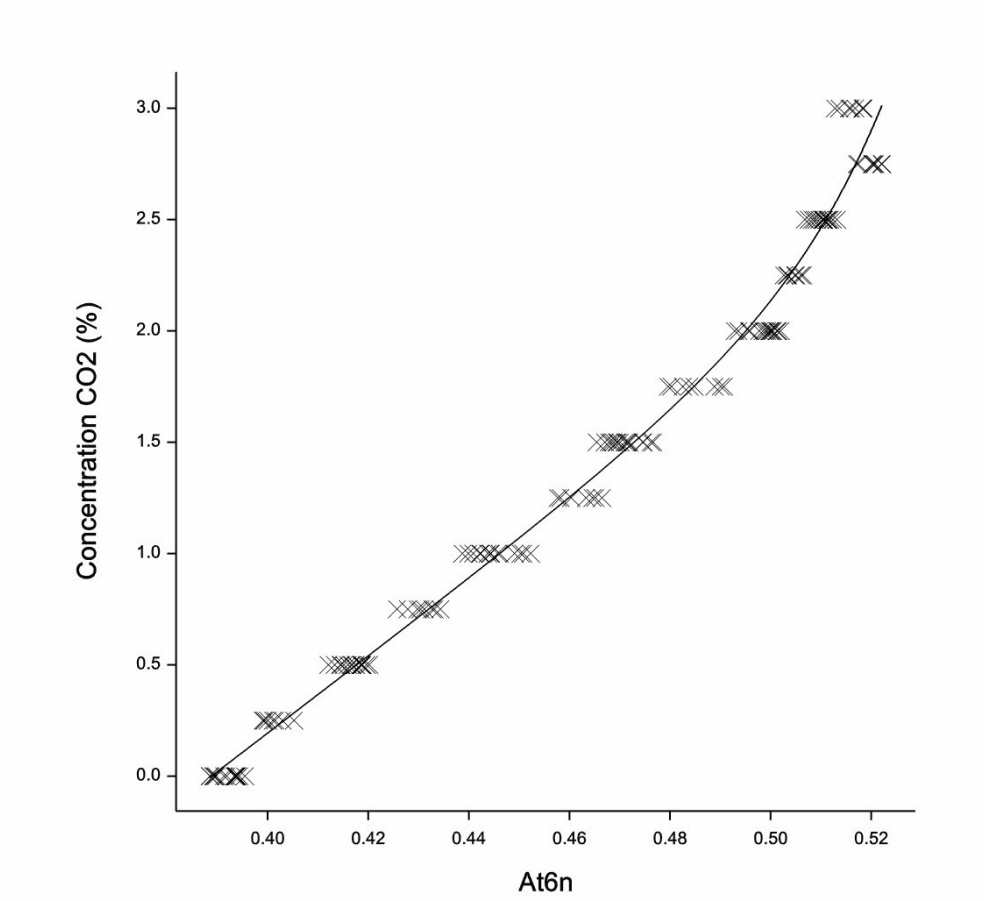


Figure A1. Fitted calibration curve for 0-3 CO_2% concentrations at 405nm with the line: CO_2% = -6.711 + $5.00x10^{-13}(2.55x10^{23}$ ^At6n) + 17.351At6n R^2= 98.60% and standard error = 0.108.

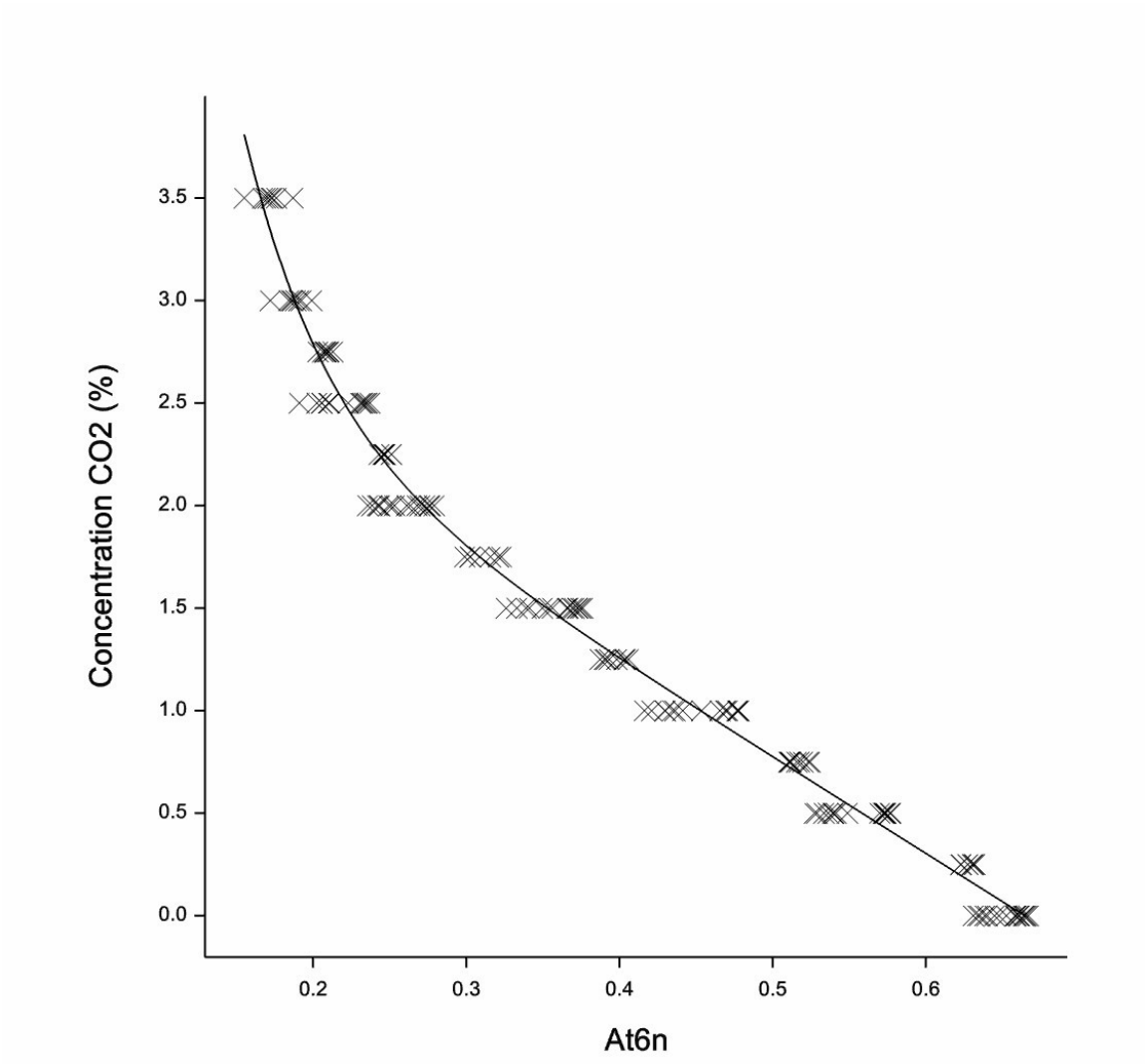


Figure A2. Fitted calibration curve for 0-3.5 CO_2% concentrations at 570nm with the line:
$CO_2\% = 3.1237 + 26.60(5.45x10^{-9}\text{^At6n}) - 4.727At6n$
R^2= 98.40% and standard error = 0.126.

Appendix B: Repeated Isothiocyanate Pot Trial Results

Figure B1. *G. pallida* hatch after exposure to ITCs for four weeks in soil. Water control (C), AITC (A), BITC (B) and PEITC (PE). Upper case = 100ppm, lower case = 5ppm. C1-C4 indicate weekly counts. Error bars represent the standard error.

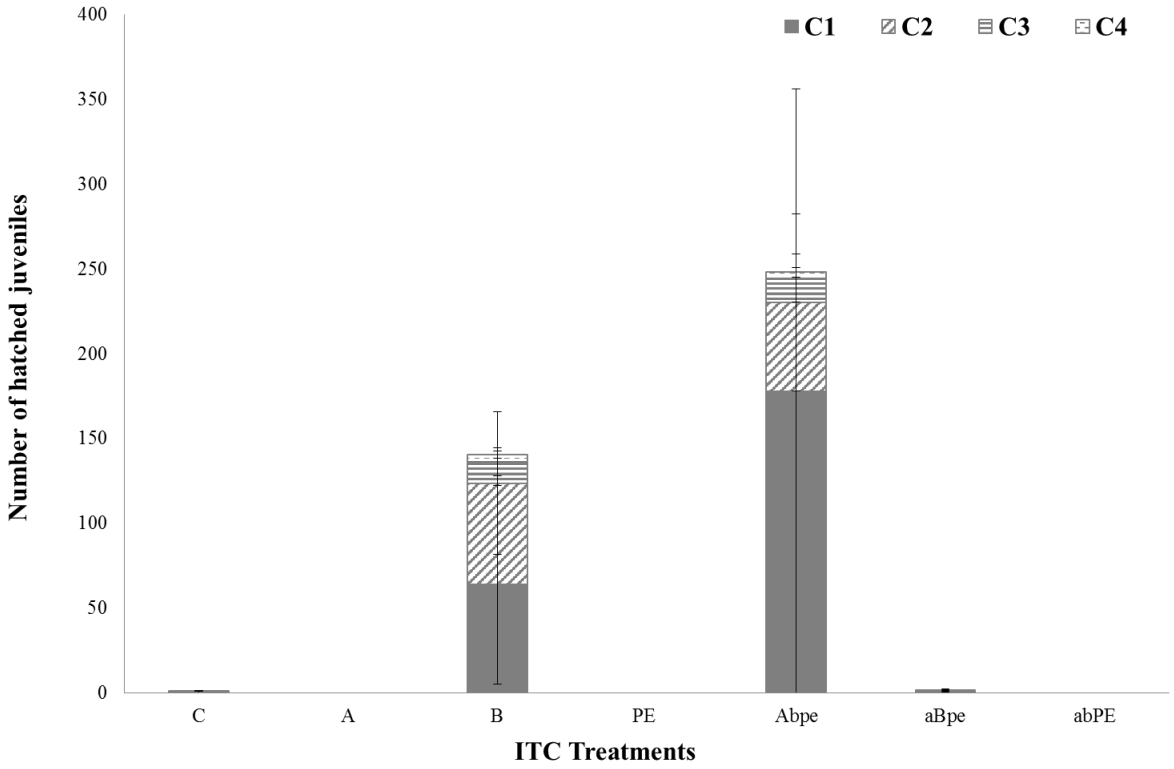


Table B1. The number of newly formed cysts sample^{-1}, eggs sample^{-1} and eggs cyst^{-1} post-multiplication after ITC exposure. Water control (C), AITC (A), BITC (B) and PEITC (PE). Upper case = 100ppm, lower case = 5ppm. Standard errors are indicated within brackets.

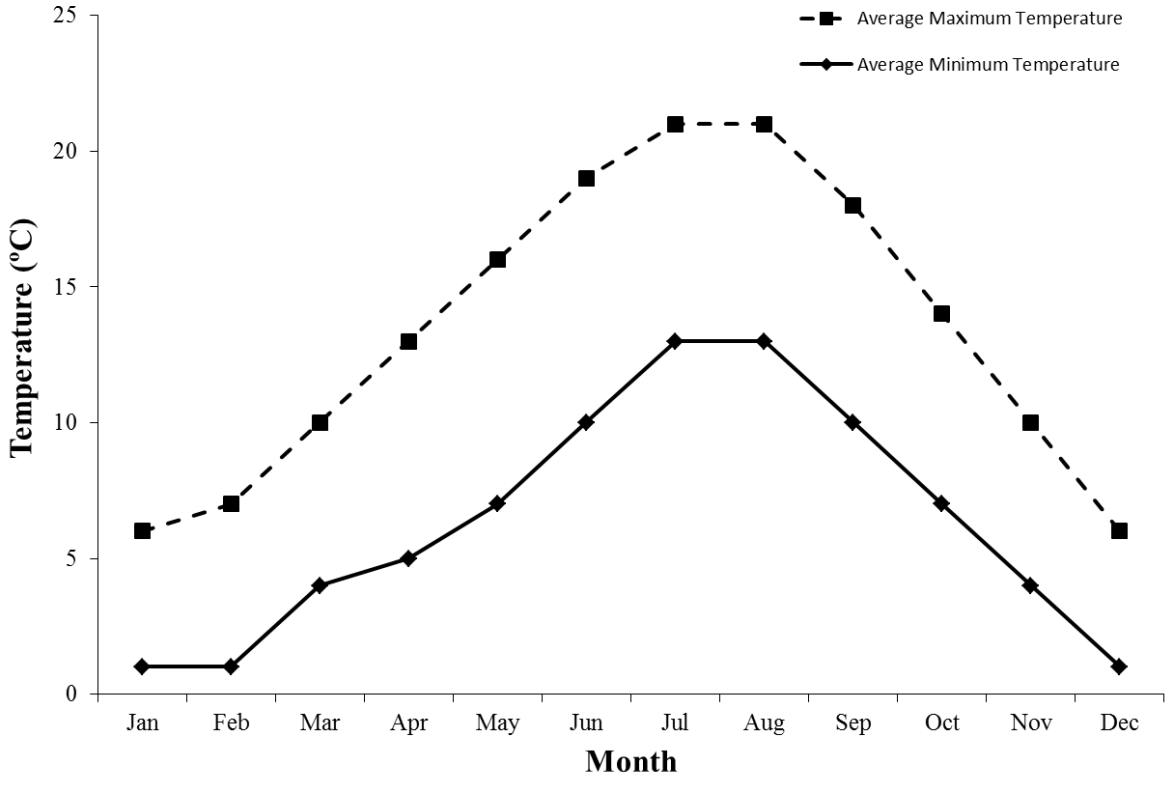
Treatment	Cysts Sample^{-1}	Eggs Sample^{-1}	Eggs Cyst^{-1}
C	67.67 (±23.45)	2762.67 (±1278.61)	33.67 (±7.58)
A	48.00 (±14.85)	1490.00 (±356.11)	30.00 (±9.00)
B	33.17 (±12.25)	1656.67 (±703.28)	42.67 (±12.57)
PE	20.33 (±8.54)	861.67 (±435.91)	38.67 (±7.11)
Abpe	21.67 (±7.03)	659.33 (±170.71)	35.33 (±7.32)
aBpe	60.33 (±14.16)	3036.33 (±1090.58)	44.33 (±9.33)
abPE	58.00 (±19.45)	2945.33 (±1134.77)	44.67 (±8.37)

Appendix C: Average Monthly Temperatures in Lincolnshire Collected from the Waddington Weather Station

Table C1. Temperatures averaged over 1961-1990 (Yr, accessed: June 2017).

Month	Temperature °C		
	Max	Average	Min
August	20.1	15.7	11.4
September	17.7	13.6	9.5
October	13.8	10.3	6.8

Figure C1. Temperatures averaged over 1981-2010 (Met Office, accessed: June 2017).



Appendix D: Identified Glucosinolate Compounds

Table D1. Intact GSLs identified within above-ground material of Brassicaceae species in Chapter 5. m/z = mass to charge ratio * = relative response factor unknown therefore treated as 1.00.

Glucosinolate	Retention Time (mins)	Primary Ion (m/z)	Relative Response Factor
Glucoraphanin	6.0	436	1.07
Glucoraphenin	6.1	434	0.90
Epi/Progoitrin	6.4	388	1.09
Glucoraphasatin	8.6	417	0.40
4-hydroxyglucobrassicin	19.0	463	0.28
Glucoerucin	22.3	420	1.04
Gluconasturtiin	22.8	422	0.95
4-methoxyglucobrassicin	22.9	477	0.25
Glucobrassicin	23.5	447	0.29
Neoglucobrassicin	23.5	477	0.20
Sinigrin	6.5	358	1.00
Glucoalyssin	7.6	450	1.07
Gluconapin	10.9	372	1.11
Glucobrassicanapin	21.9	386	1.15
Glucotropaeolin	22.2	408	0.95
Glucocapparin	8.4	332	1.25
Glucosinalbin	9.9	424	0.50
Diglucothiobeinin	13.9	600	1.00*
Glucosativin	18.2	406	1.00*
Glucolepiidin	21.5	346	1.00*
DMB	22.2	811	1.00*

Table D2. Intact GSLs identified within above-ground material of Brassicaceae species at time of incorporation in Chapter 6. m/z = mass to charge ratio * = relative response factor unknown therefore treated as 1.00.

Glucosinolate	Retention Time (mins)	Primary Ion (m/z)	Relative Response Factor
Glucoraphanin	6.0	436	1.07
Glucoraphenin	6.1	434	0.90
Epi/Progoitrin	6.4	388	1.09
Glucoraphasatin	8.6	417	0.40
4-hydroxyglucobrassicin	19.0	463	0.28
Glucoerucin	22.3	420	1.04
Gluconasturtiin	22.8	422	0.95
4-methoxyglucobrassicin	22.9	477	0.25
Glucobrassicin	23.5	447	0.29
Neoglucobrassicin	23.5	477	0.20
Sinigrin	6.5	358	1.00
Glucoalyssin	7.6	450	1.07
Gluconapin	10.9	372	1.11
Glucobrassicanapin	21.9	386	1.15
Glucotropaeolin	22.2	408	0.95
Glucocapparin	8.4	332	1.25
Glucosinalbin	9.9	424	0.50
Glucoiberin	4.4	422	1.07
Methylpentyl-GSL	23.6	402	1.00*
Hexyl-GSL	23.8	402	1.00*
Diglucothiobeinin	13.9	600	1.00*
Glucosativin	18.2	406	1.00*
Gluconapoleiferin	22.0	402	1.00*
DMB	22.2	811	1.00*

Appendix E: Field Trial Locations

Figure E1. First field trial location.

Figure E2. Second field trial location.

Appendix F: Field Trial Treatments and Plot Designs

Figure F1. Soil analysis of the first field trial completed by NRM.

SOIL ANALYSIS REPORT

Laboratory Sample Reference	Field Details			Index			mg/l (Available)		
	No.	Name or O.S. Reference with Cropping Details	Soil pH	P	K	Mg	P	K	Mg
55816/14	1	**RES-MUST** *No cropping details given*	7.4	2	2-	2	24.8	167	69

If general fertiliser and lime recommendations have been requested, these are given on the following sheets.
The analytical methods used are as described in DEFRA Reference Book 427
The index values are determined from the DEFRA Fertiliser Recommendations RB209 8th Edition (Appendix 4).

Released by *Dr R C Wilkinson* On behalf of NRM Ltd Date *11/08/14*

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nrm laboratories

MICRO NUTRIENT REPORT

DATE **11th August 2014**

SAMPLES FROM **RES-MUST**

HELEN BARKER [NF]
BARWORTH AGRICULTURE
63 CAMERON STREET
HECKINGTON
SLEAFORD
NG34 9RP

Reference: **13609/55816/14** Field Name: **RES-MUST**	Result	(*)	Deficient	Marginal	Target	Marginal	Excessive
EDTA Extractable Copper mg/l	4.4						
Hot Water Soluble Boron mg/l	1.7						
Ammonium Nitrate Extractable Sodium mg/l	10.9	1					
EDTA Extractable Zinc mg/l	1.3	2					
DPTA Extractable Iron mg/l	29.4	3					
Organic matter (LOI) %	3.4		*OM level data not available for this crop*				
Phosphate Buffer Extractable Sulphate mg/l	30.0	4					
DPTA Extractable Manganese mg/l	12.4	5					

Figure F2. Treatments and plot design in the first field trial.

1. Scala (9kg ha^{-1})
2. Ida Gold (7kg ha^{-1})
3. ISCI 99 (9kg ha^{-1})
4. Nemat (6kg ha^{-1})
5. Bento (20kg ha^{-1})
6. Fallow

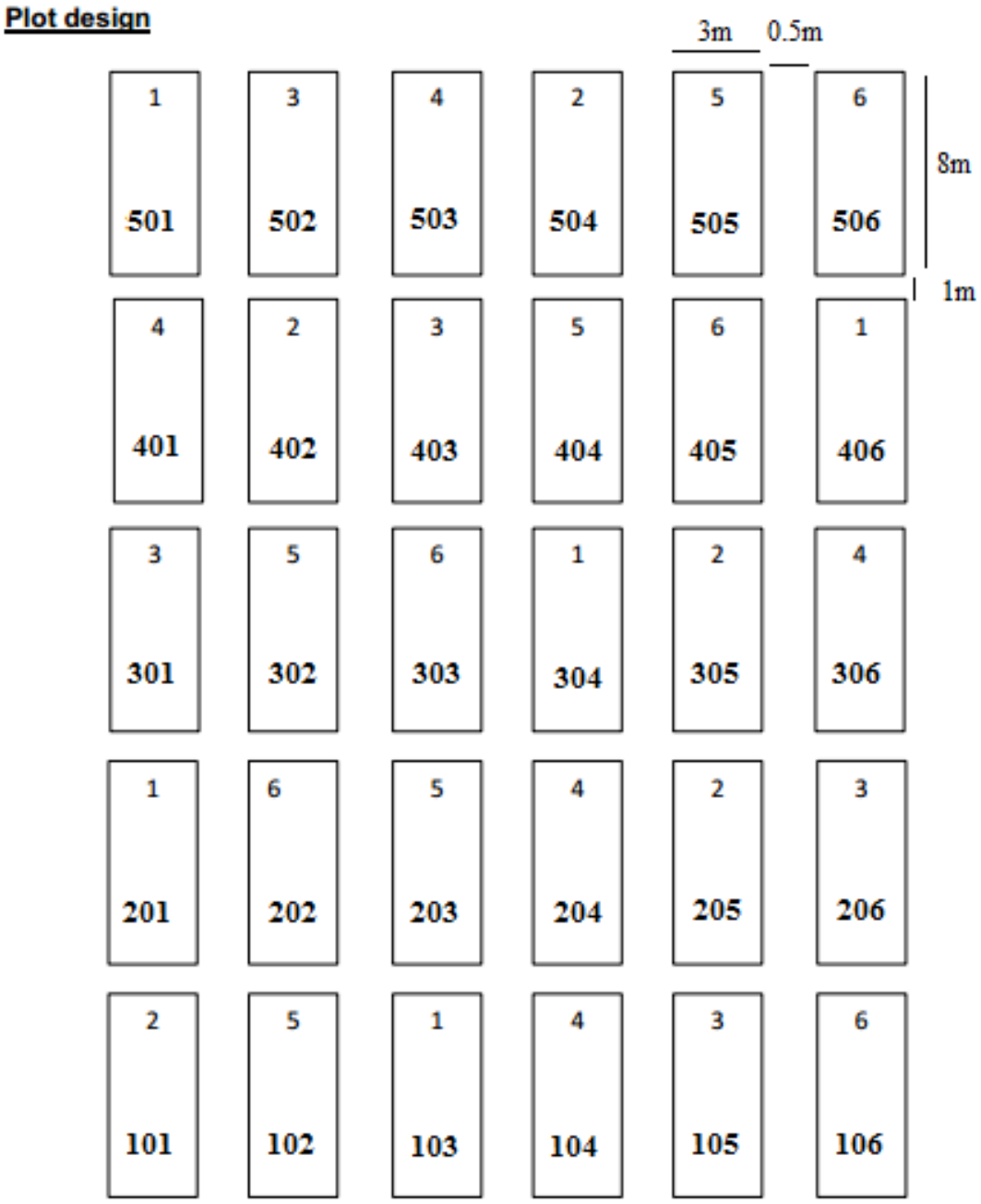


Figure F3. Nematicide split-plot design in the first field trial. Grey subplots = Nemathorin® 10G, white subplots = no nematicide.

Plot design

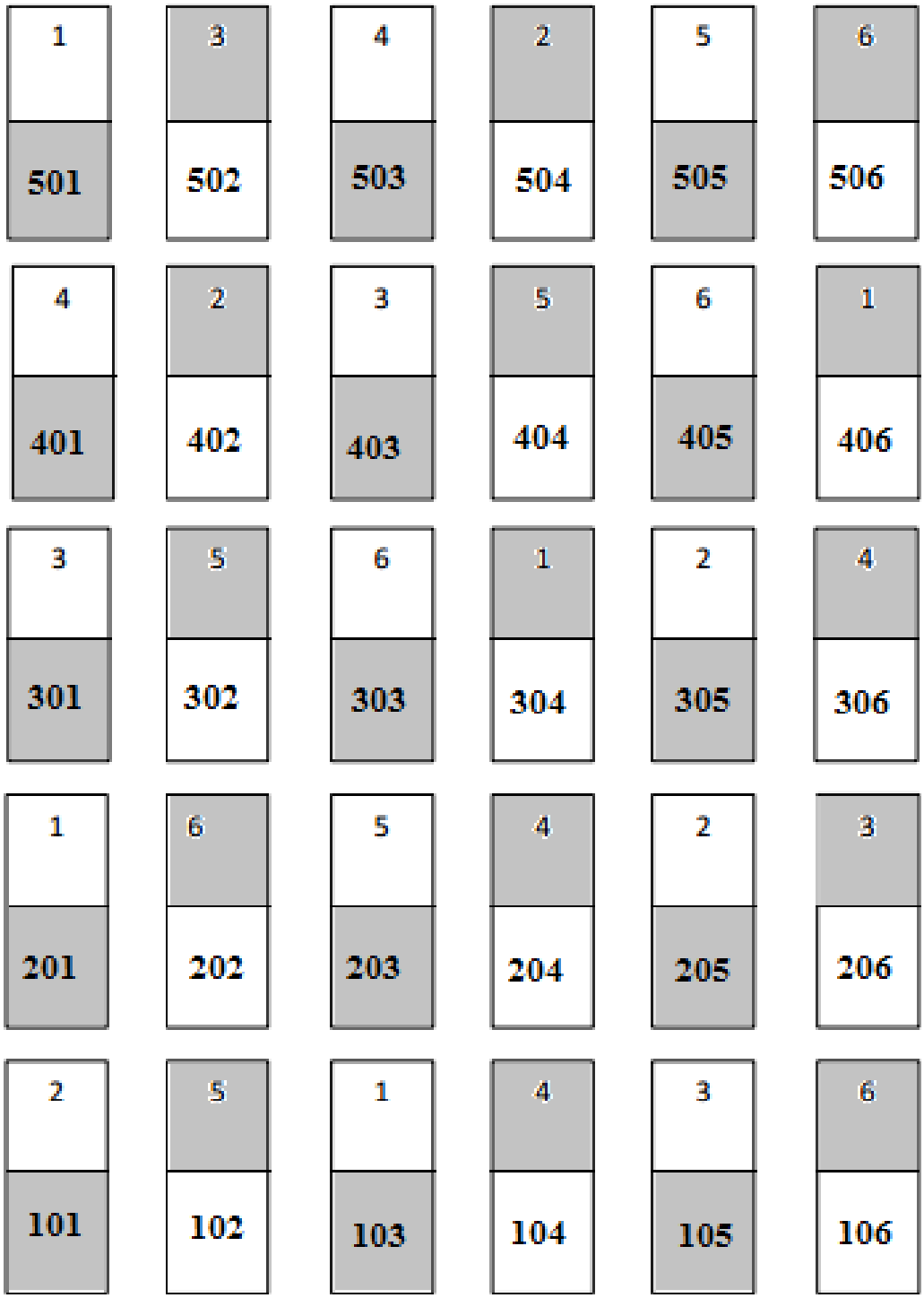
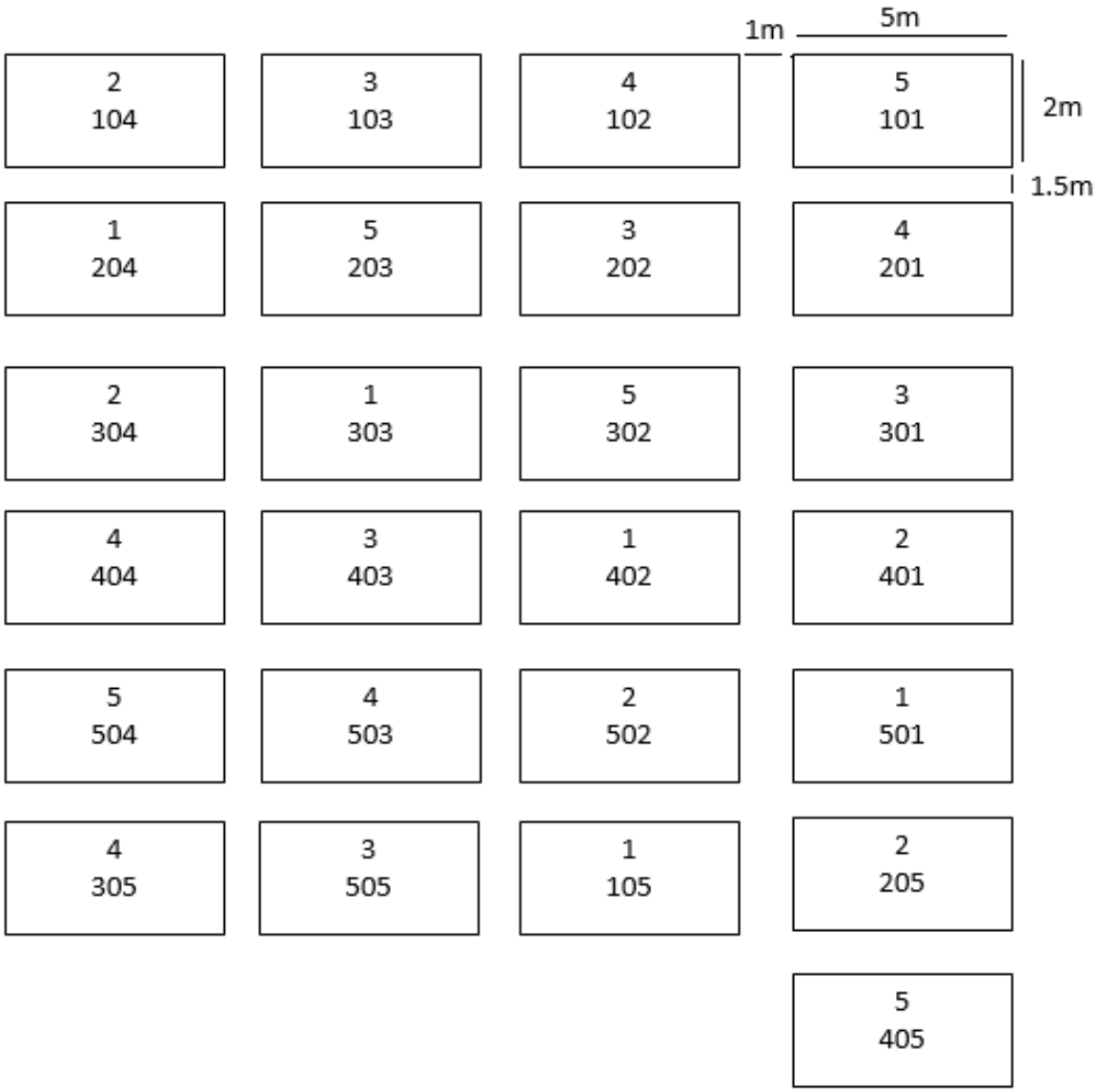


Figure F4. Treatments and plot design in the second field trial.

1. ISCI 99 (8kg ha^{-1})
2. Ida Gold (8kg ha^{-1})
3. Bento (15kg ha^{-1})
4. Bristle Oats (80kg ha^{-1})
5. Fallow

Plot design



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