

**Biofumigation for control of *Globodera pallida* and  
*Rhizoctonia solani***

James Starkey Lord

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## Abstract

Potato cyst nematodes, *Globodera pallida* and *G. rostochiensis* are a major problem for British potato growers. They are currently estimated to cost over £35 million  $y^{-1}$  in lost yield and nematicides. Due to environmental concerns, some of the most widely used nematicides in the 1990s have now been withdrawn from use within the European Union and more environmentally benign control measures are sought. The soil-borne fungal pathogen *Rhizoctonia solani* is another constraint on potato production for which current control measures are not entirely satisfactory. Biofumigation, the incorporation of brassica green manures into soil to control soil-borne pests and pathogens, has the potential to control various organisms in a number of cropping systems. The mechanism most commonly implicated in pest or pathogen suppression by brassica green mulches is the production of volatile secondary metabolites called isothiocyanates, which are produced upon enzymatic hydrolysis of parent glucosinolates. In some cases, suppression through biological mechanisms or by volatile toxins not derived from glucosinolates has also been observed. The principal aims of this project were to evaluate the potential of biofumigation to control *G. pallida* and *R. solani* and to determine the mechanisms involved in any suppression. An *in vitro* toxicity assay was developed and used to screen five isothiocyanates and aqueous leaf extracts of 22 brassica lines for toxicity to *G. pallida* second-stage juveniles (J2). *G. pallida* J2 were highly sensitive to isothiocyanates and to extracts of several of the brassica lines tested. The toxicity of benzyl, 2-phenylethyl, 2-propenyl and 3-(methylthio)propyl isothiocyanate was similar, with  $ED_{80}$  values ranging from 11 to 18  $\mu M$ , while 2-methylbutyl isothiocyanate was less toxic. The plants causing greatest suppression *in vitro* were *Raphanus sativus*, *Nasturtium officinale* and *Brassica juncea*. The fungistatic activity of the isothiocyanates toward *R. solani* was determined *in vitro*. The rank order of toxicity of the isothiocyanates to the two organisms was similar but the fungus was more tolerant than the nematode. A method was developed for quantifying viable potato cyst nematode eggs by measuring the abundance of actin mRNA and selected plants were tested for



activity toward *G. pallida* eggs and *R. solani* mycelium in soil in glasshouse trials. Glucosinolate profiles of the brassicas incorporated into soil were determined by HPLC. Brassica green manures had no consistent effect on the density of *R. solani* inoculum or on resulting disease incidence on bioassay potato plants. In contrast, several brassica green manures caused substantial mortality to *G. pallida* eggs, of which three *B. juncea* lines (Nemfix, Fumus and ISCI99) containing high concentrations of 2-propenyl glucosinolate were the most effective. These plants caused over 95 % mortality in polyethylene-covered soil and over 80 % mortality in uncovered soil. Toxicity in soil correlated with the concentration of isothiocyanate-producing glucosinolate but not total glucosinolate. The biosafety of the approach was examined by characterising the effects of biofumigation and conventional fumigation on the free-living soil nematode community. Both treatments had strong and lasting effects on free-living nematodes, reducing the abundance of sensitive taxa by almost 100 %. This study has shown that biofumigation with particular brassica varieties can potentially achieve levels of control that would make it commercially viable as part of integrated management of potato cyst nematodes.

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# Chapter 1: General Introduction

## 1.1 The potato, *Solanum tuberosum*

### 1.1.1 Economic importance of potato

The potato, *Solanum tuberosum*, is the most important dicotyledonous crop in the world. Only the monocots sugar cane, maize, wheat, rice and barley are produced in greater quantities (FAO, 2010). It is a highly productive crop with mean global yields of 17 tonnes ha<sup>-1</sup> y<sup>-1</sup> (FAO, 2010) and mean yields in the UK of 45 tonnes ha<sup>-1</sup> y<sup>-1</sup> (Potato Council, 2010a). The mean value of the potato crop over the period 2004 – 2008 was \$ 35 billion y<sup>-1</sup> globally (FOA, 2010) and £ 750 million y<sup>-1</sup> in the UK (Potato Council, 2010a). Over 30,000 people are employed in the potato industry in the UK (Kerry *et al.*, 2003).

### 1.1.2 Agronomy of potato

There are two subspecies of cultivated potato, *Solanum tuberosum* spp. *tuberosum*, grown in Europe and North America and *S. tuberosum* spp. *andigena*, grown in South America. Potatoes can be grown from true seed but most are propagated vegetatively from “seed” tubers. Since tubers are potentially one of the main routes for the spread of disease, in the UK seed potatoes are produced by specialist growers and must be certified free from certain pests and pathogens (UK government, 2006). Potatoes grow best in cool temperate regions or in tropical regions at altitudes over 2,000 m above sea level (Hooker, 1981). They require cool nights and well-drained soil with adequate moisture and prefer pH between 5 and 6 (Hooker, 1981). Potatoes are usually grown in rotation with other crops, partly due to high nutrient demand but primarily to allow natural mortality to reduce the density of soil-borne pests and pathogens.

### 1.1.3 Principal pests and diseases of potato in the UK

In the UK, pests and pathogens are estimated to reduce potato yields by 15 % (Bradshaw *et al.*, 2001). The most serious problem is late blight, caused by the oomycete *Phytophthora infestans*. This pathogen costs the UK potato industry an estimated £ 55 million y<sup>-1</sup> in lost yield (Twining *et al.*, 2009) and necessitates frequent prophylactic fungicide spraying (Bradshaw *et al.*, 2001; Twining *et al.*, 2009), costing an additional £ 20 million y<sup>-1</sup> (Potato Council, 2010b). After late blight, the next most serious problem is potato cyst nematodes (PCN), estimated to cost £ 26 million y<sup>-1</sup> in lost yield (Twining *et*



*al.*, 2009) and an additional £ 10 million  $y^{-1}$  in nematicides (Kerry *et al.*, 2003). Slugs are the third most important problem, costing an estimated £ 15 million  $y^{-1}$  in lost marketable yield, largely due to their effect on crop quality (Twining *et al.*, 2009).

Other serious diseases and their causal agents include the fungal diseases black dot (*Colletotrichum coccodes*), black scurf and stem canker (*Rhizoctonia solani*), dry rot (*Fusarium* spp.), powdery scab (*Spongospora subterranea*) and silver scurf (*Helminthosporium solani*), the bacterial diseases black leg and tuber soft rot (*Bacterium carotovora*) and common scab (*Streptomyces scabies*), and Potato virus Y (Lane *et al.*, 2000; Bradshaw *et al.*, 2001; Twining *et al.*, 2009).

## 1.2 Potato cyst nematodes (PCN), *Globodera rostochiensis* and *Globodera pallida*

### 1.2.1 PCN distribution and dispersal

Potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, occur in almost all countries in which potatoes are cultivated (Mai *et al.*, 1977; Turner & Evans, 1998). It seems likely that they were introduced to Europe after the potato famine (1845-1849), when new potato breeding material was imported from South America as a source of resistance to late blight (Evans *et al.*, 1975). Most European PCN populations are genetically similar and consist of a narrow subset of the total gene pool from South American source populations (Phillips *et al.*, 1992; Zaheer *et al.*, 1992; Trudgill *et al.*, 1996; Plantard *et al.*, 2008). South American populations have wider host ranges than European populations (Roberts & Stone, 1981; Canto Saenz & Mayer de Scurrah, 1977) and probably possess virulence alleles not present in Europe. For this reason, it is important to prevent further introductions.

PCN now occur in at least 64 % of potato fields in England and Wales (Minnis *et al.*, 2002). *G. pallida* is the dominant species, with 67 % of sampled populations being solely *G. pallida* and 25 % comprising of both species (Minnis *et al.*, 2002). There is evidence that *G. pallida* is outcompeting *G. rostochiensis* in the UK (Whitehead, 1992). Several factors probably contribute to the selection of *G. pallida*, including: cultivation of *G. rostochiensis* resistant cultivars containing the *H1* resistance gene (Cole & Howard, 1962; Whitehead & Turner, 1998); better adaptation of *G. pallida* to cool conditions (van Riel & Mulder, 1998); and later hatching of *G. pallida*, which probably reduces exposure to nematicides (Whitehead *et al.*, 1984; Byrne, 1998). The infective juvenile can only move a maximum of approximately 1 m through soil in search of a host and so PCN dispersal is primarily by passive movement (Turner & Evans, 1998). The main routes of

dispersal are movement in soil adhered to seed tubers, other vegetables, farm workers, machinery or livestock, dispersal in run-off water and irrigation water and by wind (Turner & Evans, 1998).

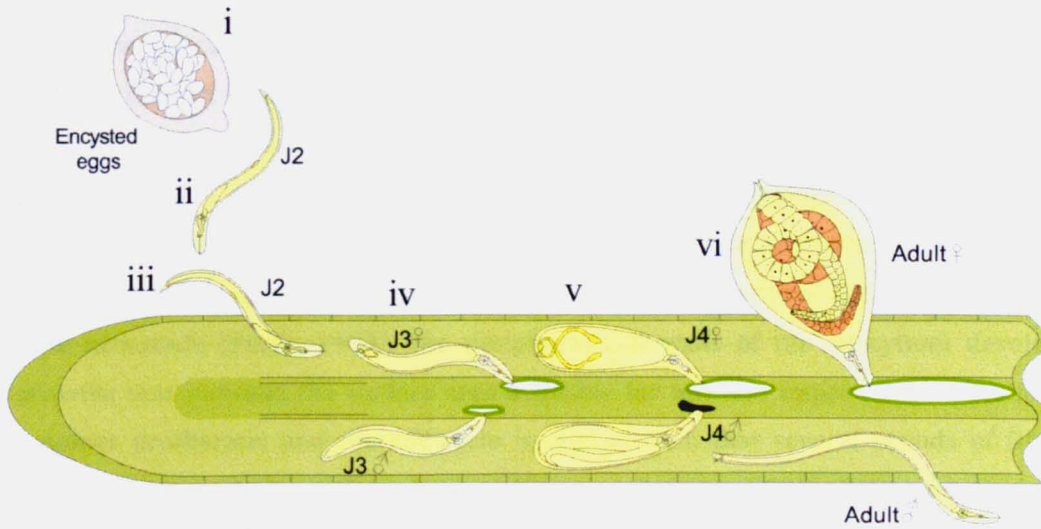
### 1.2.2 Host specificity

PCN are specialists that can only feed on a few species within the Solanaceae family (Evans & Rowe, 1998). There are three major crop hosts, *Solanum tuberosum*, *S. melongena* (aubergine) and *Lycopersicon esculentum* (tomato), and a few weed hosts, including *S. nigrum* (black nightshade), *S. dulcamara* (bitter nightshade), *S. capsicastrum* (false Jerusalem cherry) and *Atropa belladonna* (deadly nightshade) (Evans & Rowe, 1998).

### 1.2.3 Life cycle

The life cycle of the potato cyst nematode (Figure 1.1), like that of all nematodes, consists of four juvenile stages and an adult, with each stage separated by moulting of the body cuticle (Turner & Evans, 1998). A cyst is the tanned spherical cuticle of a dead female, containing 60-700 eggs (Byrne, 1998). First-stage juveniles develop and moult to become second-stage juveniles (J2) within the egg. When the female dies, the unhatched J2 become partially dehydrated, surrounded by a high concentration of the sugar trehalose in the perivitelline fluid (Perry, 1998), and can survive for up to 20 years in this state.

In the absence of external stimuli, only a small proportion of J2 will spontaneously hatch each year. This proportion varies from 13-40 % between populations (Whitehead, 1995; Ryan & Devine, 2005). Hatching factors produced by growing host roots induce hatching at greatly increased rates, although, even in the presence of hatching factors, a certain proportion of encysted J2 remain unhatched and "carry over" to subsequent years (Byrne, 1998). Hatching factors initiate an increase in permeability of the inner lipid layer of the eggshells, allowing hydration of the J2 within (Clarke *et al.*, 1978). As J2 hydrate, they become active and perforate their eggshells with their stylets, cutting a slit through which they then emerge (Byrne, 1998).



**Figure 1:** Life-cycle of the potato cyst nematode: i) eggs within a cyst, the tanned cuticle of a dead female, can survive in soil for many years; ii) eggs hatch in response to potato root exudates and second-stage juveniles (J2) migrate towards growing potato roots; iii) J2 penetrate the root epidermis with their stylets, migrate intracellularly to the vasculature and establish a feeding site (syncytium); iv) J2 moult to become sedentary third-stage juveniles (J3), by which time sex has been determined; v) females continue to feed from the syncytium through the J3, J4 and adult stages whilst males cease to feed at the J3 stage and undergo two moults inside the J2 cuticle; vi) the body of the adult female erupts from the root epidermis whilst continuing to feed from the syncytium and adult males emerge as motile filiform worms that migrate through soil to fertilise females. From Lilley *et al.* (2005).

Hatched J2 leave the cyst and move through the soil within the water phase (Decraemer & Hunt, 2006). J2 are attracted to host roots along chemical gradients, which probably include non-specific signals such as CO<sub>2</sub>, amino acids, ions and sugars (Perry, 1998), as well as specific hatching factors (Byrne, 1998). The preferred sites of entry of J2 are root tips and points of emergence of lateral roots (Turner & Evans, 1998). A J2 penetrates an epidermal cell wall with its stylet and moves intracellularly through the cortex until it reaches the vascular cylinder. It selects a cell adjacent to the vascular bundle and punctures the cell wall with its stylet (Perry, 1998). Secretions from the stylet form a feeding tube, which is inserted in the cytoplasm (Rumpfenhorst, 1984) and the nematode injects substances that bring about major structural and physiological changes within that and adjacent cells (Perry, 1998). The cell walls degrade and several cells fuse to form the syncytium, a structure with several features enabling the diversion of nutrients to the nematode (Perry, 1998): the peripheral cell walls of the syncytium develop ingrowths that increase the surface area available for nutrient import; organelles and ribosomes proliferate; and the cell-cycle is altered such that several rounds of DNA synthesis occur without nuclear division (Gheysen & Jones, 2006). Genes involved in protein and carbohydrate metabolism are induced (Gheysen & Jones, 2006).

During the transition from the motile invasive stage to the sedentary feeding stage, fibrillar material is deposited on the J2 epicuticle, which may function to anchor the nematode to its feeding site (Perry, 1998). The J2 continues to feed and becomes thicker before it moults to the J3. By this stage, the sex of the animal has been determined. Females are estimated to require 100 times more nutrition than males and sex determination depends on the nutrient status of the syncytium; the greater the resources available, the more likely the individual is to become a female (Trudgill, 1967; Turner & Evans, 1998). Males cease feeding after the second moult and undergo two more moults to become J4 and then adults. Male development takes place within the cuticles of the J2-J4 stages, which are not shed until the adult emerges. The adult male is a motile filiform worm about 1 mm long (Turner & Rowe, 2006). It lives for about 10 days, in which time it swims through the soil, attracted by sex pheromones towards females with which to mate (Turner & Rowe, 2006). *Globodera* require fertilisation for reproduction and several males can mate successfully with one female (Turner & Evans, 1998). Females remain sedentary and continue to feed from the syncytium. As the female develops, the body becomes swollen and ultimately spherical in the adult (Turner & Rowe, 2006). At the J3 stage, the female's growing body ruptures the root surface and emerges, while the head remains anchored to the feeding site by adhesive substances secreted immediately posterior to the head. After the female dies, phenolic

compounds in the cuticle undergo polymerisation reactions catalysed by polyphenol oxidases, giving the cuticle, or cyst coat, a dark tanned appearance (Ferraz & Brown, 2002). The time to complete the lifecycle is approximately 90 days, depending on environmental conditions (Byrne, 1998). The UK climate only permits one generation per year and all eggs of field populations pass through at least one winter in the state of dormancy known as diapause (Byrne, 1998).

#### *1.2.4 Dormancy*

Unhatched J2 within eggs can endure harsh conditions for long periods in one of two states of dormancy: diapause or quiescence. Diapause is a response to seasonal fluctuations in temperature and host availability whereas quiescence is a response to aseasonal adverse conditions. The essential difference between these two states is that once eggs have entered diapause, they will stay dormant for several months even if conditions are favourable for hatch while quiescence is a temporary condition that only lasts while conditions are unfavourable (Wright & Perry, 2006). Diapause appears to be triggered by signals from the host plant in response to decreasing photoperiod, signals from the cyst wall as it tans and low temperature (Hominick, 1986). Quiescence can be caused by extremes of temperature, high salt concentration, high osmotic pressure and anoxia (Perry, 1989). Comparisons with other cyst nematodes suggest that dehydration of unhatched J2 and the requirement for specific host hatching stimulants are adaptive mechanisms with a fitness advantage for specialists such as PCN (Clarke *et al.*, 1978).

#### *1.2.5 Pathology*

There are no diagnostic effects of PCN on their hosts (Hooker, 1981). Symptoms are non-specific, resulting from nutrient deficiency and water stress. This is because diversion of nutrients from the roots to the parasite results in retardation of the root system and hence poor uptake of water and nutrient ions (Turner & Evans, 1998). Lack of nutrients limits haulm growth and thus photosynthetic capacity (Turner & Evans, 1998). As the parasites develop and transpiration increases due to greater area of foliage and longer, hotter days, water stress becomes more severe and results in reduced cell expansion (Turner & Evans 1998) and early senescence of leaves (Turner & Evans 1998). In addition, rupturing of the root epidermis by emergence of females provides sites of easy penetration for pathogenic fungi (Turner & Evans, 1998). Yield losses due to potato cyst nematodes vary according to host genotype and environmental

conditions but the following estimates are typical: 6 to 8 t ha<sup>-1</sup> per 20 eggs g<sup>-1</sup> soil for the 0-40 eggs g<sup>-1</sup> soil range and 1.67 t ha<sup>-1</sup> per 20 eggs g<sup>-1</sup> soil for the 40-160 eggs g<sup>-1</sup> soil range (Brown & Sykes, 1983; Whitehead *et al.*, 1984).

### 1.3 Current management of potato cyst nematodes

Potato cyst nematodes are difficult to control because unhatched juveniles are well protected whilst dormant within the cyst, they can survive long periods in the absence of a host and because they are distributed through the soil. The multiplication rate of *G. pallida* at population densities below the damage threshold of 1–3 eggs g<sup>-1</sup> soil can be over 50-fold (Haydock *et al.*, 2006). Hence, in order to maintain population densities below the economic threshold, any combination of control measures must reduce the population density by at least 50 times between each crop of susceptible potato. Few single measures will achieve this and current control practices involve a combination of phytosanitary measures, resistant cultivars where possible, long rotations and chemical control with nematicides and fumigants (Kerry *et al.*, 2003; Turner & Rowe, 2006). In July 2010, a new EU Directive on PCN management, 2007/33/EC, came into force, requiring that a random sample of 0.5 % of ware potato land be sampled for PCN each year. Where PCN are detected, the legislation requires that growers implement formal control strategies agreed with local plant health inspectors aimed at “suppressing” PCN.

#### 1.3.1 Phytosanitary measures

Potato cyst nematodes are dispersed passively, one of the main routes being via soil adhered to tubers, farm workers or machinery (Turner & Evans, 1998). Many potato-growing areas of the UK are still free from potato cyst nematodes and so good hygiene is necessary to minimise further contamination. All seed potatoes in the UK must be from land certified free from potato cyst nematodes (UK government, 2006). Phytosanitary checks are made on plants, seeds and tubers intended for planting of all *Solanum* species entering the UK and imports from countries in which PCN occur are prohibited altogether (DEFRA, 2006).

#### 1.3.2 Rotation

In the absence of a suitable solanaceous host, populations of PCN gradually decline. Solanaceous crops besides potato are not grown in the UK and the few solanaceous

weeds that occur, such as deadly nightshade, are rare in arable fields and easily controlled (Kerry *et al.*, 2003). Simple crop rotation is therefore one of the most effective means of control. The major drawback is that PCN have low rates of natural decline, typically between 15 and 30 %  $y^{-1}$  (Whitehead, 1995). Given these rates and without other control measures, the length of rotation required to reduce population densities of PCN by 50 times would be from 11 to 24 years. Due to the high profit margins achieved with potato, farm profitability is inversely proportional to rotation length (Jatoe *et al.*, 2008) and so most producers grow potatoes more frequently than once in six years (Minnis *et al.*, 2002). Besides the economic drawbacks, rotation is not necessarily the most sustainable solution either since it increases the amount of land required to produce a given yield and may select for persistent PCN.

### 1.3.3 Natural Resistance

Genes conferring resistance to PCN have been found in South American potato cultivars and wild relatives and some have been introgressed into commercial cultivars. Most notably, *H1* from *S. tuberosum* ssp. *andigena* (Ellenby, 1954; Gebhardt *et al.*, 1993; Pineda *et al.*, 1993) was released in the 1960s in cultivars such as Maris Piper. This gene confers a high level of durable resistance to all UK populations of *G. rostochiensis* but does not affect *G. pallida* (Dale & de Scurrah, 1998). As mentioned above, the use of these cultivars has contributed to the increased prevalence of *G. pallida* in the UK (Cole & Howard, 1962; Whitehead & Turner, 1998). There are currently no commercial cultivars with full resistance to *G. pallida* (Whitehead & Turner, 1998; Philips & Blok, 2008). Cultivars with partial resistance are available (i.e. Nadine, Santé and Rocket) but are not widely adopted due to less than outstanding quality characteristics and consequent lack of market demand (Lane *et al.*, 2000). Only two cultivars with partial resistance to *G. pallida* (Nadine and Santé) were grown on more than 2,000 ha in 1999 and only 7 % of UK potato land was planted to these cultivars (Kerry *et al.*, 2003).

To date, sources of resistance to *G. pallida* have either been single dominant genes that confer resistance to only a limited subset of *G. pallida* populations or quantitative trait loci (Finlay *et al.*, 1998). Resistance conferred by multiple genes is difficult to breed into commercial cultivars without "diluting" the desirable genes from each parent since *S. tuberosum* is a tetraploid and there are many traits besides PCN resistance to consider simultaneously (Finlay *et al.*, 1998; Gebhardt & Valkonnen, 2001). Furthermore, successive cropping of some partially resistant cultivars results in selection of more

virulent *G. pallida* populations (Philips & Blok, 2008). Only a relatively small proportion of wild *Solanum* species have been exploited as sources of resistance (Gebhardt and Valkonen, 2001) and new sources of *G. pallida* resistance continue to be identified (Castelli *et al.*, 2003). Development of elite cultivars with full durable resistance to *G. pallida* is a long-term goal but is unlikely to be achieved in the near future (Kerry *et al.*, 2003).

#### 1.3.4 Chemical control of PCN

Nematicides are currently viewed as essential in maintaining the productivity of UK potato land (Kerry *et al.*, 2003). There are three main groups of nematicides: carbamates, organophosphates and fumigants (Haydock *et al.*, 2006). In 2003, four granular nematicides and one fumigant were available for control of PCN in the UK: two carbamate nematicides, aldicarb (Temik) and oxamyl (Vydate), used on 21,000 and 8,000 ha, respectively; two organophosphate nematicides, fosthiazate (Nemathorin) and ethoprophos (Mocap), used on 7,000 and 2,000 ha, respectively; and the soil fumigant 1,3-dichloropropene (Telone II), used on 800 ha (Garthwaite *et al.*, 2003; Kerry *et al.*, 2003). Due to the much greater rates applied, 1,3-dichloropropene (1,3-D) accounted for 60 % of the mass of all nematicides applied at that time (Garthwaite *et al.*, 2003).

Carbamate and organophosphate nematicides are acetylcholinesterase inhibitors, which disrupt nematode chemoreception and locomotion, preventing J2 from invading host roots (Perry, 1996; Atkinson *et al.*, 2003). They are applied as granules at the time of planting, are effective at extremely low concentrations and persist in soil long enough to affect hatched juveniles (Whitehead & Turner, 1998). Aldicarb and oxamyl can almost completely prevent multiplication of *G. pallida* on a susceptible host at doses of 9 kg active ingredient per hectare (Whitehead *et al.*, 1994). Organophosphates are less effective in highly organic soils (Whitehead *et al.*, 1994), due to their hydrophobicity and consequent sorption by organic matter while carbamates are hydrophilic and function in a wide range of soil types (Whitehead & Turner, 1998). Granular nematicides are generally less effective against *G. pallida* than *G. rostochiensis*. For instance, equivalent doses of oxamyl reduced multiplication of *G. rostochiensis* and *G. pallida* by 80 and 42 %, respectively (Whitehead *et al.*, 1984). Likewise, higher doses of ethoprophos are required to control *G. pallida* than *G. rostochiensis* (Whitehead *et al.*, 1985; Whitehead *et al.*, 1994).



1,3-D and other halogenated fumigants are general biocides thought to interfere with protein synthesis and respiration (Haydock *et al.*, 2006). They are applied several weeks or months before planting, in order to prevent phytotoxic effects (Haydock *et al.*, 2006). Under optimal conditions, 1,3-D can reduce PCN population density by over 90 % in the upper soil layers (Kerry *et al.*, 2003), although reductions of around 60 % are more typical (Storey, 1982; Been & Schomaker, 1999; Whitehead *et al.*, 1994). Methyl isothiocyanate-liberating fumigants, such as dazomet or metham sodium, are generally more effective than 1,3-D on a dose-for-dose basis (Whitehead *et al.*, 1973) but are too expensive for use in potato production (Whitehead & Turner, 1998). Gaseous fumigants such as 1,3-D diffuse through soil in the air phase and their efficacy depends on edaphic factors that influence the rate of diffusion, including texture, structure, water content and temperature. For effective fumigation, soil temperature should be above 7 or 10 °C for 1,3-D or methyl isothiocyanate liberators, respectively (Whitehead & Turner, 1998). Soil moisture is also critical; soil should be moist but not waterlogged (Whitehead & Turner, 1998). Limiting the rate of volatile loss from the soil surface is essential to achieving lethal doses of fumigants in upper soil layers (Whitehead & Turner, 1998). Highly volatile fumigants, such as 1,3-D, require a very good surface seal, ideally plastic sheeting (Haydock *et al.*, 2006), while liberators of less volatile methyl isothiocyanate require only soil surface smearing (Haydock *et al.*, 2006). 1,3-D and methyl isothiocyanate are adsorbed by organic matter and are less effective in heavy or organic soils (Whitehead & Turner, 1998). Both fumigant and granular nematicides are degraded by microbial activity and repeated application can lead to accelerated biodegradation and much reduced efficacy (Smelt *et al.*, 1987, 1996).

The use of synthetic nematicides may be one of the most effective means of control but it imposes serious financial and environmental costs. Treatment with a granular nematicide costs approximately £ 300 ha<sup>-1</sup> and a fumigant approximately £ 600 ha<sup>-1</sup> (Kerry *et al.*, 2003). When compared with mean gross margins of £ 1,900 ha<sup>-1</sup> (Kerry *et al.*, 2003), this is a substantial cost. Carbamate and organophosphate nematicides are extremely toxic compounds that are potentially very harmful to aquatic ecosystems and human health (Haydock *et al.*, 2006), as well as to birds, which can mistake granules for seed (Kerry *et al.*, 2003). Although 1,3-D leaves no detectable residues in the crop, it can leach into groundwater and is a mutagen and probable human carcinogen (Kerry *et al.*, 2003).

Due to environmental concerns, the most widely used granular nematicide in the 1990s, aldicarb, and the fumigant 1,3-D were withdrawn from use within the European Union in 2008 under the provisions of European Council Directive 91/414/EEC. This Directive has now been replaced by Regulation (EC) 1107/2009, which comes into effect in June 2011. The Regulation stipulates that all plant protection products must be risk assessed by the European Food Safety Authority and placed on a list of approved substances before they can be legally marketed or used. The criteria for approval are laid out in Annex II of the Regulation and include maximum acceptable levels for toxicity and carcinogenicity to humans, environmental persistence, bioaccumulation and toxicity to aquatic organisms. Importantly, substances failing to meet the criteria can still be approved if “exposure of humans to that active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans.” It has yet to be determined which active substances will be given approval. All of the remaining nematicides currently approved in the UK (ethoprophos, fosthiazate and oxamyl) would fail to meet the hazard criteria (Clayton *et al.*, 2008) and their approval or rejection will therefore be determined by the assessment of exposure risk. In the absence of chemical control or alternatives, gross margins of a typical potato farm on PCN-infested land would be halved for the first potato crop and would become negative after two five-year rotations (Clayton *et al.*, 2008). Alternative control measures would be required; otherwise rotations would have to be increased to one crop in every 10 years or more (Clayton *et al.*, 2008).

## 1.4 Potential management options for PCN

### 1.4.1 Engineered resistance to PCN

Transformation of potatoes with resistance (*R*) genes from other Solanaceae is a potential means of achieving broad-spectrum resistance. The *Hero* gene in tomato, *Lycopersicon esculentum*, confers >90 % resistance to *G. rostochiensis* and >80 % resistance to *G. pallida* (Ganal *et al.*, 1995, Sobczak *et al.*, 2005). However, when potato plants were transformed with *Hero* and shown to express the gene, no resistant phenotype was conferred (Sobczak *et al.*, 2005). *Hero* is a classical resistance gene (Ernst *et al.*, 2002). Lack of function in a heterologous species may be due to failure of the resistance gene product to interact either with a host protein modified by a pathogen virulence factor or with downstream components of a signal transduction

pathway. Better understanding of the basis of pathogen recognition and signal transduction by *Hero* could facilitate engineering of an entire defence pathway but simply inserting this resistance gene is not sufficient.

A great many possibilities exist for engineering PCN resistance in potatoes by incorporation of genes from distantly related taxa. Several strategies have been successfully demonstrated, including expression of genes for protease inhibitors, lectins and acetylcholinesterase inhibiting peptides (Atkinson *et al.*, 2003). One of the most promising strategies is the expression of protease inhibitors. When susceptible potato cultivar Desiree was transformed with chicken egg white cystatin, some of the transformed lines showed 70 % resistance to *G. pallida* (Urwin *et al.*, 2001). Likewise, expression of a modified rice cystatin, OclAD86, under control of the root-preferential ARSK1 promoter, resulted in 70 % resistance (Atkinson *et al.*, 2003). Transformation of partially resistant cultivars, Santé and Maria Huanca, with CaMV35S/OclAD86 resulted in a fully resistant phenotype (Urwin *et al.*, 2003). The environmental safety of these transgenic lines has been demonstrated (Cowgill, 2002a, 2002b; Celis *et al.*, 2004).

#### 1.4.2 Trap crops for control of PCN

Cultivated potato, *S. tuberosum*, can be used as a trap crop. Tubers are planted and allowed to grow, stimulating PCN to hatch and invade the roots but before the nematodes can complete their life-cycle, the crop is lifted (Lane *et al.*, 2000). A worthwhile yield can be produced from the trap crop (Halford *et al.*, 1999) but obviously, if it is lifted too late, PCN multiplication will occur (Whitehead & Turner, 1998). When managed correctly, trap cropping can reduce *G. pallida* population density by 70 to 95 % (Whitehead *et al.*, 1994; Halford *et al.*, 1999). However, the cost of establishing a commercial potato crop is as much as £ 3,500 ha<sup>-1</sup> (Kerry *et al.*, 2003). Although this can be partially offset by the value of the crop, it is still very expensive (Kerry *et al.*, 2003). A wild, non-tuber-forming relative of potato, *Solanum sysimbrifolium*, has potential as a trap crop (Kerry *et al.*, 2003). This species produces hatching factors that stimulate PCN to hatch and invade but it does not allow PCN multiplication (Scholte, 2000). As the crop is grown from seed and does not produce tubers that would have to be removed, it would be less expensive than trap-cropping with *S. tuberosum* (Kerry *et al.*, 2003). However, levels of control are inconsistent between different UK PCN populations (Kerry *et al.*, 2003).

### 1.4.3 Biological control of PCN

Broadcast application of products containing biological control agents is unlikely to be economical for commercial potato cultivation due to the quantities required to effectively treat large volumes of soil but inoculation of seed tubers with the organisms is feasible (Kerry *et al.*, 2003). Several organisms have shown potential as bio-control agents of PCN, including rhizobacteria of the genera *Agrobacterium* and *Bacillus* (Haskey-Günther *et al.*, 1998), the nematode-parasitic bacterium *Pasteuria* (Chen & Dickson, 1998), the nematode-trapping fungus *Arthrobotrys oligospora* (Cooke, 1962a; Cooke, 1962b) and fungi that attack eggs within cysts, including *Pochonia chlamydosporia* and *Paecilomyces lilacinus* (Jacobs *et al.*, 2003). Products containing some of these organisms have been developed but, to date, biological control of PCN has not been commercially successful (Kerry *et al.*, 2003).

## 1.5 *Rhizoctonia solani*

### 1.5.1 Pathology and economic importance of *Rhizoctonia solani*

*R. solani* is a soil-borne multinucleate basidiomycete fungus that is a saprophyte and a plant parasite (Menzies, 1970). On potato it causes the diseases stem canker and black scurf. Stem canker refers to the brown necrotic lesions that develop on infected stems, although stolons, roots and tubers can also be infected and develop similar lesions. Infection can result in complete girdling and killing of emerging stems before they leave the soil, resulting in delayed emergence and canopy development (Hide *et al.*, 1989). Likewise, stolon infection can kill stolons, resulting in emergence of secondary stolons and alteration of the number and size distribution of progeny tubers (Hide *et al.*, 1973). Black scurf refers to the accumulation of sclerotia on the tuber surface. Sclerotium is dense mycelium with heavily thickened cell walls. It serves as a survival structure as well as a means of dispersal with tubers.

Yield losses caused by *R. solani* are low, typically less than 1 % (Twining *et al.*, 2009). Economic damage is primarily due to reduced marketability of tubers due to black scurf, particularly in the pre-pack and seed markets (Jeger *et al.*, 1996; Twining *et al.*, 2009). Uneven tuber size due to stolon infection also reduces the value of the crop and delayed emergence due to stem canker can prevent growers from securing higher prices in the early market (Bradshaw *et al.*, 2001). Marketable yield losses of up to 30 % have been reported (Banville, 1989).

### 1.5.2 Genetics and life cycle of *R. solani*

Like all basidiomycetes, *R. solani* can reproduce vegetatively by the growth of hyphae or sexually by the formation of spores (Flentje *et al.*, 1970). The name *Rhizoctonia solani* actually describes only the asexual vegetative state, otherwise known as the imperfect state or anamorph (Parmeter & Whitney, 1970). The sexual basidiospore-forming state, the perfect state or teleomorph, is referred to as *Thanatephorus cucumeris* (Talbot *et al.*, 1970; Ahvenniemi *et al.*, 2009). Young vegetative cells of *R. solani* contain six to 21 haploid nuclei. These cells can be heterokaryotic or homokaryotic and the nuclei they contain divide simultaneously and segregate evenly such that each daughter cell contains an identical complement of nuclei (Flentje *et al.*, 1970; Cubeta & Vilgalys, 1997). When the fungus enters the perfect state, the nuclei in vegetative cells pair and septa form between the pairs of nuclei. The resulting binucleate cells undergo several rounds of mitotic division, forming cells called basidia. The pairs of haploid nuclei in each basidium fuse to form one diploid nucleus, which then undergoes meiotic division, forming four spores containing a single haploid nucleus each (Flentje *et al.*, 1970). On potato, the teleomorph *T. cucumeris* forms around the stem base just above the soil, causing the disease symptom "white collar" (Woodhall *et al.*, 2008). The spore-bearing structures are thus aerial and basidiospores are discharged ballistically. Basidiospores germinate in the soil and form multinucleate homokaryotic hyphal cells. When two hyphae meet, they can fuse in the process of anastomosis, resulting in the exchange of cytoplasm and nuclei between cells (Flentje *et al.*, 1970). In this way, exchange of nuclei results in generation of heterokaryotes (Flentje *et al.*, 1970). As well as facilitating genetic exchange, anastomosis results in the development of a hyphal network.

Anastomosis only occurs between compatible isolates and *R. solani* has been divided into 13 anastomosis groups (AG) (Carling *et al.*, 2002). Isolates within a group are able to fuse freely with one another whilst isolates from different groups are incompatible. Since anastomosis is necessary for genetic exchange, different AG are genetically isolated from one another and *R. solani* is often described as a species complex (Cubeta & Vilgalys, 1997). Genetic isolation and divergence of ribosomal RNA gene sequences between AG have led some authors to suggest that certain AG be classified as distinct species (Ahvenniemi *et al.*, 2009).

### 1.5.3 Distribution and dispersal

The *R. solani* complex is found worldwide and has possibly the largest host range of any plant pathogen (Baker, 1970). Individual AG are capable of causing disease on a diverse range of plants but have much restricted host ranges compared with *R. solani* as a whole (Baker, 1970). AG1 is often associated with rice, AG2-1 with brassicas, AG3 with Solanaceae and AG8 with cereals (Woodhall *et al.*, 2008; Budge *et al.*, 2009). At least five AG are pathogenic on potato: AG2-1, AG3, AG4, AG5 and AG8, of which AG3 is the most virulent (Woodhall *et al.*, 2008). In the UK, France and Denmark over 90 % of *R. solani* isolates sampled from diseased potato plants belonged to AG3 (Chand & Logan, 1983; Campion *et al.*, 2003; Justesen *et al.*, 2003; Woodhall *et al.*, 2007). In the UK, the remainder was almost exclusively comprised of AG2-1 isolates (Woodhall *et al.*, 2007). AG3 forms sclerotia more readily than other AG, facilitating dispersal on seed tubers (Woodhall *et al.*, 2008). A survey in 1999 found that 80 % of potato crops and 20 % of all potato plants sampled in England and Wales were infected with *R. solani* and that the disease was found in all regions (Bradshaw *et al.*, 2001).

Routes of dispersal of *R. solani* include transport on seed tubers, movement of infected soil between fields and wind dispersal of basidiospores. Identification of clonal isolates in potato fields separated by 250 km suggests that long-distance dispersal of vegetative propagules is occurring, probably on seed tubers (Justesen *et al.*, 2003). Both seed-borne and soil-borne inoculum are capable of infecting potato and the presence of both has an additive effect in disease severity (Tsrer & Peretz-Alon, 2005). The extent to which aerial spores serve to disperse *R. solani* is unknown but Jeger *et al.* (1996) reasoned that since formation of the sexual stage occurs after potato canopy closure, dispersal of basidiospores by wind must be limited. Homokaryotic mycelium from single basidiospores is generally less vigorous than heterokaryotic mycelium, showing reduced virulence as a pathogen and reduced competitiveness as a saprophyte (Flentje *et al.*, 1970). Thus, colonization of new areas by basidiospores may be limited. Cubeta & Vilgalys (1997) suggested that the primary function of the teleomorph is to generate genetic diversity through meiotic recombination and to facilitate genetic exchange between local populations.

### 1.5.4 Interaction between *R. solani* and PCN

*R. solani* interacts with PCN in terms of disease development (Back *et al.*, 2006; Bhattarai *et al.*, 2009). The incidence of stem and stolon infection by *R. solani* in

experimentally inoculated field grown potato plants was positively related to the population density of *G. rostochiensis* (Back *et al.*, 2006) and *G. pallida* (Bhattarai *et al.*, 2009). In soil infested with *G. pallida* and *R. solani*, nematicide treatment reduced the quantity of *R. solani* sclerotia on the surface of progeny tubers (Bhattarai *et al.*, 2009). Inoculation of potato tubers with *R. solani* has a positive effect on the number of *Globodera* J2 infecting potato roots (Bhattarai *et al.*, 2009) but a negative effect on multiplication rate (Back *et al.*, 2006). It appears that infection with either parasite interferes with host resistance toward the other but that competition between the parasites has a negative effect on PCN multiplication.

## **1.6 Management of *Rhizoctonia solani***

### **1.6.1 Phytosanitary and cultural measures for control of *R. solani***

Avoidance is the best means of control and since tuber-borne inoculum is thought to be the primary means of dispersal (Justesen *et al.*, 2003), the main method for controlling *R. solani* and other skin diseases is the use of clean seed (Twining *et al.*, 2009). Cultivation on inoculum-free land and application of fungicides are used to minimise the occurrence of *R. solani* on seed tubers (Tsrer, 2010). Thiophanate-methyl, mancozeb, fludioxinil, flutolanil, pencycuron, azoxystrobin and thiabendazole give some control of *R. solani* (Wicks *et al.*, 1995; Wharton *et al.*, 2007; Twining *et al.*, 2009). The most commonly used fungicides for treatment of seed tubers in the UK are imazalil (43 %) and pencycuron (32 %) (Garthwaite *et al.*, 2003). Despite these measures, a significant proportion of UK seed tubers (~6 %) are infected with *R. solani* (Bradshaw *et al.*, 2001).

Potatoes are most vulnerable to *R. solani* before emergence and under cool, wet conditions. Planting in warmer, drier soil and to a shallow depth can limit infection (Jeger *et al.*, 1996; Wharton *et al.*, 2007; Djébalí & Belhassen, 2010). Early harvest limits black scurf development and removing potato residues limits the density of soil-borne inoculum (Wharton *et al.*, 2007).

### **1.6.2 Resistant cultivars for control of *R. solani***

Some UK potato varieties, such as the cultivar Blue Danube, have high levels of resistance to *R. solani* (Carnegie *et al.*, 2010). As with PCN, however, cultivars are chosen primarily for tuber quality characteristics in order to satisfy market demand and not for

disease resistance (Lane *et al.*, 2000). The most popular cultivars, Maris Piper, Estima and Cara, show moderate levels of resistance to *R. solani* (Potato Council, 2010c).

### 1.6.3 Rotation for control of *R. solani*

Continuous cropping with potato results in increasing levels of soil-borne *R. solani* inoculum and resulting disease (Larkin & Honeycutt, 2006). Crop rotation reduces the incidence of disease, rotations with barley and canola being particularly effective (Larkin & Honeycutt, 2006). Rotations of at least three years are required to reduce disease caused by soil-borne inoculum (Tsrör, 2010). Rotation will rarely provide full control, however. Reasonably good saprophytic growth and a wide host range, including several crops and weeds found in potato fields, allow *R. solani* AG3 to survive the long intervals between potato crops (Tsrör, 2010).

### 1.6.4 Fungicides for control of *R. solani*

Fungicide treatment of seed tubers is an important aspect of the control of *R. solani* and other tuber-borne fungal diseases (see section 1.6.1). Soil-borne inoculum is less amenable to chemical control due to its distribution through the soil (Jeger *et al.*, 1996). However, modern fungicides, such as azoxystrobin, flutolanil or pencycuron, applied at the time of planting can dramatically reduce the incidence of black scurf on progeny tubers (Tsrör & Peretz-Alon, 2005; Wharton *et al.*, 2007; Djébalı & Belhassen, 2010), although even high doses of flutolanil do not completely prevent black scurf formation when initial inoculum density is high (Tsrör & Peretz-Alon, 2005).

Azoxystrobin and other strobilurin fungicides bind to the Qo centre of the cytochrome bc1 complex, blocking electron transport in respiration (Brandt *et al.*, 1988). Flutolanil interferes with respiration by binding another mitochondrial enzyme complex, succinate dehydrogenase complex II (Motoba *et al.*, 1988) and pencycuron is thought to bind to tubulin, interfering with microtubule formation (Leroux *et al.*, 1990). Since these compounds have single target sites, it is likely that resistant mutants could arise relatively easily and repeated application of the same active ingredient is discouraged in order to reduce selection of resistant strains (Bartlett *et al.*, 2002).



## 1.7 Brassica green manures for control of pests and diseases

### 1.9.1 The glucosinolate-myrosinase system

The biocidal effects of brassica green manures are often attributed to toxins derived from glucosinolates (Matthiessen & Kirkegaard, 2006). Glucosinolates are secondary metabolites produced by plants within the order Brassicales, especially the families Brassicaceae, Capparaceae and Caricaceae (Fahey *et al.*, 2001; Agerbirk *et al.*, 2008). The glucosinolate structure consists of a beta-*D*-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximosulfate ester and a variable R group (Halkier & Gershenzon, 2006) (Figure 1.2). The double bonded nitrogen and carbon atoms of the oxime, together with the R group, are derived from the amino acid precursors of glucosinolates, aliphatic forms deriving from Ala, Leu, Ile, Met or Val, aromatic forms from Phe or Tyr and indole forms from Trp (Halkier & Gershenzon, 2006). In addition to variation due to the parent amino acid, the R group may be modified during biosynthesis (Grubb & Abel, 2006) and over 120 different glucosinolates have been identified in plants (Fahey *et al.*, 2001).

Glucosinolates themselves are relatively biologically inactive (Lazzeri *et al.*, 1993, 2004b; Manici *et al.*, 1997; Buskov *et al.*, 2002) and chemically and thermally stable (Holst & Williamson, 2004; Tsao *et al.*, 2000). Hydrolysis occurs under the action of myrosinase, a  $\beta$ -thioglucosidase that occurs in glucosinolate-producing plants. Myrosinase cleaves the thioglucoside linkage to produce glucose and unstable thiohydroximate-*O*-sulphates (Bones & Rossiter, 2006). These unstable aglycones spontaneously rearrange to form a variety of bioactive compounds, including isothiocyanates, nitriles and elemental sulphur, thiocyanates, oxazolidine-2-thiones and epithioalkanes (Holst & Williamson, 2004; Grubb & Abel, 2006) (Figure 1.3). Which derivative is produced depends upon pH, the glucosinolate R-group, the concentration of ferrous ions and the presence of myrosinase-interacting proteins and cofactors (Grubb and Abel, 2006). In the absence of other factors and at intermediate pH, isothiocyanates are formed (Halkier & Gershenzon, 2006).  $\beta$ -hydroxy isothiocyanates are unstable and spontaneously cyclise to form oxazolidine-2-thiones (Halkier & Gershenzon, 2006). Indole isothiocyanates are also unstable and undergo lysis to form alcohols (Holst & Williamson, 2004). Nitriles and epithionitriles form a major component of the glucosinolate hydrolysis products in some plants (Halkier & Gershenzon, 2006). *In vitro*, they are produced at pH <3 or in the presence of Fe<sup>2+</sup> ions (Halkier & Gershenzon, 2006) whereas, *in vivo*, they are produced in plants containing the myrosinase-interacting epithiospecifier protein (Halkier & Gershenzon, 2006).

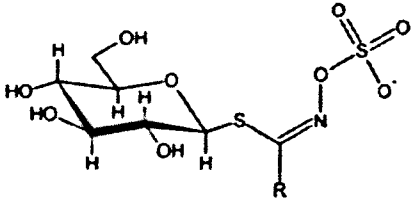


Figure 1.2: Glucosinolate structure.

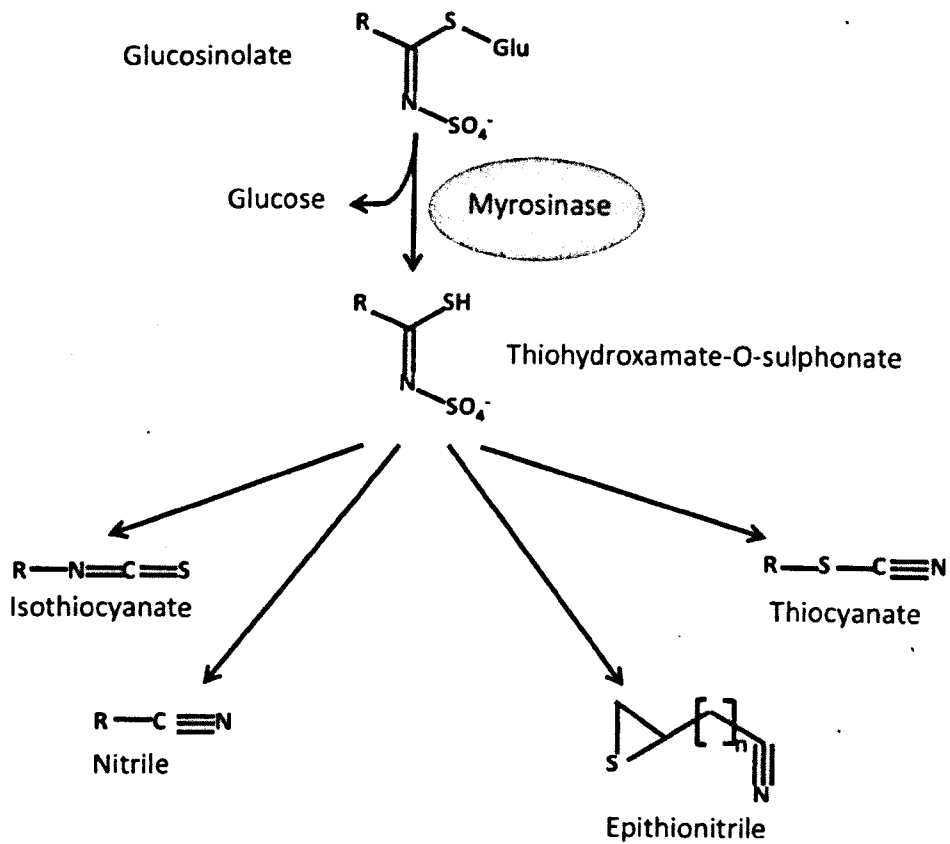


Figure 1.3 Glucosinolate hydrolysis reactions.

In a few species, thiocyanates are formed from benzyl-, allyl- or 4-methylsulfinylbutyl-glucosinolate in the presence of specific protein factors (Halkier & Gershenzon, 2006). One such protein has recently been identified in *Lepidium sativum* (Burow *et al.*, 2007). Like the epithiospecifier protein, the thiocyanate specifying protein directs the formation of its respective glucosinolate hydrolysis product only in the presence of myrosinase (Burow *et al.*, 2007).

In order to avoid production of toxic glucosinolate degradation products within the plant, glucosinolates and myrosinase are stored separately. The glucosinolate substrate is stored within protein storage vacuoles, whilst the enzyme myrosinase is expressed and stored within guard cells and specialised idioblasts lacking glucosinolate (Kelly *et al.*, 1998; Thangstad *et al.*, 2004).

### 1.9.2 Toxicity of glucosinolate hydrolysis products

Hydrolysis products of glucosinolates are toxic to a wide variety of organisms, including bacteria (Tierens *et al.*, 2001), fungi (Drobnica *et al.*, 1967a, 1967b; Angus *et al.*, 1994; Sarwar *et al.*, 1998; Manici *et al.*, 1997, 2000), insects (Blau *et al.*, 1978; Borek *et al.*, 1997; Matthiessen & Shackleton, 2005) and nematodes (Buskov *et al.*, 2002; Serra *et al.*, 2002; Zasada & Ferris, 2003, 2004, 2009; Lazzeri *et al.*, 1993, 2004a). *In vitro* toxicity assays have shown almost all isothiocyanates studied to be more toxic than other glucosinolate hydrolysis products (Angus *et al.*, 1994; Vaughn & Boydston, 1997; Manici *et al.*, 1997, 2000; Buskov *et al.*, 2002; Lazzeri *et al.*, 2004a). The most toxic isothiocyanates have LD<sub>50</sub> to plant-parasitic nematodes and fungi at concentrations as low as 10 µM (Sarwar *et al.*, 1998; Zasada & Ferris, 2003). Table 1.1 summarises results of studies of glucosinolate hydrolysis product toxicity to plant parasitic nematodes. Target species, experimental system, exposure duration and units used to describe toxicity vary between studies so comparison is not straightforward but an attempt has been made to list the compounds in ascending order of toxicity. 2-phenylethyl and benzyl isothiocyanates, both aromatic compounds, were consistently the most toxic.

Glucosinolate hydrolysis products besides isothiocyanates have been associated with pathogen suppression in some situations (Smolinska *et al.*, 1997). For instance, 5-vinylloxazolidine-2-thione, the hydrolysis product of 2-hydroxy-3-butenyl glucosinolate, was amongst the active components of *B. napus* seed meal in inhibition of *Aphanomyces euteiches* root rot of pea (Smolinska *et al.*, 1997). Due to its high aqueous solubility

**Table 1.1:** The toxicity of glucosinolate hydrolysis products toward plant parasitic nematodes.

Class *	Parent glucosinolate	Hydrolysis product **	Nematodes tested	Toxicity	Reference
G	2-Phenylethyl	Isothiocyanate	<i>G. rostochiensis</i> <i>M. incognita</i> <i>T. semipenetrans</i> <i>M. javanica</i>	2.4 mM for 16 h caused 100 % mortality LD <sub>50</sub> (48h) = 13 µM ED <sub>50</sub> (48h) = 4 µM ED <sub>50</sub> (48h) = 22 µM	Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (2004) Zasada & Ferris (2003)
G	Benzyl	Isothiocyanate	<i>H. schactii</i> <i>G. rostochiensis</i> <i>M. incognita</i> <i>T. semipenetrans</i> <i>M. javanica</i>	1.2 mM for 24 h caused 93 % mortality 2.4 mM for 16 h caused 93 % mortality LD <sub>50</sub> (48h) = 15 µM ED <sub>50</sub> (48h) = 0.01 mM ED <sub>50</sub> (48h) = 0.04 mM	Lazzeri <i>et al.</i> (1993) Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (2004) Zasada & Ferris (2003)
D	2-Propenyl (Allyl)	Isothiocyanate	<i>H. schactii</i> <i>G. rostochiensis</i> <i>M. incognita</i>	1.3 mM for 24 h caused 100 % mortality 2.5 mM for 16 h caused 20 % mortality LC <sub>50</sub> (48h) = 34 µM	Lazzeri <i>et al.</i> (1993) Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (2004)
A	4-(Methylsulfinyl)butyl	Isothiocyanate	<i>T. semipenetrans</i> <i>M. javanica</i>	ED <sub>50</sub> (48h) = 0.02 mM ED <sub>50</sub> (48h) = 0.22 mM	Zasada & Ferris (2003)
A	4-(Methylthio)butyl	Isothiocyanate	<i>M. incognita</i>	LD <sub>50</sub> (24h) = 21 µM	Lazzeri <i>et al.</i> (2004)
B	Ethyl	Isothiocyanate	<i>T. semipenetrans</i> <i>M. javanica</i>	ED <sub>50</sub> (48h) = 0.14 mM ED <sub>50</sub> (48h) = 0.11 mM	Zasada & Ferris (2003)
B	n-Butyl	Isothiocyanate	<i>T. semipenetrans</i> <i>M. javanica</i>	ED <sub>50</sub> (48h) = 0.11 mM ED <sub>50</sub> (48h) = 0.26 mM	Zasada & Ferris (2003)
A	4-Methylthio-3-butenyl	Isothiocyanate	<i>H. schactii</i> <i>M. incognita</i>	1.2 mM for 24 h caused 94 % mortality LC <sub>50</sub> (48h) = 59 µM	Lazzeri <i>et al.</i> (1993) Lazzeri <i>et al.</i> (2004)
D	3-Butenyl	Isothiocyanate	<i>H. schactii</i> <i>G. rostochiensis</i> <i>M. incognita</i>	1.3 mM for 24 h caused 93 % mortality 2.5 mM for 24 h caused 13 % mortality LD <sub>50</sub> (48h) = 77 µM	Lazzeri <i>et al.</i> (1993) Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (2004)
G	Phenyl	Isothiocyanate	<i>T. semipenetrans</i> <i>M. javanica</i>	ED <sub>50</sub> (48h) = 0.25 mM ED <sub>50</sub> (48h) = 0.24 mM	Zasada & Ferris (2003)
A	3-(Methylsulfinyl)propyl	Isothiocyanate	<i>M. incognita</i>	LD <sub>50</sub> (48h) = 0.6 mM	Lazzeri <i>et al.</i> (2004)
G	2(R)-Hydroxy-2-phenylethyl	Oxazolidine-2-thione	<i>G. rostochiensis</i> <i>H. schactii</i>	2.4 mM for 24 h caused 9 % mortality 1.2 mM for 24 h caused 5 % mortality	Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (1993)
G	4-Hydroxybenzyl	Alcohol	<i>G. rostochiensis</i> <i>M. incognita</i>	2.4 mM for 24 h caused 4 % mortality LD <sub>50</sub> (48h) = 0.8 mM	Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (2004)
D	2(S)-2-Hydroxy-3-butenyl	Oxazolidine-2-thione	<i>G. rostochiensis</i> <i>M. incognita</i>	2.5 mM for 24 h caused 5 % mortality LD <sub>50</sub> (48h) = 0.9 mM	Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (2004)
A	4-Methylsulfinyl-3-butenyl	Isothiocyanate	<i>H. schactii</i> <i>G. rostochiensis</i> <i>M. incognita</i>	1.2 mM for 24 h caused 3 % mortality 2.5 mM for 24 h caused 3 % mortality LD <sub>50</sub> (48h) = 1.3 mM	Lazzeri <i>et al.</i> (1993) Buskov <i>et al.</i> (2002) Lazzeri <i>et al.</i> (2004)
E	2-Hydroxy-2-methylpropyl	Oxazolidine-2-thione	<i>M. incognita</i>	LD <sub>50</sub> (48h) = 5.7 mM	Lazzeri <i>et al.</i> (2004)

\* as defined by Fahey *et al.* (2001): A = sulphur-containing side chains, B = straight chain aliphatic, C = branched chain aliphatic, D = olefins, E = aliphatic alcohols, F = aliphatic straight chain ketones, G = aromatic, H = ω-hydroxyalkyl (benzoates), I = indole, J = multiply glycosylated and other.

\*\* Buskov *et al.* (2002) and Lazzeri *et al.* (2004) tested glucosinolates with myrosinase and determined the resultant hydrolysis products. Lazzeri *et al.* (2004) tested pure isothiocyanates.

compared with isothiocyanates, this compound and another oxazolidine-2-thione were by far the most abundant glucosinolate hydrolysis products in aqueous extracts of the seed meal and accounted for the majority of the activity of such extracts toward *A. euteiches* (Smolinska *et al.*, 1997). The extent to which glucosinolate catabolites are involved in pest and pathogen suppression by brassica green manures in field situations is uncertain (see section 1.9.4.3).

### 1.9.3 Mode of action and structure-activity relationships of isothiocyanates

Isothiocyanates are powerful electrophiles and react with free amino and thiol groups of proteins and oxidatively cleave disulphide bonds (Kawakishi & Kaneko, 1985, 1987). Schultz *et al.* (2005) found a strong relationship between the toxicity of aromatic isothiocyanates to *Tetrahymena pyriformis* and their reactivity towards the thiol group of the cysteine residue of glutathione. Toxicity and glutathione reactivity were proportional to the predicted electronegativity of the carbon atom in the isothiocyanate group (-NCS) (Schulz *et al.*, 2005). The authors propose that the bioactivity of aromatic isothiocyanates, and thus probably also aliphatic isothiocyanates, is due to addition reactions with cellular thiols. Thus, the toxicity of isothiocyanates is thought to be due to non-specific reaction with proteins, including essential enzymes.

The structure of the isothiocyanate variable group affects the chemical and biological properties of the compound (Drobnica *et al.*, 1967a, 1967b; Lazzeri *et al.*, 1993, 2004; Angus *et al.*, 1994; Sarwar *et al.*, 1998; Manici *et al.*, 1997, 2000; Buskov *et al.*, 2002; Zasada & Ferris, 2003, 2004). Besides reactivity towards nucleophiles, other important determinants of isothiocyanate toxicity are aqueous solubility and volatility (Sarwar *et al.*, 1998; Drobnica *et al.*, 1967a, 1967b). Nastruzzi *et al.* (1996) found that isothiocyanates with greater antiproliferatory activity towards human erythroleukemic cells tended to be more lipid-soluble, a property that facilitates the permeation of phospholipid membranes (Holst & Williamson, 2004). The toxicity of synthetic benzyl isothiocyanate analogues to fungi is directly related to their lipid solubility (Drobnica *et al.*, 1967a). Likewise, 2-phenylethyl isothiocyanate analogues with polar substituents, which are consequently more water soluble, are also less toxic to fungi (Drobnica *et al.*, 1967b). Within chemically similar sub-sets of naturally occurring isothiocyanates, similar trends can be observed but there is no clear overall trend (Drobnica *et al.*, 1967a; Manici *et al.*, 1997; Sarwar *et al.*, 1998), presumably because factors besides solubility mask its effects.

When fungi are exposed to isothiocyanates dissolved in the growing medium, the aromatic compounds, such as 2-phenylethyl and benzyl isothiocyanate, are generally more toxic than the aliphatics (Drobnica *et al.*, 1967a; Sarwar *et al.*, 1998). In contrast, when fungi are exposed to isothiocyanates via the air, aliphatic compounds tend to be more toxic (Sarwar *et al.*, 1998). The greater activity of aliphatic isothiocyanates than aromatic ones when introduced into the headspace of sealed containers is due to their relatively high volatility (Sarwar *et al.*, 1998). Within the aliphatic compounds 2-propenyl, 3-butenyl and 4-pentenyl isothiocyanate, molecular mass is inversely proportional to volatility and toxicity to fungi (Sarwar *et al.*, 1998).

Other observations on the structure-activity relationships of isothiocyanates include that branched-chain saturated aliphatic isothiocyanates tend to be less toxic than straight-chain ones and both tend to be less toxic than unsaturated aliphatic isothiocyanates (Schultz & Comeaux, 1996). Isothiocyanates containing a sulphur atom but no double bond in the variable group tend to be amongst the most toxic to fungi whether exposure is via the aqueous or air phase (Drobnica *et al.*, 1967a; Manici *et al.*, 1997, 2000).

#### 1.9.4 Biofumigation

##### 1.9.4.1 Definition of biofumigation and the behaviour of isothiocyanates in soil

The term "biofumigation" was originally used by Kirkegaard *et al.* (1993) to describe suppression of pests and pathogens by incorporation of isothiocyanate-producing plant tissues into soil. The term derives from the fact that isothiocyanates are volatile and because the principal active product of the synthetic fumigants metham sodium and Dazomet is methyl isothiocyanate. Methyl isothiocyanate, like other isothiocyanates but unlike gaseous fumigants, such as methyl bromide and 1,3-dichloropropene, is a liquid at ambient temperature and has a distribution ratio of approximately 99:1 in the water and air phases of soil, respectively (Frick *et al.*, 1998; Smelt & Leistra, 1974). The vertical distribution profile of methyl isothiocyanate several days after injection into soil resembles that of the gaseous fumigant 1,3-D, albeit less well dispersed (Leistra & Smelt, 1974). This suggests that despite the small fraction that exists in the air phase, methyl isothiocyanate behaves similarly to gaseous fumigants, dispersing through the continuous air-filled pore spaces in soil (Lembright, 1990). However, when there is water percolation through the soil, methyl isothiocyanate moves downwards with the flow and losses to leaching can occur (Frick *et al.*, 1998; Laegdsmand *et al.*, 2007).

#### 1.9.4.2 Results of field trials of biofumigation

The high toxicity of compounds derived from brassica tissues towards many soil-borne pests and pathogens has been demonstrated *in vitro* using pure compounds (Lazzeri *et al.*, 1993, 2004; Manici *et al.*, 1997, 2000; Potter *et al.*, 1998; Sarwar *et al.*, 1998; Buskov *et al.*, 2002; Serra *et al.*, 2002; Smith & Kirkegaard, 2002; Zasada & Ferris, 2003; Zasada *et al.*, 2009) and brassica tissues (Angus *et al.*, 1994; Potter *et al.*, 1998; McLeod & Steel, 1999; Olivier *et al.*, 1999; Zasada & Ferris, 2004; Fan *et al.*, 2008). Likewise, experiments using soil microcosms have shown that brassica green manures can have dramatic effects on the incidence of disease and on population densities of pathogens (Smolinska *et al.*, 1997; Shetty *et al.*, 2000; Cohen & Mazzola, 2006; Yulianti *et al.*, 2007; Motisi *et al.*, 2009a) and plant-parasitic nematodes (Mojtahedi *et al.*, 1991; Al-Rehiayani *et al.*, 1999; Ploeg & Stapleton, 2001; Aballay *et al.*, 2004; Mazzola *et al.*, 2007). Despite this potential biocidal activity, levels of pest or pathogen suppression observed in field studies have been inconsistent. Biofumigation has in some cases provided moderate to high levels of control (Subbarao *et al.*, 1999; Blok *et al.*, 2000; Hao & Subbarao, 2003; Lazzeri *et al.*, 2003; Wiggins & Kinkel, 2005; Kirkegaard *et al.*, 2007; Larkin & Griffin, 2007; Ochiai *et al.*, 2007; Snapp *et al.*, 2007; Friberg *et al.*, 2009; Motisi *et al.*, 2009b) but at least as often it has had no detectable effect on disease incidence or pest or pathogen population density (Johnson *et al.*, 1992; Hao & Subbarao, 2003; Hartz *et al.*, 2005; Wiggins & Kinkel, 2005; Monfort *et al.*, 2007; Mattner *et al.*, 2008; Njoroge *et al.*, 2008; Friberg *et al.*, 2009).

Results of field trials in which significant pest or pathogen suppression has been observed include up to an 80 % reduction in the incidence of disease caused by *Ralstonia solanacearum* on tomato by incorporation of *Brassica juncea* at 5 % (w/w) (Kirkegaard *et al.*, 2007), a 75 % reduction in the density of *Verticillium dahliae* micro-sclerotia and severity of *Verticillium* wilt disease on cauliflower by incorporation of 50 t ha<sup>-1</sup> broccoli residue (Subbarao *et al.*, 1999) and up to 40 and 80 % reductions, respectively, in the incidences of powdery scab and black scurf on potato tubers by incorporation of 5-7 t d.w. ha<sup>-1</sup> of *B. juncea*, *Brassica napus* or *Sinapis alba* (Larkin & Griffin, 2007). Broccoli or rye amendment with plastic tarping reduced the inoculum density of *V. dahliae*, *R. solani* and *F. oxysporum* by several orders of magnitude (Blok *et al.*, 2000). Use of broccoli as a rotation crop reduced the incidence of lettuce drop disease caused by *Sclerotinia minor* by 0 to 40 % depending on the site and the year (Hao & Subbarao, 2003). Incorporation of *B. oleracea* var. *botrytis* (broccoli) or the non-brassic *Pisum sativum* (pea) or *Sorghum bicolor* reduced the incidence of *Verticillium*

wilt on potatoes (Ochiai *et al.*, 2007). *Verticillium* wilt severity on potatoes has also been reduced by green manures of *B. napus* and the non-brassica *Fagopyrum esculentum* (buckwheat), although these had no effect on *Streptomyces scabies* (Wiggins & Kinkel, 2005). Biofumigation with *B. juncea* reduced the incidence of disease caused by *R. solani* on *Daucus carota* (carrot) by 33 % (Friberg *et al.*, 2009) and on *Beta vulgaris* (sugar beet) by 45 % (Motisi *et al.*, 2009b) but did not affect *Fusarium* spp. (Friberg *et al.*, 2009). Biofumigation with *B. juncea* also reduced the incidence of unspecified diseases on strawberry plants and increased yield by 15 % (Lazzeri *et al.*, 2003).

In contrast, Hartz *et al.* (2005) observed no reduction in inoculum density of *V. dahliae* or *Fusarium* spp. or the incidence of resulting diseases on tomato, despite following recommended biofumigation practices and generating of up to 150 mol ha<sup>-1</sup> glucosinolate. Incorporation of *B. juncea* or *B. napus* green manures had no effect or even a positive effect on the inoculum density of *Fusarium oxysporum* and *Pythium* spp. (Njoroge *et al.*, 2008). Incorporation of 7.6 t d.w. ha<sup>-1</sup> of a *B. napus*/*B. rapa* mixture had no effect on viability of buried inoculum of *Phytophthora cactorum* or *Cylindrocarpon destructans* (Mattner *et al.*, 2008). Incorporation of 30-60 t ha<sup>-1</sup> of *B. napus* had no effect on the population density of *Meloidogyne* spp. (Johnson *et al.*, 1992). Likewise, Monfort *et al.* (2007) tested a wide range of *Brassica* spp. for suppression of *Meloidogyne incognita* but found no consistent suppression.

In some of these studies, lack of efficacy may have been due to use of cultivars producing low quantities of effective glucosinolates (Johnson *et al.*, 1992; Njorge *et al.*, 2008). For instance, *B. juncea* cv. Cutlass employed by Njorge *et al.* (2008) contained only 5.2 µmol g<sup>-1</sup> d.w. in the epigeal parts, compared with 31.3 µmol g<sup>-1</sup> d.w. in *B. juncea* cv. Nemfix (Gimsing & Kirkegaard, 2006). Attempts to control root-knot nematodes, *Meloidogyne* spp., with brassica green manures have sometimes failed due to multiplication of the nematode on the growing crop, which has negated the reduction in population caused by incorporation of the brassica tissue (Mojtahedi *et al.*, 1999; Monfort *et al.*, 2007). In many cases, the reasons underlying the efficacy or inefficacy of biofumigation are poorly understood. Investigation of the mechanisms involved is necessary to develop and improve the technique in a directed fashion rather than by simple trial and error (Matthiessen & Kirkegaard, 2006).



### 1.9.4.3 Mechanisms of pest or pathogen suppression by brassica green manures

Several studies have found correlations between the concentration of isothiocyanates produced by brassica tissues and suppression of fungal growth or nematode motility *in vitro*. The concentration of 2-propenyl isothiocyanate released from tissues of *B. juncea* or *Brassica nigra* was directly proportional to the degree of inhibition of growth of *Verticillium dahliae*, *Helminthosporium solani* and *Fusarium sambucinum* (Mayton *et al.*, 1996; Olivier *et al.*, 1999). Likewise, aqueous extracts or volatiles from a *B. juncea* cultivar containing a high concentration of glucosinolate possessed significantly greater fungistatic activity towards *Pythium irregulare* and *R. solani* than those from a *B. juncea* cultivar containing a low concentration of glucosinolate (Lazzeri *et al.*, 2004). Paralysis of root-knot nematode *Meloidogyne javanica* exposed to volatiles from brassica tissues correlated with the concentration of glucosinolates those tissues (McLeod & Steel, 1999).

Some experiments in soil microcosms have also found evidence for the involvement of glucosinolate hydrolysis products in suppression of pests, pathogens or weeds. Smolinksa *et al.* (1997) found that aqueous extracts from high glucosinolate content rapeseed meal reduced infection of pea by the fungal pathogen *A. euteiches* in soil microcosms. Extracts from low glucosinolate content rapeseed meal or from autoclaved high glucosinolate content rapeseed meal, in which myrosinase-catalysed glucosinolate hydrolysis was impaired, did not inhibit *A. euteiches* infection of pea (Smolinksa *et al.*, 1997). Similar experiments were carried out to determine the active components of rapeseed meal in inhibition of seed germination (Brown & Morra, 1995). Intact rapeseed meal inhibited germination of lettuce seeds by 90 % whilst rapeseed meal from which glucosinolate hydrolysis products had been removed lost all activity (Brown & Morra, 1995). Brassicas producing high concentrations of 2-propenyl glucosinolate were more effective than those with low glucosinolate concentrations at controlling bacterial wilt caused by *R. solanacearum*, although significant suppression was still observed with low glucosinolate content cultivars (Matthiessen & Kirkegaard, 2006). The degree of reduction in inoculum density of *Sclerotium rolfsii* and *Pythium ultimum* was correlated with the concentration of isothiocyanates and aldehydes produced by cabbage residues under plastic tarping (Gamliel & Stapleton, 1993).

Other studies with soil microcosms have found suppression of pests and pathogens by brassica green manures to be unrelated to glucosinolate content. The number of *M. javanica* recovered from soil treated with brassica green manures was reduced by up to 90 % relative to untreated soil (McLeod & Steel, 1999). Yet, in contrast to results with

the same nematodes and brassicas *in vitro*, degree of suppression was not proportional to glucosinolate concentration (McLeod & Steel, 1999). Nor was there a significant correlation between recovery of *Pratylenchus neglectus* from soil amended with brassica tissues and glucosinolate concentrations (Potter *et al.*, 1998), although in this case, there did appear to be a relationship and lack of statistical significance may have been due to the small sample size tested. Incorporation of rapeseed meal into apple orchard soil prior to replanting resulted in three to thirty-fold reduction in the number of *Pratylenchus* spp. recovered from apple roots at the end of the first growing season (Cohen *et al.*, 2005). However, soybean meal was even more effective, excluding a role for glucosinolates (Cohen *et al.*, 2005).

Alternative mechanisms of pest or pathogen suppression by brassica green manures include the generation of toxins unrelated to glucosinolates. Microbial decomposition of organic matter under anaerobic conditions generates toxic short chain fatty acids (Oka, 2010). Toxic nitrogenous species, such as ammonia (NH<sub>3</sub>) and nitrous acid (HNO<sub>2</sub>), are produced during decay of organic matter with low C/N ratios, such as brassicas (Oka, 2010). Besides the glucosinolate catabolites, decomposing brassica tissues produce other volatile sulphur-containing toxins, including methyl sulphide, dimethyl disulphide, carbon disulphide and methanethiol (Lewis & Papavizas, 1970; Forney *et al.*, 1991; Gamliel & Stapleton, 1993; Bending & Lincoln, 1999; Wang *et al.*, 2009a). These compounds are produced by microbial degradation of sulphur-containing organic compounds, such as sulphoxides, which are typically highly abundant in brassicas (Brown & Morra, 1997; Bending & Lincoln, 1999; Wang *et al.*, 2009). Peak concentrations of methanethiol and dimethyl disulphide were 80 and 30 times greater than that of the dominant isothiocyanate, 2-propenyl isothiocyanate, in the headspace above *B. juncea* leaf pieces decomposing in soil (Bending & Lincoln, 1999). Based on the results of Morra & Kirkegaard (2002) and Gimsing & Kirkegaard (2006), peak isothiocyanate concentrations would be expected to have been approximately 20 times greater had the tissue been thoroughly pulverised but nevertheless, these non-glucosinolate-related sulphur compounds are clearly produced in significant quantities.

Methanethiol and hydrogen sulphide are both more toxic than 2-propenyl isothiocyanate towards *Verticillium dahliae* in soil (Down *et al.*, 2004). Concentrations of methanethiol, hydrogen sulphide and 2-propenyl isothiocyanate required to significantly reduce the density of colony-forming units were 50, 125 and 200 µg g<sup>-1</sup> soil, respectively (Down *et al.*, 2004). Carbon disulphide acts synergistically with

methyl isothiocyanate in toxicity to fungi (Canesa & Morrell, 1995). Despite the potential importance of these compounds in biofumigation, they have been studied very little in comparison to the glucosinolate catabolites. A recent study found that the concentration of methyl sulphide and dimethyl disulphide in headspace under plastic tarping after amendment of soil with brassica green manures was inversely proportional to the rate of infection of subsequently planted potato plants by *Verticillium dahliae* (Wang *et al.*, 2009a). The relationship was weak ( $R^2 = 0.31$ ) and so other factors must have influenced the toxicity of the amendments and concentrations of isothiocyanates were not measured.

Biological mechanisms have also been implicated in suppression of microbial pathogens during biofumigation. Organic amendments can alter the microbial community in such a way as to increase soil suppressiveness to plant pathogens. For instance, Weindling (1932) found that acidification of soil favoured *Trichoderma* antagonistic towards *R. solani* and reduced damping off of citrus seedlings. Wright (1941) found that incorporation of high C/N ratio plant residues reduced the incidence of damping-off of broad-leaf tree seedlings. Damping off incidence was directly proportional to soil nitrate concentration and application of glucose reduced both nitrate and damping-off. Snyder *et al.* (1959) also found that incorporation of high C/N plant materials reduced the incidence of *Rhizoctonia* infection whilst low C/N plant material had the reverse effect.

Wiggins & Kinkel (2005) observed that suppression of *V. dahliae* by incorporation of green manures was associated with an increase in the proportion of antagonistic *Streptomyces* spp. in the microbial community. Cohen *et al.* (2005) found that incorporation of rapeseed meal into apple orchard soil caused populations of *Streptomyces* spp. to increase 10-fold or more and that the proportion of *Streptomyces* isolates possessing antibiotic activity towards *R. solani* increased from 25 to 70 %. Addition of rapeseed meal caused a significant reduction in the incidence of *R. solani* infection of apple trees (Cohen *et al.*, 2005). Despite the increase in antagonistic Streptomyces, saprophytic growth of *R. solani* was not inhibited. Instead, the mechanism of disease suppression appears to have been induction of host resistance. Apple trees were grown with one half of the root system in field soil artificially infected with *R. solani* and the other half in field soil that was either untreated or treated with rapeseed meal. Addition of rapeseed meal caused a highly significant reduction in the incidence of infection from 64 to 36 %. Interestingly, soybean meal did not reduce the incidence of *R. solani* infection of apple (Cohen *et al.*, 2005).

### 1.9.5 Factors affecting the concentration of isothiocyanates in soil during biofumigation

Unsurprisingly, the main determinant of the concentration of isothiocyanate achieved in soil during biofumigation is the amount of glucosinolate in the incorporated material (Gimsing & Kirkegaard, 2006), which is determined by genetic, ontogenic and environmental factors (Kirkegaard & Sarwar, 1998; Sarwar & Kirkegaard, 1998). The proportion of the glucosinolate that is hydrolysed to isothiocyanate can vary greatly and is influenced largely by the extent of cellular disruption of the brassica tissue (Morra & Kirkegaard, 2002; Matthiessen & Kirkegaard, 2006). Soil factors influence effluxes of isothiocyanate through volatilisation, sorption, leaching and degradation (Frick *et al.*, 1998; Gimsing *et al.*, 2007a, 2007b).

#### 1.9.5.1 Factors affecting the concentration of glucosinolate in brassicas

Of the 120 or more naturally occurring plant glucosinolates (Fahey *et al.*, 2001), approximately 20 are common (Kirkegaard & Sarwar, 1998). Types of glucosinolate produced vary greatly between species (Kirkegaard & Sarwar, 1998; Fahey *et al.*, 2001; Agerbirk *et al.*, 2008) and even between cultivars of the same species (Bellostas *et al.*, 2007; Padilla *et al.*, 2007). There are also large differences in the amounts of glucosinolate produced by different brassicas (Kirkegaard & Sarwar, 1998; McLeod & Steel, 1999; Potter *et al.*, 2000; Bellostas *et al.*, 2007; Padilla *et al.*, 2007). Tissue glucosinolate concentration and plant biomass can each vary by at least an order of magnitude between species, resulting in more than 50-fold variation in total glucosinolate yield (moles of glucosinolate produced per area) (Kirkegaard & Sarwar, 1998).

Within the same plant, there is considerable variation in the quantity and type of glucosinolates present in different organs and developmental stages (Sarwar & Kirkegaard, 1998; Brown *et al.*, 2003). The distribution of glucosinolates among organs generally corresponds to the predictions of the optimal defence hypothesis (McKey, 1974), those organs with the greatest fitness value containing the highest concentrations (Brown *et al.*, 2003). Seeds of *Arabidopsis thaliana* contain approximately 100 times the glucosinolate concentration of senescent rosette leaves (Brown *et al.*, 2003). In the vegetative phase, the younger, inner rosette leaves of *A. thaliana* contain higher concentrations than the outer leaves (Brown *et al.*, 2003). Similar patterns are observed in *Brassica napus* (Clossais-Besnard & Larher, 1991; Fieldsend & Milford, 1994). The mean leaf concentration increases throughout the

vegetative growth phase until it peaks at the initiation of flowering and then declines (Rosa *et al.*, 1996; Sarwar & Kirkegaard, 1998; Brown *et al.*, 2003). For instance, the shoot and root glucosinolate concentrations of eight field-grown brassica accessions declined from around 35  $\mu\text{mol g}^{-1}$  d.w. at the buds-raised stage to less than 1  $\mu\text{mol g}^{-1}$  d.w. at maturity (Sarwar & Kirkegaard, 1998). Biomass continues to increase after the initiation of flowering and total glucosinolate yield peaks shortly after the initiation of flowering (Sarwar & Kirkegaard, 1998).

Environmental factors such as season (Rosa *et al.*, 1996; Sarwar & Kirkegaard, 1998), day length, temperature, light wavelength (Engelen-Eigles *et al.*, 2006), sulphate availability (Kopsell *et al.*, 2007) and attack by herbivores (Travers-Martin & Müller, 2007) or pathogens (Rostas *et al.*, 2002) can have a strong effect on glucosinolate profiles. Glucosinolate content is often greater in summer-grown than winter-grown brassicas (Rosa *et al.*, 1996; Kirkegaard & Sarwar, 1998) and plants grown under long-day length conditions (16 h d<sup>-1</sup>) contain higher concentrations than those grown under standard day length (8 h d<sup>-1</sup>) (Engelen-Eigles *et al.*, 2006). Schonhof *et al.* (2007) propose that the greater production of glucosinolate under long-day conditions may be due to the fact that the first committed step in glucosinolate biosynthesis is catalysed by a light-dependent cytochrome P450 monooxygenase. Both biotic and abiotic stressors induce glucosinolate accumulation. Insect herbivory and mechanical wounding can induce 100 % increases in leaf glucosinolate content (Travers-Martin & Müller, 2007). Heat and cold stress induce glucosinolate accumulation and there is a strong correlation between concentrations of alkyl glucosinolate and the plant stress indicator proline in broccoli exposed to varying temperature (Schonhof *et al.*, 2007).

#### 1.9.5.2 Factors affecting the efficiency of glucosinolate hydrolysis

Measurement of isothiocyanate concentrations in soil revealed that the proportion of potentially isothiocyanate producing glucosinolate that is actually converted to isothiocyanate is typically less than 5 % (Morra & Kirkegaard, 2002). Since the substrate and enzyme are separately sequestered, tissue disruption is a key determinant of the rate of hydrolysis (Matthiessen & Kirkegaard, 2006). Freezing and thawing *B. juncea* leaf disks prior to incorporation into soil resulted in approximately 400-fold greater peak isothiocyanate concentrations than generated with untreated leaf disks (Morra & Kirkegaard, 2002). Incorporation into waterlogged soil resulted in doubling of peak isothiocyanate concentrations relative to moist soil for both frozen and untreated leaf disks (Morra & Kirkegaard, 2002).

### 1.9.5.3 Losses of isothiocyanate from the soil

Frick *et al.*, (1998) quantified the losses to volatilisation, adsorption and leaching from sterile soil columns injected with methyl isothiocyanate and inferred the losses to degradation from the mass balance. Measurements were made using four soils under three watering regimes. Losses to volatilisation varied greatly, from less than 0.1 % under a constant 25 mm water per day to 30 % when initial watering was delayed by 35 days, out of a total of 52 days. Volatilisation loss was proportional to the air-filled porosity of the soil and addition of water was proposed to reduce volatilisation loss by inhibiting gas-phase diffusion and by reducing the methyl isothiocyanate pool due to leaching (Frick *et al.*, 1998). Losses to leaching were much greater with more frequent watering; 70 % under 25 mm water per day compared with 40 % under 25 mm water every four days (Frick *et al.*, 1998). Despite the low aqueous solubility of isothiocyanates (Holst & Williamson, 2004), their principal route of transport is in the water phase, so long as there is adequate percolation (Frick *et al.*, 1998).

The strength of sorption of a compound onto clay minerals and organic matter is inversely proportional to its aqueous solubility (Bailey & White, 1964). Glucosinolates are highly soluble and only very weakly sorbed by the key soil components aluminium hydroxide ( $\text{Al}(\text{OH})_3$ ), goethite ( $\alpha\text{-FeOOH}$ ), kaolinite, montmorillonite and humic acid (Gimsing *et al.*, 2007b). Isothiocyanates are hydrophobic (Cooper *et al.*, 1997) and are therefore predicted to be strongly sorbed. Frick *et al.* (1998) calculated losses of methyl isothiocyanate to sorption to be very low, less than half a percent. However, this estimate was based on the amount of methyl isothiocyanate that could be extracted and it is likely that the compound was irreversibly bound to soil solids. Smelt & Leistra (1974) estimated the extent of sorption of methyl isothiocyanate by determining the headspace concentration over wetted soil in sealed vials, calculating the aqueous concentration from the water-air partition coefficient and calculating the sorbed fraction by subtraction. Smelt & Leistra (1974) found methyl isothiocyanate to be strongly sorbed and that the extent of sorption was directly proportional to the organic matter content of the soil.

Methyl isothiocyanate is the most volatile isothiocyanate (Mattheissen & Shackleton, 2005). Less volatile isothiocyanates are even more strongly sorbed, especially at low temperatures. This was illustrated by the experiment of Mattheissen & Shackleton (2005). These authors determined dose-response curves for two relatively volatile aliphatic (methyl and 2-propenyl) and two relatively non-volatile aromatic isothiocyanates (benzyl and 2-phenylethyl) against the soil insect *Naupactus leucoloma*

*in vitro* and in three soils of varying organic matter content at four temperatures (5, 10, 15 and 20°C). In the assays, the test organism was always separated from the isothiocyanate by air, whether or not the compound was in soil. Consistent with the results of other vapour exposure tests, the most volatile compound, methyl isothiocyanate was found to be the most toxic under all conditions. The effect of soil was to exacerbate the lower availability and hence activity of less volatile isothiocyanates. For all four compounds, LD<sub>95</sub> was proportional to soil organic matter content but the effect was much more marked for the less volatile aromatics, especially at lower temperatures (Matthiessen & Shackleton, 2005). For instance, at 20°C the LD<sub>95</sub> of methyl isothiocyanate was 28 times greater in peat (31.6% organic matter) than *in vitro*, whereas for 2-phenylethyl isothiocyanate the difference was a factor of 446. At 5 °C the toxicity reducing effect of soil was even more pronounced, with LD<sub>95</sub> values 49 and over 12,500 times greater in peat than *in vitro* for methyl and 2-phenylethyl isothiocyanate, respectively. Within both the aromatic and aliphatic isothiocyanates, the shorter chain compound was found to be more toxic and less affected by organic matter. The authors propose that sorption by organic matter is more pronounced for aromatic isothiocyanates due to their lower volatility and possibly greater reactivity with organic substrates. They suggest that aliphatic isothiocyanates are more available to act on organisms and thus more effective in soil due to their greater volatility. Matthiessen & Kirkegaard (2006) agree that brassicas producing short-chain aliphatic isothiocyanates, such as the commonly produced 2-propenyl isothiocyanate, are more likely to be cause effective pest suppression under field conditions. This hypothesis may well be true for organisms within the air phase of soil but has yet to be tested on those inhabiting the water, such as nematodes. Given the high water/air partition coefficients of isothiocyanates, it is expected that organisms within the water phase would be exposed to greater quantities of isothiocyanate and that isothiocyanate volatility has less of an influence on toxicity than for organisms within the air phase.

Isothiocyanates in the environment are predominantly degraded by microbial processes, rather than chemicals ones (Warton *et al.*, 2003; Rumberger & Marshner, 2003). Repeated application of the same organic pesticide to the same soil can lead to selection and proliferation of microbial populations adapted to detoxify that xenobiotic or even utilize it as a nutrient or energy source (Matthiessen & Kirkegaard, 2006; Haydock *et al.*, 2006). Isothiocyanates are no exception; they are degraded primarily through biological processes and enhanced biodegradation has been reported to reduce the amount of methyl isothiocyanate produced by a given quantity of metham sodium by over 50 % (Warton *et al.*, 2003). Careful management may be required to

prevent this from occurring, including monitoring of isothiocyanate residence times and alternating biofumigation with other pest control strategies (Matthiessen & Kirkegaard, 2006).

### **1.10 Aims**

The overall aim of this project was to evaluate the potential of biofumigation for control of PCN and *Rhizoctonia solani* in potato cultivation. Brassicas and isothiocyanates were screened for activity towards the target organisms *in vitro*. Brassicas with the greatest activity *in vitro* were selected and the effects of green manures of these plants on the viability of the target organisms in soil were quantified. In order to determine whether or not glucosinolates play a role in the observed pest suppression, the glucosinolate profiles of these plants were characterised and the relationship between glucosinolate concentration and pest suppression was analysed. Part of the rationale for biofumigation rather than conventional fumigants is that the former is supposedly less harmful to ecosystems. This assumption was tested by examining the effects of biofumigation on non-target nematodes.



## **Chapter 2: In vitro activity of isothiocyanates and brassica leaf extracts toward *Globodera pallida* second-stage juveniles**

### **2.1 Introduction**

The first aim of this project was to identify brassica species that possess activity against PCN. Since the principal nematicidal components of brassica green manures are thought to be isothiocyanates (Buskov *et al.*, 2002; Lazzeri *et al.*, 2004b) and since there is a great variety of brassicas producing different isothiocyanates, often with multiple compounds produced by the same plant (Fahey *et al.*, 2001; Bellostas *et al.*, 2007; Padilla *et al.*, 2007), it was decided to first determine the toxicity of pure isothiocyanates. The range of brassicas to be screened would be narrowed if certain compounds were particularly toxic. Isothiocyanates were selected (Table 2.1) to maximise coverage of the structural classes defined by Fahey *et al.* (2001), on the assumption that compounds within a class would be more likely to share similar physico-chemical and biological properties than compounds from different classes. A toxicity assay was developed and the toxicity of several isothiocyanates to *Globodera pallida* second-stage juveniles was determined. Based on the results of these assays, brassicas were selected and extracts of their leaves were screened for toxicity using the same assay. The total glucosinolate concentration of the leaves was quantified in order to determine the relationship between any anti-nematode activity and glucosinolate.

#### **2.1.1 Toxicity assays with nematodes**

The toxicity of a compound to an organism can be described by the dose-response curve, the relationship between the concentration to which a population is exposed and the proportion of the population responding. The response under consideration is either mortality or a sub-lethal effect, such as paralysis. Due to the shape of the typical dose-response curve, the greatest change in response for a given change in concentration is at the point where 50 % of the population is affected. Given that there will be sampling error in determining the proportion responding, the concentration on the dose-response curve that can be most accurately defined is that required to elicit a response in 50 % of the population. This is the classical measure of toxicity, the effective dose for 50 % of the population (ED<sub>50</sub>). As a measure of the potency of a pesticide ED<sub>50</sub> is entirely suitable.

**Table 2.1:** Structural classes of isothiocyanates tested.

Structural class of parent glucosinolate <sup>1</sup>		Isothiocyanate tested
A	Aliphatic, sulphur containing	3-(methylthio)propyl
B	Aliphatic, straight chain	Propyl
C	Aliphatic, branched chain	2-methylbutyl
D	Olefins	2-propenyl
E	Alcohols	$\beta$ -hydroxy-isothiocyanates are highly labile (Halkier & Gershenzon, 2006)
F	Ketones	Not commercially available
G	Aromatics	Benzyl and 2-phenylethyl
H	Benzoates	Not commercially available
I	Indoles	Indole isothiocyanates are highly labile (Holst & Williamson, 2004)
J	Multiply glycosylated and other	Not commercially available

<sup>1</sup> According to Fahey *et al.* (2001)

Methods to study the effects of toxins on nematodes can measure mortality (Lazzeri *et al.*, 1993; McLeod & Steel, 1999; Buskov *et al.*, 2002; Serra *et al.*, 2002) or inhibition of particular functions, such as chemoreception (Bargmann *et al.*, 1993), motility (Thomas & Lockery, 1991; Schroeder, 2007) or hatching (Twomey *et al.*, 2000). Interference with chemoreception can be measured by observing the proportion of a population that moves towards an attractant compound after exposure to the toxin (Bargmann *et al.*, 1993). Interference with locomotion can be measured by placing nematodes in the centre of an agar plate and measuring distance from the origin (Thomas & Lockery, 1991) or total distance moved (Schroeder, 2007) after a given period or by scoring the frequency of sinusoidal body movements (Zasada *et al.*, 2009). The proportion of nematodes able to move through a column of sand can also be used to assess motility (Zasada & Ferris, 2003).

The majority of published reports of the toxicity of glucosinolate hydrolysis products to plant-parasitic nematodes have measured mortality, employing the following method. Free-swimming second-stage juveniles (J2) are exposed to a test compound in Petri dishes or a similar container and the proportion of dead individuals is monitored after a given exposure period. Individuals are considered dead if they fail to respond to gentle heating under an incandescent light bulb and mechanical stimulation with a pin or if they fail to revive after transferral to distilled water (Lazzeri *et al.*, 1993; McLeod & Steel, 1999; Buskov *et al.*, 2002; Serra *et al.*, 2002). In an alternative method,

nematodes are exposed to a test compound in sealed containers with soil or sand then the containers are opened and motile individuals are allowed to escape through mesh and collected for counting (Zasada & Ferris, 2003).

### 2.1.2 Aims

1. To develop an assay to quantify the effects of toxins on motility of *G. pallida* J2.
2. To determine the ED<sub>50</sub> of isothiocyanates to *G. pallida* J2.
3. To identify brassica species with activity towards *G. pallida* J2.
4. To identify from the literature and validate an assay for quantifying glucosinolate.
5. To determine the relationship between brassica glucosinolate content and activity towards *G. pallida* J2.

## 2.2 Methods

### 2.2.1 Culture and hatching of *Globodera pallida*

#### 2.2.1.1 Culture of *Globodera pallida*

Soil infested with *G. pallida* Pa2/3 at 290 eggs g<sup>-1</sup> soil was diluted with loam soil (Norfolk loam, Bailey's of Norfolk) to give a density of 20 eggs g<sup>-1</sup> soil. Nine kg was dispensed into each of 40 10" pots and a single seed tuber (*Solanum tuberosum* L. var. Desiree, grade E2) was placed into each pot. The pots were placed outdoors on trays to prevent infection of the surrounding plot at Grove House Gardens Experimental Station, University of Leeds. The potatoes were grown from April until September when haulms were removed and the soil was spread out on trays in a glasshouse to dry for two weeks. *G. pallida* cysts were extracted by floatation and separated from organic material by rolling on an inclined metal tray. Cysts were allowed to dry at room temperature then stored at 4 °C.

#### 2.2.1.2 Collection of potato root diffusate

Tubers of *Solanum tuberosum* cv. Desirée were grown in 5" diameter pots containing perlite. When the plants were 27-48 days old, 50-100 ml tap water was passed through the pots each day and collected. This potato root diffusate (PRD) was stored in plastic bottles at 4°C in the dark for no longer than nine months then filter sterilised and diluted four times in sterile tap water before use.

#### 2.2.1.3 Preparation of *Globodera pallida* second-stage juveniles

Cysts of *G. pallida* Pa2/3 were surface-sterilised according to the method of Urwin *et al.* (1997). Cysts were then rinsed with sterile tap water in a side-arm conical flask and incubated in the dark at 20 °C in PRD to stimulate hatch. Second-stage juveniles (J2) were collected every two days for use in toxicity assays. The number of J2 in 50 µl aliquots were counted in a Pieters counting slide under a microscope (Heerbrugg Wild) at 25x magnification and the suspension was diluted to approximately 2.5 J2 µl<sup>-1</sup> and the J2 in five 50 µl aliquots were counted to obtain an accurate estimate of density.

## **2.2.2 Development of an assay to determine the toxicity of substances to *Globodera pallida***

The initial experimental design was based on that of Zasada & Ferris (2003), in which exposure to isothiocyanate and separation of motile and immotile individuals were carried out in sand in one container. Several preliminary experiments were carried out to develop the protocol.

### ***2.2.2.1 Comparison of two sizes of experimental chamber***

Initially, two different sizes of experimental chamber following the same design were tested in order to determine which gave the greatest recovery rate of nematodes. The ends were removed from 2 ml or 20 ml plastic syringes (Terumo, UK) and 50 µm pore diameter nylon mesh (John Stanier Ltd., UK) was attached by melting the cut ends on a hot plate and pressing onto the mesh. Then, 0.7 or 2.8 g autoclaved silica sand (BDH Laboratory Supplies, UK) were added to the 2 ml or 20 ml tubes. The tubes were placed into 15 ml or 50 ml centrifuge tubes (Falcon, UK) and autoclaved tap water was added until the surface of the water touched the sand and water moved to the surface of the sand by capillary action. Approximately 300 or 600 J2 were inoculated onto 16 or 100 mm<sup>2</sup> squares of GF/A filter paper (Whatman, UK) in 10 µl or 20 µl potato root diffusate, for the smaller and larger set-ups, respectively. The filter papers were placed onto the sand and the tops of the tubes were sealed with laboratory film (Pechiny Plastic Packaging, Chicago, USA). The tubes were incubated in the dark at room temperature for 72 h. After 72 h, the water in the centrifuge tubes was transferred to a Pieters counting slide and J2 were counted under a microscope at 25x magnification. There were five replicates of each set-up.

### ***2.2.2.2 Comparison of two methods for attaching nylon mesh to polypropylene cylinders to make sand columns***

The protocol was the same as for the larger experimental set-up described in 2.2.2.1 except for the following: there were 250 J2 per sample; nylon mesh was attached to half of the syringes by melting as before and to the other half using Araldite Precision Glue (Bostik, UK); tubes were incubated at 20 °C for 48 h. There were six replicates.

### *2.2.2.3 Determination of a non-lethal concentration of methanol*

A test was carried out to establish whether or not methanol is toxic to J2 at the concentration that would be used to deliver isothiocyanate (0.75 %). The protocol was the same as described in 2.2.2.2 using the melting method except for the following: the mesh covered ends of the inner tubes were sealed with laboratory film (Pechiny Plastic Packaging, Chicago, USA) before being placed into the outer tubes, allowing the volume of water in the sand to be controlled; prior to adding J2, 665  $\mu$ l autoclaved tap water was added to the sand, followed by a further 5  $\mu$ l autoclaved tap water or methanol; after 48 h incubation at 20 °C, the laboratory film was removed from the bottoms of the tubes and the tubes were incubated for a further 38 h for J2 to move through the mesh to be collected and counted.

### *2.2.2.4 Testing the effects of 2-phenylethyl isothiocyanate*

Having established that 0.75 % methanol did not affect nematode recovery rate, a test was carried out to determine the effects of 1 % methanol and of 18 mM 2-phenylethyl isothiocyanate in 1 % methanol. At this stage, only one concentration of one isothiocyanate was tested simply to determine whether or not the assay would detect an effect. The protocol was the same as described in 2.2.2.3 except that 750  $\mu$ l autoclaved tap water was added to the sand, followed by a further 7.6  $\mu$ l autoclaved tap water, methanol or methanol containing 14.4  $\mu$ mol 2-phenylethyl isothiocyanate prior to adding J2 in 40  $\mu$ l potato root diffusate.

### *2.2.2.5 Determination of a dose-dependent effect of 2-phenylethyl isothiocyanate*

Next, several concentrations of 2-phenylethyl isothiocyanate were tested to determine whether or not the assay could detect a dose-dependent effect. The protocol was the same as described in 2.2.2.4 except that sufficient amounts of 2-phenylethyl isothiocyanate were added in 7.5  $\mu$ l methanol to achieve final concentrations of 0, 6, 18 or 54 mM 2-phenylethyl isothiocyanate.

### *2.2.2.6 Comparison of the method described in 2.2.2.1 with a new method*

The initial toxicity assay protocol described in section 2.2.2.1 was flawed (see Results section 2.3.1.5). A novel method was developed, in which J2 were exposed to isothiocyanate in micro-centrifuge tubes then transferred to sand columns for

separation of motile and immotile individuals. The original experimental design was repeated as described in 2.2.2.3 and compared with the new method, which was as follows. Approximately 250 J2 in 50  $\mu$ l potato root diffusate were added to 1.5 ml micro-centrifuge tubes (Maxymum Recovery, Axygen) containing 950  $\mu$ l autoclaved tap water. Then 7.5  $\mu$ l autoclaved tap water, methanol or methanol containing 18  $\mu$ mol 2-phenylethyl isothiocyanate was added. The tubes were incubated in the dark at 20 °C for 48 h. To separate motile and immotile nematodes, the contents of the tubes were carefully pipetted onto the surface of sand in sand columns. Sand columns consisted of 18 mm deep x 10 mm internal diameter polypropylene cylinders sealed at the bottom with 50  $\mu$ m pore size nylon mesh (John Stanier Ltd., UK) containing 0.7 g autoclaved silica sand (BDH Lab Supplies). Prior to addition of J2, sand columns were placed into microtiter plate (Cellstar, Greiner Bio-One) wells containing 400  $\mu$ l sterile tap water. After addition of J2, microtiter plates were incubated in the dark at 20 °C for 62 h. Sand columns were gently removed and J2 were counted directly in the wells under a microscope at 25x magnification.

#### *2.2.2.7 Determination of a dose-dependent effect of 2-phenylethyl isothiocyanate using the method described in 2.2.2.6*

A test was performed to determine whether or not the method described in 2.2.2.6 could distinguish the effects of different concentrations of isothiocyanate. The protocol was as described in 2.2.2.6 except that the final concentrations of 2-phenylethyl isothiocyanate were 20 and 200  $\mu$ M. There were five replicates of each concentration and no controls were performed due to a temporary shortage of J2.

#### *2.2.2.8 Optimisation of the assay protocol described in 2.2.2.6*

The effects on J2 recovery rate of reducing the depth of sand in sand columns or adding potato root diffusate to microtiter plate wells as an attractant were tested. The protocol was as described in 2.2.2.6 except for the following: sand columns contained either 0.3 or 0.7 g sand; microtiter plate wells contained either 500  $\mu$ l sterile tap water or 500  $\mu$ l potato root diffusate.

### 2.2.3 Toxicity of isothiocyanates to *Globodera pallida* second stage juveniles

#### 2.2.3.1 The finalised toxicity assay protocol

Suspensions of 90-110  $\mu\text{l}$  containing  $250 \pm 22$  (SD) J2 were transferred to 1.5 ml micro-centrifuge tubes (Maximum Recovery, Axygen) and sterile tap water was added to a total volume of 1.00 ml. Isothiocyanate dilution series were prepared in methanol so that the required amounts were delivered to the tubes containing the J2 in 7.5  $\mu\text{l}$ . Control treatments received 7.5  $\mu\text{l}$  methanol alone. J2 were exposed to isothiocyanates or methanol for 24 h in the dark in a rotary incubator at 19 °C and 200 rpm. After the 24 h exposure period, J2 were transferred to sand columns for separation of motile and immotile individuals. Sand columns consisted of 14 mm deep x 10 mm internal diameter polypropylene cylinders sealed at the bottom with 100  $\mu\text{m}$  aperture diameter nylon mesh containing  $0.30 \pm 0.02$  g autoclaved silica sand of particle diameter 212 – 300  $\mu\text{m}$  (BDH Laboratory Supplies, 330945E). Prior to addition of J2, sand columns were placed into microtiter plate (Cellstar, Greiner Bio-One, 677180) wells containing 600  $\mu\text{l}$  sterile tap water. After addition of J2, microtiter plates were incubated for 48 h at 20 °C in the dark. Motile J2 that accumulated in the microtiter plate wells were counted on a Pieters counting slide under a dissecting microscope at 25x magnification.

#### 2.2.3.2 Approximation of the dose-response curves for six isothiocyanates to *Globodera pallida* J2

The finalised protocol (2.2.1) was used to determine the dose-response curves for benzyl, 2-phenylethyl, 2-propenyl, 2-methylbutyl and 3-(methylthio)propyl isothiocyanate. Preliminary experiments with at least three concentrations of each isothiocyanate were used to approximate dose-response curves. To verify reproducibility, 2-phenylethyl isothiocyanate was included in each preliminary experiment. Dose-response curves were fitted to the data using Probit analysis in SPSS 16.0. For each experiment, the mean number of J2 recovered in controls was calculated. This value was taken to be 100% recovery and was used as the “total” parameter in Probit analysis. For each sample, the number of individuals responding to treatment was calculated by subtracting the number of individuals recovered in that sample from the “total” value. In other words, the individuals responding to treatment were those that were not recovered from the sand columns but that were predicted to be recovered had they been exposed to methanol alone. This is equivalent to using Abbott’s formula for correcting mortality (Abbott 1925).



### 2.2.3.3 Accurate determination of the dose-response curves for six isothiocyanates to *Globodera pallida* J2

Using the dose-response curves approximated in 2.2.2, the lower 95 % confidence interval of the ED<sub>20</sub> and the upper 95 % confidence interval of the ED<sub>80</sub> were determined for each isothiocyanate. In order to accurately define dose-response curves within the range 20 to 80 % suppression, each compound was tested in a two-fold dilution series over a range of concentrations that encompassed the 95 % confidence intervals of the approximated ED<sub>20</sub> and ED<sub>80</sub>, with five replicates at each concentration. In order to facilitate statistical comparison, the different compounds were tested at the same concentrations when possible. Hence, 2-phenylethyl and benzyl isothiocyanate were tested at 0.5, 1, 2, 4, 8, 16 and 32 µM, 2-propenyl isothiocyanate was tested at 0.5, 1, 2, 4, 8, 16, 32 and 64 µM, 3-(methylthio)propyl isothiocyanate was tested at 1, 2, 4, 8, 16, 32 and 64 µM, propyl isothiocyanate was tested at 2, 4, 8, 16, 32, 64 and 128 µM and 2-methylbutyl isothiocyanate was tested at 4, 8, 16, 32, 64 and 128 µM.

## 2.2.4 Toxicity of brassica leaf extracts to *Globodera pallida*

### 2.2.4.1 Selection of species/cultivars for screening

Seed samples of over fifty brassica species were obtained, including several cultivars marketed as biofumigant crops or known to have high levels of effective glucosinolates and a subset of 22 cultivars was selected for screening, in addition to the non-brassica, *Triticum aestivum* (Table 2.1). These plants were selected based on the results of toxicity assays with pure isothiocyanates and their glucosinolate profiles known from the literature. Some of the lines tested were known to contain very low levels of glucosinolate and were included as controls.

### 2.2.4.2 Plant growth conditions

Ten individuals of each of the 23 plants (Table 2.2) were grown in a glasshouse (25-30 °C) in a mixture of three parts peat-based compost (Sinclair Horticultural Ltd.) to one part horticultural grade sharp sand (J. Arther Bowers) in 90 mm diameter pots with 16 h/d illumination from a 400 W bulb (Hortilux Schreder) approximately 1.5 m above bench surface (150-250 moles photons s<sup>-1</sup> m<sup>-2</sup> at top of foliage). The soil was kept moist by watering.

#### 2.2.4.3 Preparation of freeze-dried brassica leaf material

Leaves were harvested when plants were at the initiation of flowering or when they were eight weeks old if inflorescences had not already begun to develop. All the leaves from at least five plants were immediately frozen in liquid nitrogen then ground to a powder with a pestle and mortar. The powdered tissue was transferred to a pre-cooled 50 ml tube (Greiner, 227261) and freeze-dried in a vacuum chamber attached to a high vacuum pump (Edwards) and condenser (Modulyo, Edwards). The pressure inside the vacuum chamber was <3 mbar and the temperature of the condenser was -45 °C. Samples were freeze-dried for three to four days and dryness was verified by weighing sub-samples before and after incubation at 65 °C. Freeze-dried material was stored at -20 °C in 30 mg aliquots in 500 µl micro-centrifuge tubes.

#### 2.2.4.4 Preparation of leaf tissue extracts for use in toxicity assays

Extracts were prepared immediately prior to each assay by placing 30 mg freeze-dried leaf tissue in a 15 ml centrifuge tube with 15 ml sterile tap water and incubating for 200 min in a rotary incubator at 37 °C and 200 rpm. The extract was then passed through a 0.20 µm filter (Sartorius Stedim, 16532-K) to remove insoluble debris and micro-organisms.

#### 2.2.4.5 Determination of the toxicity of brassica leaf extracts to *Globodera pallida* J2

Suspensions of 90-110 µl containing  $250 \pm 22$  (SD) J2s were transferred to 1.5 ml micro-centrifuge tubes (Maxymum Recovery, Axygen). Nine volumes of leaf extract were added so that the final concentration was equivalent to 1.8 mg dry leaf tissue ml<sup>-1</sup>. Incubation with the test substance and measurement of J2 motility were carried out as described for pure isothiocyanates (section 2.2.1). On each experimental occasion, four randomly selected leaf extracts were tested with seven replicates of each, as well as ten replicates of a water control and ten replicates of *B. juncea* cv. Nemfix, used as a reference control to verify reproducibility between experimental occasions. Each extract was tested on at least three such occasions so that there was a total of twenty-one replicates with each extract. Numbers of J2 recovered through sand columns were expressed as a proportion of the mean recovery from controls performed on the same experimental occasion. Proportion data were arcsin square-root transformed and analysed by two-way analysis of variance with treatment and experimental block as fixed factors and Games Howell post-hoc test using SPSS 16.0 (IBM, Somers, NY, USA).

**Table 2.2:** Plants screened for efficacy against *Globodera pallida* J2.

Plant	Source	Rationale for selection
<i>Brassica juncea</i> cv. Nemfix	Dr. John Kirkegaard, CSIRO, Australia	Commercial biofumigant. Contains high levels of 2-propenyl glucosinolate (Gimsing & Kirkegaard, 2006).
<i>Brassica juncea</i> cv. Fumus	Dr. John Kirkegaard, CSIRO, Australia	Commercial biofumigant. Contains 2-propenyl glucosinolate (Kirkegaard, pers. comm.).
<i>Brassica juncea</i> cv. Arid	Dr. John Kirkegaard, CSIRO, Australia	Contains 2-propenyl and 3-butenyl GST at low levels (Kirkegaard, pers. comm.). Included as a control.
<i>Brassica napus</i> cv. Nemcon	Dr. John Kirkegaard, CSIRO, Australia	Commercial biofumigant. Implied anti-nematode activity is unsubstantiated (Kirkegaard, pers. comm.).
<i>Brassica napus</i> cv. Maxima Plus	Dr. John Kirkegaard, CSIRO, Australia	Contains low levels of glucosinolate (Kirkegaard, pers. comm.). Included as a control.
<i>Raphanus sativus</i> cv. Weedcheck	Dr. John Kirkegaard, CSIRO, Australia	Developed as a trap crop for <i>Heterodera schachtii</i> and has useful agronomic properties: large seed, resistance to insects and general vigour (Kirkegaard, pers. comm.).
<i>Brassica carinata</i>	Joordens seeds, Denmark	Commercial biofumigant. Contains 2-propenyl glucosinolate (Bellostas <i>et al.</i> , 2007).
<i>Brassica juncea</i> high GST	Joordens seeds, Denmark	Commercial biofumigant. Contains 2-propenyl glucosinolate (Bellostas <i>et al.</i> , 2007).
<i>Eruca sativa</i> , #9	Joordens seeds, Denmark	Commercial biofumigant. Contains 3-(methylthio)-propyl and 4-(methylthio)butyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Eruca sativa</i> , #8	Joordens seeds, Denmark	Contains low levels of glucosinolate (Dr. P. Jongelengen, pers. comm.). Included as a control.
<i>Eruca sativa</i> cv. Nemat	Dr. Ekaterini Riga, Washington State University, USA	Commercial biofumigant. Poor host for <i>Meloidogyne hapla</i> (Melakeberhan <i>et al.</i> , 2006). Contains 3-(methylthio)propyl and 4-(methylthio)butyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Brassica rapa</i> cv. 132	Dr. Maria Cartea, CSIC, Spain	Contains high levels of 3-butenyl glucosinolate (Carrea <i>et al.</i> , 2007).
<i>Nasturtium officinale</i>	Jardin Botanique de Caen	Contains 2-phenethyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Moricandia moricandioides</i>	National Botanical Garden of Belgium	Contains 2(R)-2-hydroxy-3-butenyl glucosinolate (Fahey <i>et al.</i> , 2001). Chosen as a substitute for <i>M. arvensis</i> , which did not germinate but which contains benzyl and phenethyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Sisymbrium austriacum</i>	National Botanical Garden of Belgium	Contains 3-(methylthio)propyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Sinapis alba</i>	Botanische Garten der Universitat Bonn	Contains benzyl glucosinolate (Fahey <i>et al.</i> , 2002).
<i>Cardaria draba</i>	Jardin Botanique de Caen	Contains 2-propenyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Lepidium latifolium</i> IS21	Jardin Botanique de Bordeaux	Contains benzyl and 3-(methylthio)propyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Lepidium latifolium</i> IS22	Jardin Botanique de Caen	
<i>Lepidium sativum</i>	National Botanical Garden of Belgium	Contains 2-phenethyl and 2-propenyl glucosinolate (Fahey <i>et al.</i> , 2001).
<i>Barbarea vulgaris</i>	Jardin Botanique de Bordeaux	Contains 2-phenethyl and 3-(methylthio)propyl glucosinolate, as well as several indole glucosinolates (Fahey <i>et al.</i> , 2001).
<i>Hesperis matronalis</i>	Humboldt-Universitat zu Berlin	Contains a wide variety of glucosinolates, including several unusual multiply glycosylated forms (Fahey <i>et al.</i> , 2001; Larsen <i>et al.</i> , 1992).
<i>Triticum aestivum</i>	RAGT Seeds, UK	Spring wheat was included as a non-brassica control as it is grown preceding potatoes in rotations.

## 2.2.5 Quantification of total glucosinolate content of leaves

### 2.2.5.1 Extraction of glucosinolate

The freeze-dried leaf material used in glucosinolate analysis was from the same stock as that used in the toxicity assays described above. Glucosinolates were extracted in phosphoric acid, following a modified version of the method of Van Doorn *et al.* (1998). For each cultivar, 0.50 g freeze-dried powdered leaf tissue was suspended in 20 ml 2% phosphoric acid and incubated overnight in a shaking incubator (New Brunswick Scientific, Excella E25) at 28 °C and 200 rpm. The suspension was passed through a Miracloth filter (Calbiochem, 475855) into a 15 ml centrifuge tube (Greiner, 188271). The filtrate was centrifuged at 2,547 rcf for 20 min at 21 °C in a Hermle Z400K refrigerated centrifuge. 12 ml of the supernatant was decanted into a fresh 15 ml centrifuge tube and neutralised to pH 7.00 ± 0.04 (SD) by addition of 1.7 ml 5 M KOH. The sample was centrifuged at 2,547 rcf for 10 min at 4 °C to remove precipitates. The supernatant was transferred to a fresh 15 ml centrifuge tube and heated in a boiling water bath for 15 min to denature any dissolved proteins, particularly myrosinase. The sample was cooled to room temperature then centrifuged at 2,547 rcf for a further 10 min at 4 °C to remove precipitates. The clear supernatant was transferred to a fresh 15 ml tube and stored in the dark at -20 °C until use.

### 2.2.5.2 Quantification of total glucosinolate

Glucosinolate was quantified using a modified version of the method of Van Doorn *et al.* (1999), in which free glucose was degraded enzymatically, glucosinolate was hydrolysed with myrosinase and the released glucose was quantified in a colorimetric assay. Triplicate samples from several of the plants were spiked with sodium phosphate, pH 7, containing various concentrations of 2-propenyl glucosinolate (Sigma, 85440-250MG) to give final concentrations of exogenous 2-propenyl glucosinolate in the samples of 0, 0.5, 1.0 or 2.0 mM. These spiked samples would form a standard curve to relate the optical density from the colorimetric assay to glucosinolate concentration.

In a 1.5 ml micro-centrifuge tube (Sarstedt, D-51588), 285 µl glucose oxidase solution (MP Biomedicals, 0219519610) (103 U ml<sup>-1</sup>) was mixed with 190 µl glucosinolate sample and incubated for four hours in the dark at room temperature to remove free glucose. After the glucose oxidase incubation, the samples were heated in a heat block at 100 °C for 20 min to destroy glucose oxidase activity and degrade the hydrogen peroxide formed. The samples were allowed to cool to room temperature (~23 °C)

before 25  $\mu\text{l}$  myrosinase (Sigma, T4528-25UN) ( $1.33 \text{ U ml}^{-1}$  in 100 mM sodium phosphate, pH 7) or 100 mM sodium phosphate, pH 7 alone was mixed into the samples. The samples were incubated at 25 °C for 20 hours to allow glucosinolate hydrolysis. For the colorimetric assay, 500  $\mu\text{l}$  glucose assay mixture containing 100 mM sodium phosphate, pH 7, 156  $\text{ng ml}^{-1}$  4-aminoantipyrine (Sigma, A4382), 5  $\text{U ml}^{-1}$  peroxidase (VWR International, 1.16216.0001), 65  $\text{U ml}^{-1}$  glucose oxidase (MP Biomedicals, 0219519610) and 1  $\text{mg ml}^{-1}$  phenol (Sigma, P1037-25G) was added to each sample. Samples were incubated in the dark at 25 °C for one hour before the absorbance of light of wavelength 520 nm was measured using a Jenway 6715 UV/Vis. spectrophotometer.

For each extract, the mean optical density of three myrosinase-free controls was subtracted from the mean value of three test samples with myrosinase. The concentration of glucosinolate in the sample was calculated from its optical density using the standard curve. Leaf glucosinolate concentration was calculated from the sample glucosinolate concentration considering the amount of leaf material per sample.

#### *2.2.5.3 Comparison of the glucose quantification method and HPLC for measuring glucosinolate*

When glucosinolate profiles of ten brassicas were determined by HPLC (section 3.2.3), the opportunity was taken to check the accuracy of the glucose assay method. The same material used in HPLC was tested with the method outlined above. Measurements according to each method were plotted on a graph and the relationship analysed by linear regression in SPSS 16.0.

## 2.3 Results

### 2.3.1 Development of an assay to determine the toxicity of substances to *Globodera pallida*

Several experiments were carried out to develop an initial experimental design based on that of Zasada & Ferris (2004), in which J2 were exposed to isothiocyanate in sand and then motile individuals were extracted and counted (see methods section 2.2.2).

#### 2.3.1.1 Comparison of two sizes of experimental chamber

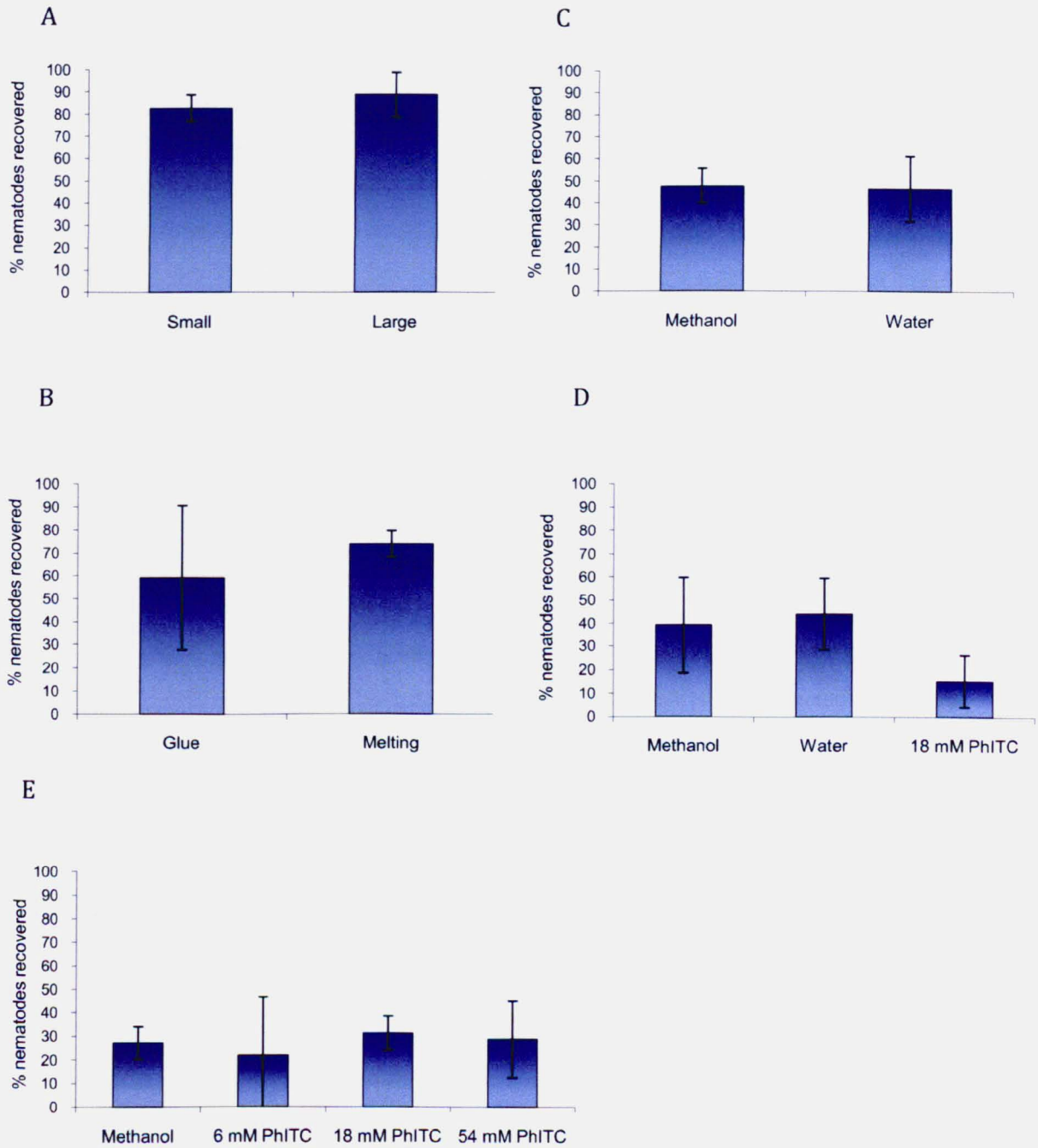
A comparison between larger and smaller experimental chambers showed no significant effect of chamber size on J2 recovery rate (t-test,  $t = -1.23$ ,  $P = 0.262$ ) (Figure 2.1A). The larger set-up was chosen, as it was more practical to use.

#### 2.3.1.2 Comparison of two methods for attaching nylon mesh to polypropylene cylinders to make sand columns

Initially, it was difficult to get an uninterrupted seal between the mesh and syringe end by melting the syringe end. This allowed sand to escape and interfered with counting J2. Glue consistently provided an uninterrupted seal. The effects of gluing and melting were compared in case the glue was toxic to nematodes. Fewer J2 were recovered from tubes made with glue and the variance was greater (Figure 2.1B), although the difference between the means was not significant (t-test,  $t = 1.13$ ,  $P = 0.307$ ). The melting method was retained and in further experiments, only containers with a good seal between mesh and syringe were used.

#### 2.3.1.3 Determination of a non-lethal concentration of methanol

Since isothiocyanates are poorly soluble in water, dilution series were prepared in methanol. An experiment was carried out to test if 0.75 % methanol interfered with J2 locomotion. No significant difference in J2 recovery rate was detected between water and methanol treatments (t-test,  $t = 0.246$ ,  $P = 0.812$ ) (Figure 2.1C).



**Figure 2.1:** Percentage recovery of *Globodera pallida* second stage juveniles after treatment with water, 1 % methanol or 2-phenylethyl isothiocyanate using a modified version of the protocol of Zasada & Ferris (2003). **A)** nematodes mock treated with water in larger or smaller experimental containers; **B)** nematodes mock treated with water in experimental containers sealed with glue or by melting plastic; **C)** nematodes treated with 0.75 % methanol or water; **D)** nematodes treated with 18 mM 2-phenylethyl isothiocyanate in 1 % methanol, 1 % methanol alone or water; **E)** nematodes treated with a range of concentrations of 2-phenylethyl isothiocyanate in 1 % methanol or methanol alone. Error bars show SEM. N ≤ 5.

#### 2.3.1.4 Testing the effects of 2-phenylethyl isothiocyanate

Next, a test was carried out to determine whether or not the assay would detect the effects of a putatively lethal concentration of isothiocyanate. Buskov *et al.* (2002) found that 2.4 mM 2-phenylethyl glucosinolate with myrosinase caused 100 % mortality to *G. rostochiensis* J2 after 16 h exposure and that the hydrolysis product of 2-phenylethyl glucosinolate was the corresponding isothiocyanate. Thus, 18 mM 2-phenylethyl isothiocyanate for 24 hours was expected to be more than sufficient to cause 100 % inhibition of *G. pallida* J2. Contrary to this prediction, 15 % of nematodes were recovered after this treatment (Figure 2.1D). This was significantly less than from the methanol controls (Mann-Whitney U test,  $U = 2.00$ ,  $P = 0.010$ ).

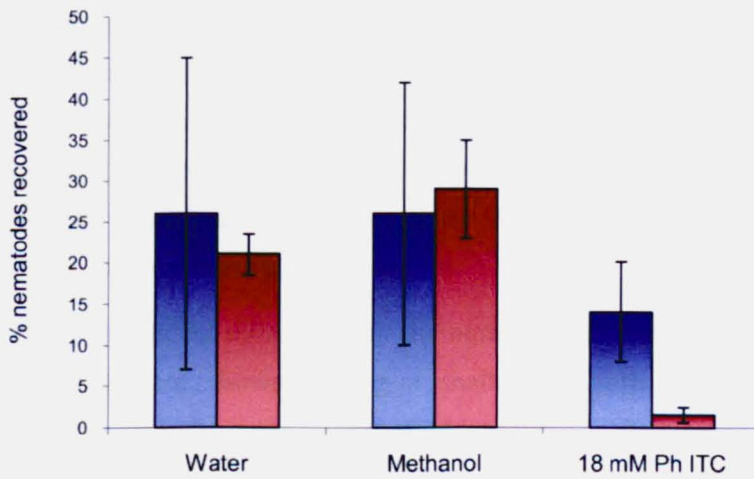
#### 2.3.1.5 Determination of a dose-dependent effect of 2-phenylethyl isothiocyanate

A range of concentrations of 2-phenylethyl isothiocyanate was tested to determine whether or not the assay would detect a dose-response relationship. Isothiocyanate concentration had no significant effect on J2 recovery rate (Figure 2.1E) (ANOVA,  $F = 0.336$ ,  $P = 0.800$ ). Despite the fact that the previous experiment had detected significant suppression by 18 mM 2-phenylethyl isothiocyanate, in this experiment, no significant reduction in J2 recovery was observed. It was observed that nematodes collected after isothiocyanate treatments were immotile whilst those from controls were moving normally. Thus, it seemed that immotile individuals were being recovered from the experimental chambers and counted as motile.

#### 2.3.1.6 Comparison of the method described in 2.2.2.1 with a new method

The initial toxicity assay protocol (see section 2.2.2.1) was repeated alongside a novel protocol, in which isothiocyanate exposure and separation of motile and immotile nematodes were carried out in separate containers (see section 2.2.2.6). On this occasion, the protocol described in section 2.2.2.1 did detect a reduction in J2 recovery rate after treatment with 18 mM 2-phenylethyl isothiocyanate (Figure 2.2). However, the variance was high and this difference was not significant (Kruskal-Wallis test,  $\chi^2 = 2.257$ ,  $P = 0.323$ ). With the new microtiter plate protocol (see section 2.1.3.1), the recovery rate after treatment 18 mM 2-phenylethyl isothiocyanate was less than 2 % and this was significantly less than from controls (Kruskal-Wallis test,  $\chi^2 = 18.5$ ,  $P < 0.001$ ) (Figure 2.2).





**Figure 2.2:** Comparison of two protocols for determining the effects of isothiocyanates on motility of *Globodera pallida* second stage juveniles. A modified version of the protocol of Zasada & Ferris (2003) (blue columns) was compared with a novel protocol (red columns) in which nematodes were exposed to compounds in micro-centrifuge tubes and then transferred to sand columns for separation of motile and immotile individuals (section 2.2.2.6). Nematodes were exposed to 18 mM 2-phenylethyl isothiocyanate in methanol, methanol alone or water. The methanol concentration was 1 % in the first protocol and 0.75 % in the novel protocol. Error bars show SEM. N = 6.

### *2.3.1.7 Determination of a dose-dependent effect of 2-phenylethyl isothiocyanate using the novel toxicity assay protocol*

Using the novel toxicity assay protocol (section 2.2.2.6), significant differences were observed between the effects of 20 and 200  $\mu\text{M}$  2-phenylethyl isothiocyanate (Mann-Whitney U test,  $U = 2.00$ ,  $P = 0.027$ ) (Figure 2.3). The new experimental design was capable of distinguishing the effects of different concentrations of isothiocyanate.

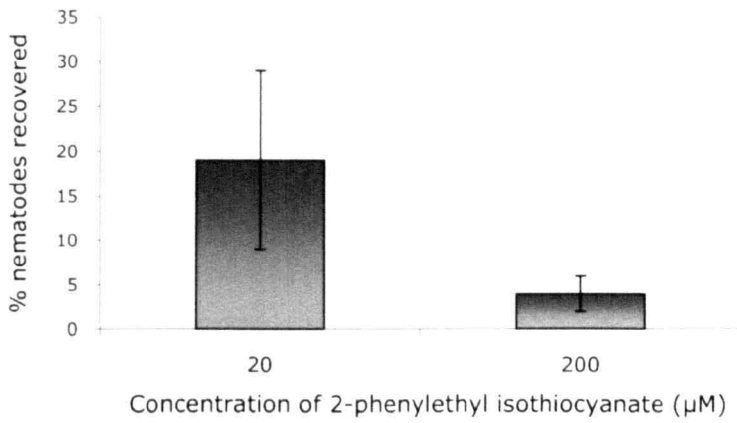
### *2.3.1.8 Optimisation of the toxicity assay protocol described in 2.2.2.6*

Modifications were made with the aim of maximising the number of juveniles recovered from controls in order to minimise the effects of other variables besides isothiocyanate treatment on nematode motility. It was thought that a shallower depth of sand, larger pore sizes between sand particles, larger pore size in nylon mesh, use of younger J2 (24 h hatching period, rather than 48 h), using a more dilute J2 suspension or using potato root diffusate instead of water in sand columns might all increase the rate of recovery and/or decrease the variance.

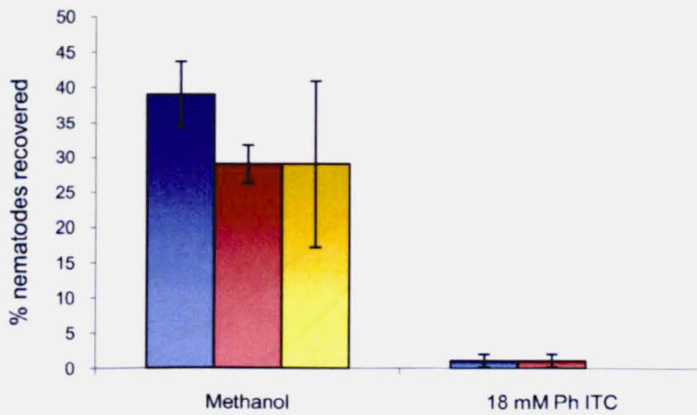
There were no significant differences in the number of J2 from concentrated or dilute suspensions (data not shown). Using a shallower depth of sand was found to significantly increase the proportion of J2 recovered after mock treatment with methanol (Figure 2.4). The shallower depth of sand did not cause any increase in the proportion of J2 recovered after treatment with 18 mM 2-phenylethyl isothiocyanate, indicating that dead or immobilised J2 were unable to pass through. The use of potato root diffusate did not affect mean recovery rate in control treatments but increased the variance (Figure 2.4).

## **2.3.2 Toxicity of isothiocyanates to *Globodera pallida* second stage juveniles**

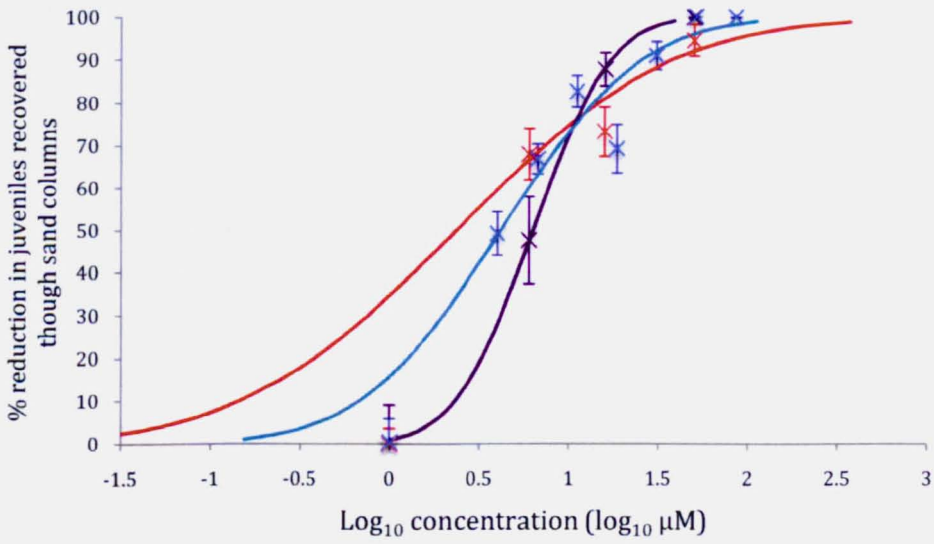
The overall mean J2 recovery rate after mock treatment with methanol was  $124 \pm 36.3$  (SD) out of 250 individuals. The toxicity assay for 2-phenylethyl isothiocyanate was performed three times. The same concentrations of 2-phenylethyl isothiocyanate caused similar degrees of suppression independently of experimental occasion (Figure 2.5), showing that the assay gives reproducible results. The  $\text{ED}_{50}$  value was well defined since most of the experimental data are for concentrations causing between 50 and 100 % inhibition. As with all regression analyses, Probit should not be used to extrapolate dose-response curves beyond the range of experimental data and so the lower part of



**Figure 2.3:** Percentage recovery of *Globodera pallida* second stage juveniles through sand columns after treatment with 20 or 200 μM 2-phenylethyl isothiocyanate in 0.75 % methanol. Error bars show SEM. N = 5.



**Figure 2.4:** Percentage recovery of *Globodera pallida* second stage juveniles through sand columns after treatment with 18 mM 2-phenylethyl isothiocyanate in 0.75 % methanol or 0.75 % methanol alone. The effects of sand depth in sand columns and use of potato root diffusate as an attractant in microtiter plate wells beneath sand columns were determined. Separation of motile and immotile nematodes was carried out in sand columns with 0.3 g sand and water in the microtiter plate wells (blue columns), 0.7 g sand in the columns and water in the wells (red columns) or 0.7 g sand in the columns with potato root diffusate in the wells (yellow columns). Error bars show SEM. N = 6.



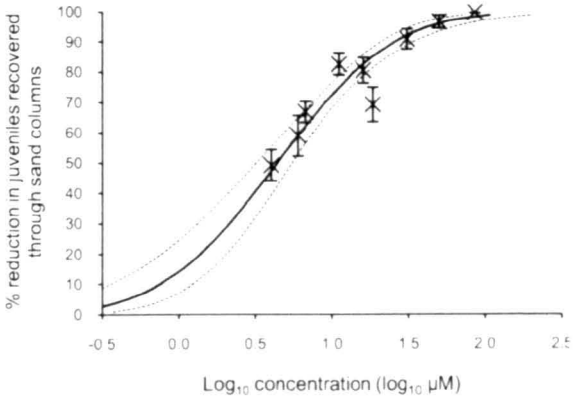
**Figure 2.5:** Dose-response curves for *Globodera pallida* second-stage juveniles exposed to a range of concentrations of 2-phenylethyl isothiocyanate in three separate experiments (red, blue and purple represent data from separate experiments). Probit analysis was used to estimate dose-response curves (solid lines) from experimental data (crosses with error bars, representing mean and SEM of  $\geq 6$  replicates).

the dose-response curve cannot be accurately estimated from these data. For instance, estimates of  $ED_{50}$  differed considerably, being 2.3, 4.2 and 6.3  $\mu\text{M}$ . In order to more accurately define the dose-response curves, assays were performed using concentrations estimated to cause suppression in the range 20-80 %. The results of these experiments are shown in Figure 2.6. The  $ED_{50}$  values for each compound except for 2-propenyl isothiocyanate are well defined. The descending rank order of toxicity was 2-phenylethyl > benzyl > 2-propenyl > 3-(methylthio)propyl > 2-methylbutyl isothiocyanate.  $ED_{50}$  values ranged from 4.17 to 21.9  $\mu\text{M}$  (Table 2.3). The sulphur-containing side-chained 3-(methylthio)propyl isothiocyanate was significantly less toxic than the aromatic isothiocyanates and the branched side-chained 2-methylbutyl isothiocyanate was significantly less toxic than all of the others ( $P < 0.05$ ).

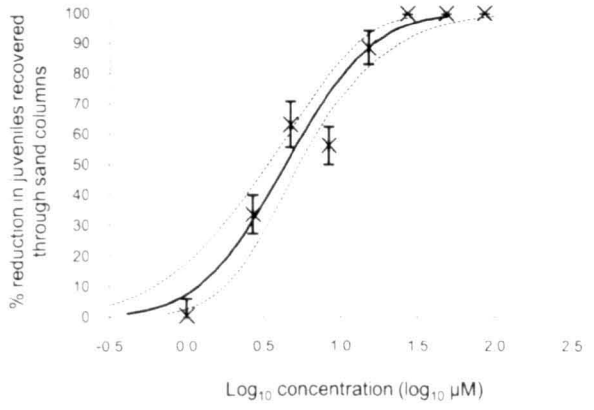
### 2.3.3 Toxicity of brassica leaf extracts to *Globodera pallida* second-stage juveniles

The effects of brassica leaf extracts on motility of second-stage juveniles (J2), measured as movement through sand columns, ranged from almost total inhibition to a stimulatory effect (Figure 2.7). Leaf extracts from the majority of the plants tested, including the non-brassica, *Triticum aestivum* cv. Paragon (spring wheat), caused significant suppression relative to treatment with water alone. Cultivars of three plant species, *Raphanus sativus* cv. Weedcheck (radish), *Nasturtium officinale* (cress) and *Brassica juncea* cv. Nemfix (Indian mustard), were particularly potent, causing 97%, 93% and 89% inhibition, respectively. The following cultivars all caused between 55 and 75 % suppression: *Eruca sativa* cv. Nemat (arugula), *Brassica rapa* (turnip), *Sinapis alba* (white mustard), *Moricandia moricandioides* and all of the *B. juncea* cultivars, even the low-glucosinolate line "Arid". *Eruca sativa* cv. 9 and *Brassica napus* cv. Nemcon (rape), which are commercially marketed as biofumigant crops, performed relatively poorly against *G. pallida*, causing 25 and 34 % suppression, respectively. This was no better than the non-brassica, wheat, which caused 34 % suppression. The increase in J2 recovery caused by *Hesperis matronalis* may have been due to a repellent effect of the leaf extracts since there would have been a gradient of concentration, decreasing towards the microtiter plate wells from which J2 were recovered.

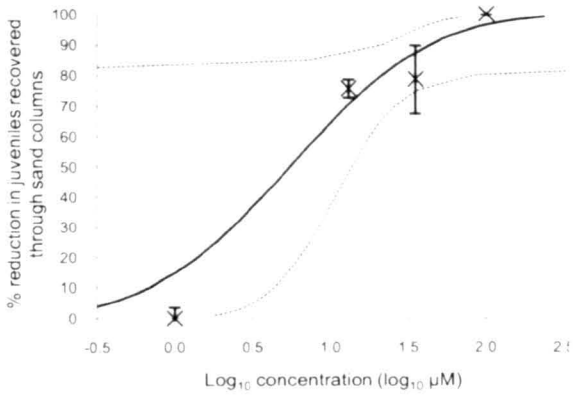
## 2-phenylethyl isothiocyanate



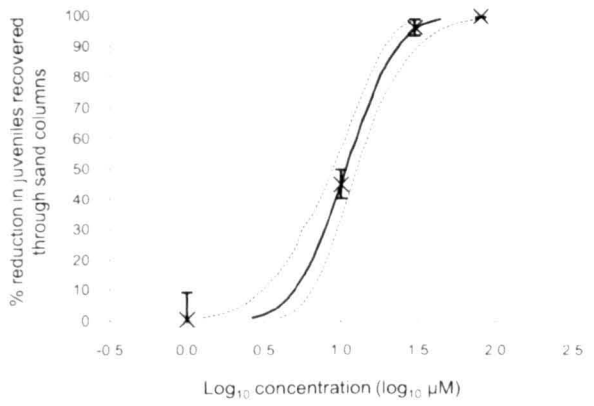
## Benzyl isothiocyanate



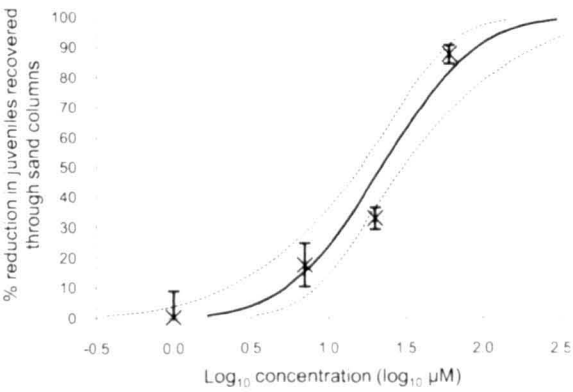
## 2-propyl isothiocyanate



## 3-(methylthio)propyl isothiocyanate



## 2-methylbutyl isothiocyanate

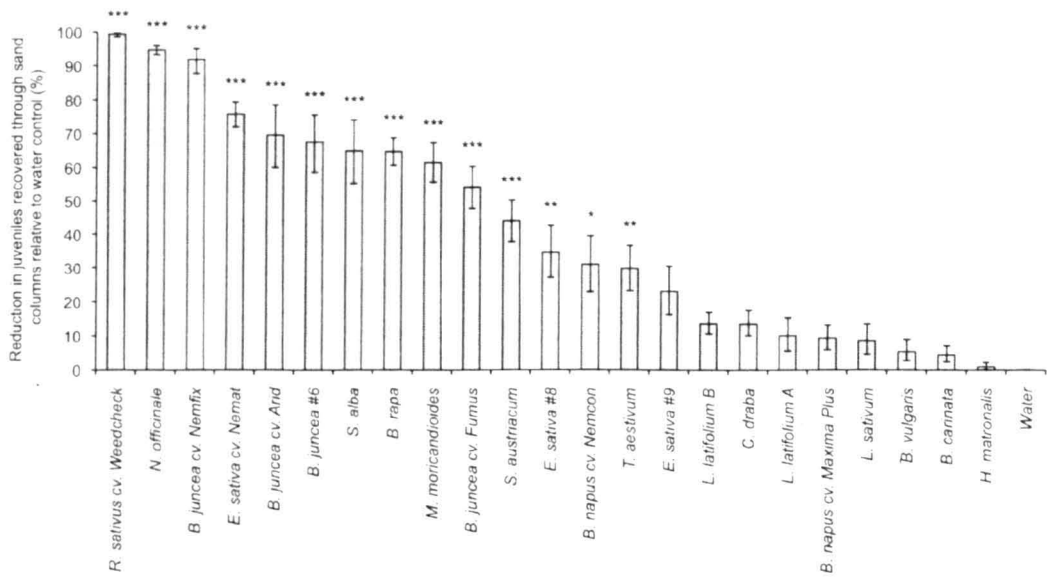


**Figure 2.6:** Dose-response curves for *Globodera pallida* second-stage juveniles exposed to a range of concentrations of five isothiocyanates. Probit analysis was used to estimate dose-response curves (solid lines) and 95 % confidence intervals (dotted lines) from experimental data (crosses). Error bars represent mean  $\pm$  SEM.  $N \geq 6$ .

**Table 2.3:** Concentrations of isothiocyanates causing 50 % and 80 % reductions in the proportion of *Globodera pallida* second-stage juveniles recovered through sand columns after 24 h exposure to isothiocyanates. Values in parentheses indicate 95 % confidence intervals.

<b>Isothiocyanate</b>	<b>ED<sub>50</sub></b>	<b>ED<sub>80</sub></b>
2-phenylethyl	4.17 (2.69-5.62)	13.8 (11.2-17.4)
<b>Benzyl</b>	<b>4.37 (3.39-5.37)</b>	<b>10.5 (8.51-13.5)</b>
2-propenyl	6.31 (0.00-13.5)	12.3 (0.01-85.1)
<b>3-(methylthio)propyl</b>	<b>10.7 (8.91-12.6)</b>	<b>17.8 (15.1-22.4)</b>
2-methylbutyl	21.9 (16.2-30.2)	56.2 (38.9-107)





**Figure 2.7:** Percentage reduction of *G. pallida* second-stage juveniles recovered from sand columns after 24 h exposure to extracts from leaves of 22 brassica lines or *Triticum aestivum* (1.8 mg dry leaf tissue ml<sup>-1</sup>) relative to water only control. Error bars show SEM. Significant differences from water control are highlighted with asterisks (Games-Howell test). N ≥ 20.

### 2.3.4 Glucosinolate concentration in leaf tissues

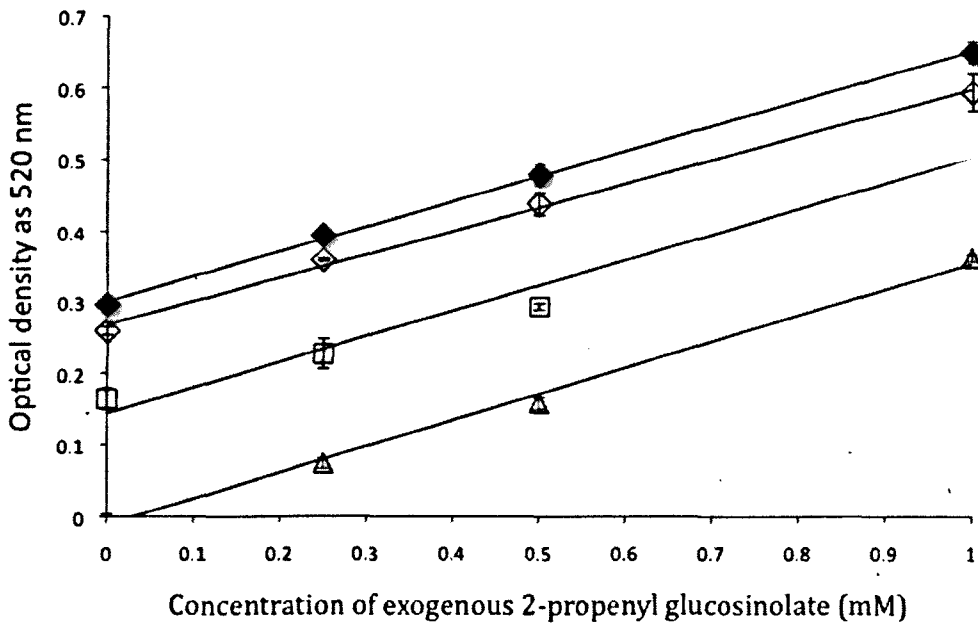
#### 2.3.4.1 Relationship between total glucosinolate content of leaves and suppression of *Globodera pallida* J2

There was a linear relationship between the concentration of exogenously supplied 2-propenyl glucosinolate and absorbance at 520 nm (Figure 2.8), indicating that the assay was quantitative over this range, which encompasses the range of concentrations in the test samples. The slope of the line was independent of sample type and experimental occasion so the mean slope of the three parallel lines in Figure 2.8 was used as a standard curve to determine the concentration of glucosinolate in test samples.

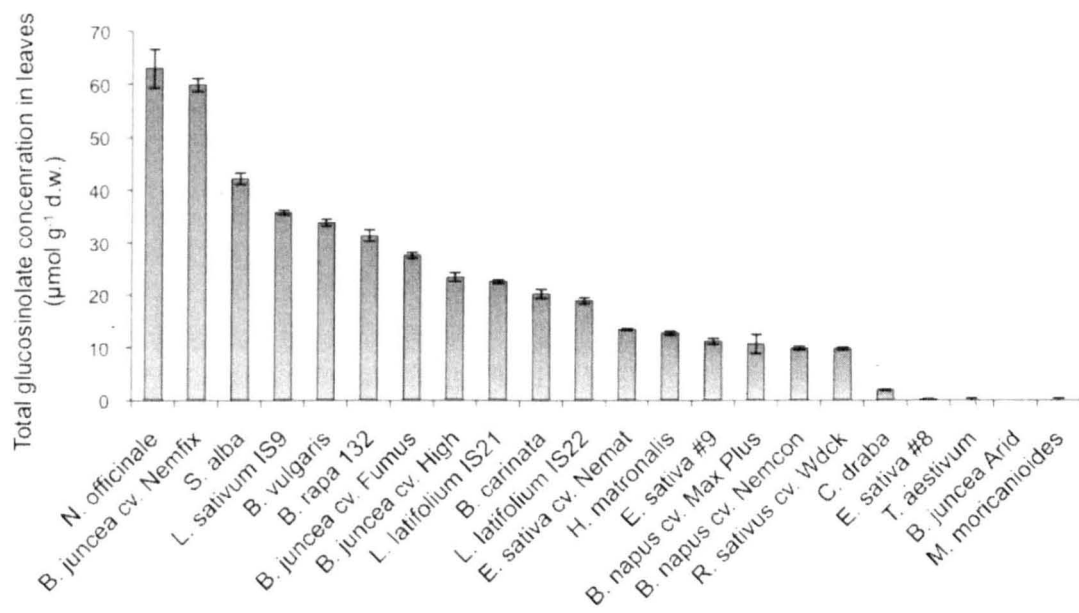
The concentration of glucosinolate in leaf tissues varied considerably among the brassica species (Figure 2.9). The non-brassica, wheat, and the brassicas *B. juncea* cv. Arid and *M. moricandioides* contained no detectable glucosinolate. Several brassica cultivars contained less than 15  $\mu\text{mol g}^{-1}$  dry leaf tissue, including *E. sativa* cv. Nemat, *R. sativus* cv. Weedcheck and *B. napus* cv. Nemcon. There was a continuous spectrum of plants spanning the range 18 - 42  $\mu\text{mol g}^{-1}$ , from *L. latifolium* IS22 with 18.6  $\mu\text{mol g}^{-1}$  to *S. alba* with 42.1  $\mu\text{mol g}^{-1}$ . Two plants, *B. juncea* cv. Nemfix and *N. officinale*, contained substantially higher levels, with 59.8 and 63.0  $\mu\text{mol g}^{-1}$  dry leaf tissue, respectively.

Contrary to predictions, there was no significant relationship between glucosinolate content and efficacy of leaves against *G. pallida* J2s (Figure 2.10). Some plants with a high level of activity against J2 contained relatively low levels of total glucosinolate. For instance *R. sativus* cv. Weedcheck and *E. sativa* cv. Nemat, caused 97% and 74% inhibition of motility despite containing only 9.7 and 13.2  $\mu\text{mol glucosinolate g}^{-1}$  dry leaf tissue, respectively. Moreover, *B. juncea* cv. Arid and *M. moricandioides* both caused over 60% suppression despite containing no detectable glucosinolate. Even the non-brassica wheat, which does not produce glucosinolates, caused a significant degree of nematode suppression. Some plants lacking nematostatic activity contained high levels of glucosinolate. For instance, *B. vulgaris* and *L. sativum* were ineffective against J2 yet contained 33.8 and 35.8  $\mu\text{mol glucosinolate g}^{-1}$  dry leaf tissue, respectively.

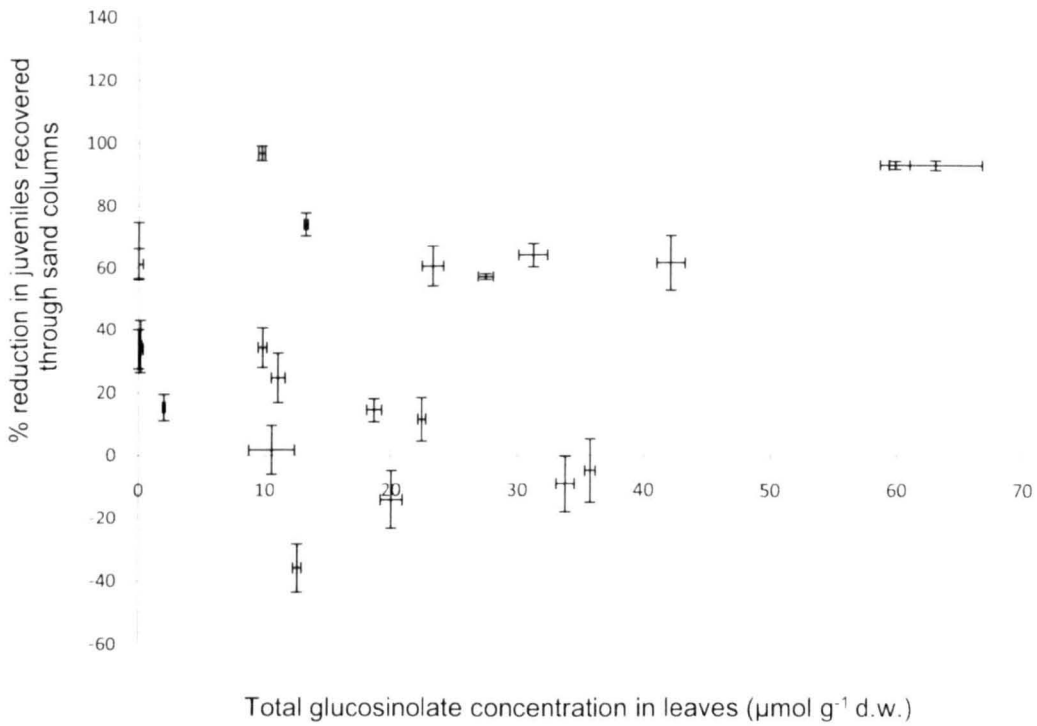
Some of the plants did follow the expected relationship. *B. juncea* cv. Nemfix and *N. officinale* caused around 90 % inhibition and contained the highest levels of glucosinolate of all the plants tested. Several of the ineffective plants, including *C. draba*, *B. napus* cv. Maxima Plus, *B. napus* cv. Nemcon and *E. sativa* 9 did indeed contain low levels of glucosinolate, below 12  $\mu\text{mol g}^{-1}$  d.w.



**Figure 2.8:** Mean optical density at 520 nm in triplicate colorimetric assays for total glucosinolate content of leaf extracts with added concentrations of exogenous 2-propenyl glucosinolate: triangles, *E. sativa* #8; squares, *E. sativa* #9, solid and open diamonds, *L. latifolium* IS22 on two separate occasions). The difference in elevation between the regression lines represents the difference in glucosinolate levels between cultivars. The mean slope of the three parallel lines is  $y = 0.357x$ . Error bars show SEM.



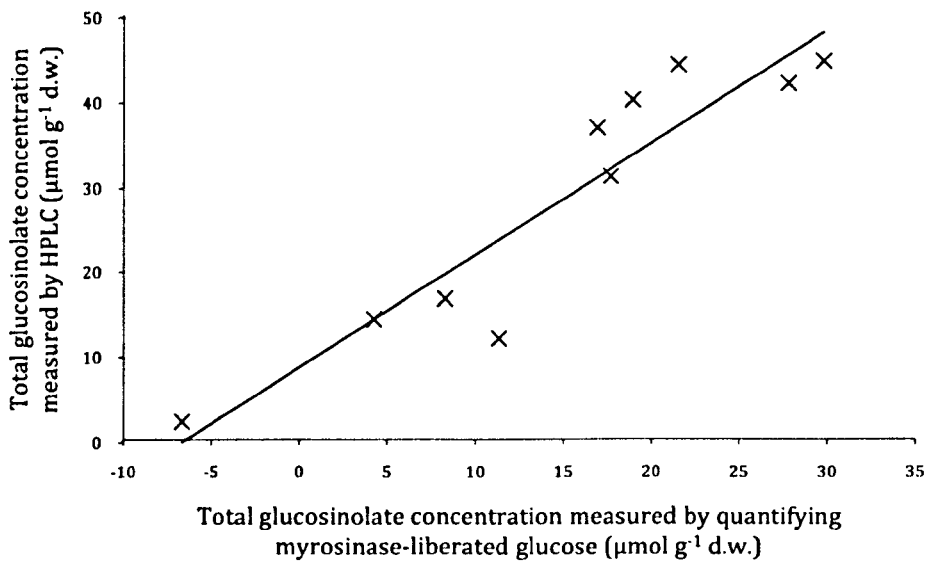
**Figure 2.9:** Total glucosinolate concentration in leaves of brassica lines screened for activity against *G. pallida*, as determined by quantification of glucose liberated by myrosinase. Leaves of *Triticum aestivum* (wheat) were tested to verify the absence of glucosinolate. Error bars show SEM. N = 3.



**Figure 2.10:** Relationship between the percentage of second-stage juveniles recovered from sand columns after 24 h exposure to extracts from brassica leaves and the total glucosinolate content of those leaves. Regression analysis showed no significant relationship ( $R^2 = 0.076$ ,  $P > 0.05$ ). Error bars show SEM.

#### *2.3.4.2 Comparison of the glucose quantification method and HPLC for measuring glucosinolate*

Glucosinolate profiles of ten brassicas were determined by HPLC as part of a subsequent glasshouse trial (Chapter 3, sections 3.23 and 3.3.2.3, Figure 3.7). Total glucosinolate concentrations in these samples were also determined using the glucose assay method in order to check the accuracy of this method. There was a strong correlation between total glucosinolate measured by the glucose assay method and by HPLC ( $R^2 = 0.865$ ,  $P < 0.001$ ) although the glucose assay method tended to underestimate the total glucosinolate concentration (Figure 2.11).



**Figure 2.11:** Correlation between results of two methods for quantifying total glucosinolate. The leaf material tested was from ten different brassica lines used in pot experiment 2. There is a significant relationship ( $R^2 = 0.865$ ,  $P < 0.001$ ).

## 2.4 Discussion

### 2.4.1 Development of a toxicity assay for *Globodera pallida* second stage juveniles

It was decided to measure motility for two main reasons. Firstly, the method allows large sample sizes to be tested, reducing sampling error. Secondly, in the field, sufficient inhibition of motility to prevent root invasion would result in death of the juvenile nematode. After several rounds of modification, a useful protocol was developed that reproducibly quantified inhibition of J2 locomotion. Carrying out exposure to test compounds and separation of motile and immotile individuals in separate containers allowed exposure duration and distance travelled through sand columns to be rigorously standardised. This reduced the variance in recovery rate and the number of false results in which immotile individuals were recovered and counted as motile.

Values of suppression caused by a toxin can be normalised for variation in incidental mortality or any other response by treating the proportion responding in controls as the total number of individuals exposed to the toxin (Abbott, 1925). However, incidental responses should be minimised, due to the potential for synergistic effects between the toxin and other stressors. In other words, if the subjects of the assay are not healthy individuals then the toxicity of the test substance may be overestimated. Modifications were made to the protocol that increased the recovery rate of nematodes in control treatments to approximately 50 %.

In the soil, isothiocyanate concentrations have been found to peak immediately after incorporation of a brassica green manure and then to decrease logistically, with half-lives of between approximately eight and sixty hours (Borek *et al.*, 1995, *unpublished* & Kirkegaard, 2006). An exposure duration of 24 hours was considered to be appropriate.

### 2.4.2 Toxicity of isothiocyanates to *Globodera pallida* second stage juveniles

Toxicity assays with pure isothiocyanates showed that these compounds are highly toxic to *G. pallida*, with ED<sub>50</sub> values ranging from 4.2 to 22 µM (Table 2.3). The most potent compounds were 2-phenylethyl, benzyl, 2-propenyl and 3-(methylthio)propyl isothiocyanate. The ED<sub>50</sub> value for 24 h exposure to 2-phenylethyl isothiocyanate was 5 µM, compared with 4 µM for *Tylenchulus semipenetrans* and 22 µM for *Meloidogyne javanica* exposed for 48 h (Zasada & Ferris, 2003). The LD<sub>50</sub> for *M. incognita* exposed to 2-phenylethyl glucosinolate with myrosinase for 24 h was 13 µM (Lazzeri *et al.*, 2004).



Zasada & Ferris (2003) suggest that *T. semipenetrans* is more sensitive than *M. javanica* because the former is a specialist of citrus while the latter has a broad host range, including some brassicas. *Globodera* spp. are also specialists that would not have evolved under selective pressure for tolerance of isothiocyanates. From these results, *Globodera* appears to be even more sensitive than *Tylenchulus*.

Concentrations required to inhibit motility of *G. pallida* in the present study were much lower than those found to be lethal to *G. rostochiensis*. Buskov *et al.* (2002) found that 24 h exposure of *G. rostochiensis* J2 to 2.5 mM 2-propenyl glucosinolate with myrosinase caused 29 % mortality. In the present study, the ED<sub>30</sub> of 2-propenyl isothiocyanate was 3.3 µM. Serra *et al.* (2002) found that 24 h exposure of *G. rostochiensis* to 710 µM 2-phenylethyl glucosinolate with myrosinase caused 47 % mortality whilst the ED<sub>50</sub> to *G. pallida* in the present study was 4.2 µM. Buskov *et al.* (2002) found that all of the 2-propenyl glucosinolate was converted to isothiocyanate. It seems unlikely that there are great differences in sensitivity to isothiocyanates between the two species of PCN. Therefore, the reason that isothiocyanates were active at much lower concentrations in the present study was either because interference with motility occurs at much lower concentrations than required for lethality or because in the studies of Buskov *et al.* (2002) and Serra *et al.* (2002), exposure to test compounds was carried out in unsealed Petri dishes, allowing isothiocyanate to escape by volatilisation.

It is not known if the isothiocyanate concentrations found to inhibit motility in the present study were sufficient to cause mortality but Lazzeri *et al.* (2004) determined the concentrations of glucosinolates required, in the presence of myrosinase, to paralyse or to kill *M. incognita* J2. After treatment with test compounds, they transferred paralysed J2 to distilled water to determine whether or not they would revive. For the isothiocyanate-producing glucosinolates, ED<sub>50</sub> and LD<sub>50</sub> values were similar if not identical, whilst for glucosinolates degrading to oxazolidine-2-thiones, concentrations required for paralysis were several times lower than required for lethality. The fact that paralysed J2 did not recover is consistent with the fact that isothiocyanates react irreversibly with proteins (Kawakishi & Kaneko 1985, 1987). The concentrations found to paralyse or kill *M. incognita* (Lazzeri *et al.*, 2004) were similar to those required to prevent recovery of *M. javanica* from sand columns (Zasada & Ferris, 2003) in a similar assay to that used in the present study. Thus, the much greater lethal concentrations for *G. rostochiensis* found by Buskov *et al.* (2002) and Serra *et al.* (2002) than for *G. pallida* were possibly due to escape of volatile isothiocyanates from experimental chambers, which would not have occurred in the present study.

The rank order of toxicity of isothiocyanates (Table 2.3) was similar to that found for other nematodes (Table 1.1).

It is interesting that small changes in isothiocyanate chemical structure can have profound effects on biological activity. For instance, phenyl isothiocyanate differs from benzyl isothiocyanate only in that the carbon chain between the isothiocyanate group and the benzene ring is shorter by one carbon, yet phenyl isothiocyanate is six times less toxic to *M. javanica* and 25 times less toxic to *T. semipenetrans* than benzyl isothiocyanate (Zasada & Ferris, 2003). Likewise, 4-methylthio-3-butenyl glucosinolate differs from 4-methylsulphinyl-3-butenyl glucosinolate only by the oxidation of the sulphur atom in the side chain, yet the isothiocyanates derived from the former and the latter caused 94 % and 3 % mortality, respectively, to *H. schachtii* after 24 hours exposure (Lazzeri *et al.*, 1993).

#### 2.4.3 Toxicity of brassica leaf extracts to *Globodera pallida* and relationship with glucosinolate content

Seed samples of over fifty brassica species were obtained, including several cultivars commercially marketed as biofumigant crops. Of these, a subset of 22 cultivars was selected for screening (Table 2.3). These plants were chosen because they contain glucosinolates known to produce highly nematicidal hydrolysis products, such as 2-phenylethyl isothiocyanate, because they contained glucosinolates producing hydrolysis products of unknown activity or because they are already marketed as commercial biofumigants. Since non-brassica-specific components of green manures have been found to suppress pests and pathogens, brassica lines known to contain very low levels of glucosinolate and a non-brassica, wheat, were included as controls. Wheat was chosen as it often precedes potatoes in rotations. Leaves were evaluated because they have been found to contain higher concentrations of glucosinolate and to be more effective against nematodes than root tissues (Potter *et al.*, 1998) and because above-ground tissues generally comprise over 90 % of the biomass of brassicas (Kirkegaard & Sarwar, 1998).

Leaf extracts of the 23 plants tested ranged in activity from highly potent to totally ineffective against *G. pallida* J2 (Figure 2.5). Estimates of total leaf glucosinolate (Figure 2.7) were similar to those found previously. Gimsing & Kirkegaard (2006) found 31.3  $\mu\text{mol g}^{-1}$  d.w. in epigeal tissues of *B. juncea* cv. Nemfix, compared with 60.0  $\mu\text{mol g}^{-1}$  d.w. in leaves in this study. Padilla *et al.* (2007) found between 57 and 74  $\mu\text{mol g}^{-1}$  d.w. in

leaves of *B. rapa* cv. 132, compared with  $31.2 \mu\text{mol g}^{-1}$  d.w. in the present study.

Total glucosinolate content was not a good predictor of efficacy for the 22 brassicas tested. There was no significant relationship ( $R^2 = 0.076$ ,  $P = 0.142$ ) between glucosinolate content of leaves and the percentage of J2 recovered from sand columns after exposure to extracts of those leaves (Figure 2.8). While the second and third most potent plants, *N. officinale* and *B. juncea* cv. Nemfix, both contained high concentrations of glucosinolate, the plant with the strongest activity, *R. sativus* cv. Weedcheck, contained six-fold less glucosinolate (Figure 2.7). Likewise, *B. juncea* cv. Arid, *M. moricandioides* and the non-brassica, *T. aestivum*, caused greater levels of suppression (66, 61 and 34%, respectively) than expected for plants containing no detectable glucosinolate. *Lepidium sativum* and *Barbarea vulgaris* contained high concentrations of glucosinolate but had no effect on nematodes.

There are several reasons why total leaf glucosinolate content is a poor predictor of efficacy. Clearly, toxicity varies between isothiocyanates, as shown in the toxicity assays with pure compounds. Secondly, there may have been variation in the proportion of glucosinolate converted to isothiocyanate. Specific myrosinase-interacting proteins can alter the fate of glucosinolate hydrolysis. Epithiospecifier protein causes nitriles and epithionitriles to be produced (Bones & Rossiter, 2006) and thiocyanate specifying protein causes nitriles and thiocyanates to be produced (Burow *et al.*, 2007). Lack of myrosinase activity could also limit glucosinolate hydrolysis. The myrosinase activity concentration in freeze-dried mature *Arabidopsis thaliana* leaves was found to be  $0.07 \text{ nmol glucosinolate min}^{-1} \text{ mg}^{-1}$  fresh weight at  $23 \text{ }^\circ\text{C}$  (Travers-Martin *et al.*, 2008). Assuming that dry weight was one tenth of fresh weight as it was in other experiments (Figure 3.6), this would equate to  $0.7 \text{ nmol glucosinolate min}^{-1} \text{ mg}^{-1}$  dry weight. Thus, at least  $140 \text{ nmol glucosinolate mg}^{-1}$  dry tissue could be hydrolysed in the 200 min incubation at  $37 \text{ }^\circ\text{C}$  allowed for preparation of leaf extracts in the present study. This is more than twice the concentration of glucosinolate in the most concentrated of leaf tissues tested (Figure 2.7). However, myrosinase activity varies between brassicas (Travers-Martin *et al.*, 2008) and may have been limiting in some of the plants.

For instance, *Lepidium sativum* contains only benzyl and traces of 4-hydroxybenzyl glucosinolate in the above-ground organs (Burow *et al.*, 2007) and was found to contain sufficient glucosinolate to produce a concentration of benzyl isothiocyanate equal to the  $\text{ED}_{98}$  against *G. pallida* J2. *L. sativum* produces thiocyanate-specifying protein in seedlings, flowers and seeds but autolysates of leaves contain benzyl isothiocyanate as the sole glucosinolate hydrolysis product (Burow *et al.*, 2007). The most likely explanation for the lack of efficacy of this plant seems to be low myrosinase activity. The

fact that, *B. juncea* cv. Arid, *M. moricandioides* and the non-brassica, *T. aestivum*, caused significant suppression of J2 despite containing no detectable glucosinolate shows that non-glucosinolate components of leaf extracts also confer anti-nematode properties.

*R. sativus* cv. Weedcheck and *Eruca sativa* cv. Nemat were also more active than predicted from their total glucosinolate content. Either these species produce anti-nematode compounds unrelated to glucosinolates or glucosinolates that give rise to particularly potent hydrolysis products. The leaf glucosinolates of these cultivars were later identified by HPLC (Figure 3.7). *R. sativus* cv. Weedcheck contained 4-(methylthio)-3-butenyl (53 % of total), 4-(methylsulfinyl)-3-butenyl (31 %) and 4-methoxy-indol-3-ylmethyl (15 %). *E. sativa* cv. Nemat contained a glucosinolate that formed a dimer during HPLC, putatively identified as 4-mercaptobutyl glucosinolate (75 %), an unidentified glucosinolate (13 %), 4-(methylsulfinyl)butyl (7 %) and 4-(methylthio)butyl glucosinolate (5 %). These glucosinolates are all capable of producing isothiocyanate and *Raphanus sativus* does not produce epithiospecifier protein (O'Hare *et al.*, 2008). Interestingly, brocolli, *Brassica olerifera*, also produces 4-(methylthio)-3-butenyl and 4-(methylsulphanyl)-3-butenyl glucosinolate but contains epithiospecifier protein and so does not produce the equivalent isothiocyanates (O'Hare *et al.*, 2008).

The toxicity to nematodes of the isothiocyanates derived from the glucosinolates of *R. sativus* cv. Weedcheck are moderate to low (Table 1.1). In this study, the total glucosinolate content of *R. sativus* cv. Weedcheck leaves was quantified at 16.5  $\mu\text{mol g}^{-1}$  dry tissue. Later, total glucosinolate content of another batch of the same cultivar was quantified by the glucose assay method and by HPLC, giving measurements of 8.3 and 16.5  $\mu\text{mol g}^{-1}$  dry tissue, respectively. The concentration of leaf tissue in the extracts used in toxicity assays was 1.8  $\text{mg ml}^{-1}$ . Thus, there was potentially 29.7  $\mu\text{M}$  isothiocyanate in the hydrolysed plant extract. 4-(methylthio)-3-butenyl glucosinolate is hypothesised to be very readily hydrolysed (Visentin *et al.*, 1992) and so hydrolysis efficiency may well have been virtually 100%. Even so, this concentration of isothiocyanate would not have been expected to cause the observed level of suppression. *R. sativus* cv. Weedcheck caused 97% inhibition of J2 motility and the  $\text{ED}_{97}$  for the most toxic ITC tested, benzyl ITC, was 37  $\mu\text{M}$  with a lower 95% confidence limit of 28  $\mu\text{M}$ . As mentioned earlier, the evidence suggests that the rank order of toxicity of different isothiocyanates is similar for all plant parasitic nematodes so it would seem unlikely that 4-(methylthio)-3-butenyl and 4-(methylsulfinyl)-3-butenyl isothiocyanate are as toxic as benzyl isothiocyanate to *G. pallida*. Therefore, it may be that other components of the leaf extract have anti-nematode activity.

For those plants, such as *B. juncea* cv. Nemfix and *N. officinale*, which possessed high glucosinolate content and potent activity toward *G. pallida* J2, comparison of the amounts of glucosinolate available with the dose-response curves for the corresponding isothiocyanates, suggests that isothiocyanate could have been responsible for the activity of these plants. For instance, the glucosinolate concentration in *B. juncea* cv. Nemfix was determined to be 59.8  $\mu\text{mol g}^{-1}$  dry weight. This may have been an over-estimate, since later measurements by the glucose assay method and by *in vivo* gave measurements of 17.7 and 31.1  $\mu\text{mol g}^{-1}$  dry weight, respectively (Chapter 2) and Gimsing & Kirkegaard (2006) determined the glucosinolate content of shoots of this cultivar to be 31.3  $\mu\text{mol g}^{-1}$ . Since the concentration of leaf tissue in the extracts used in toxicity assays was 1.8  $\text{mg ml}^{-1}$ , the amount of glucosinolate available for hydrolysis would have been between 60 and 110  $\mu\text{mol ml}^{-1}$ . *B. juncea* cv. Nemfix contains 2-propenyl glucosinolate as the sole glucosinolate (Figure 3.7) and caused 93 % suppression. The  $\text{ED}_{93}$  for 2-propenyl isothiocyanate was estimated as 52  $\mu\text{M}$ , less than the amount of glucosinolate present.

## 2.5 Summary

The main outcomes from this chapter are as follows:

1. An assay protocol for determining the effects of toxins on motility of *G. pallida* second-stage juveniles (J2) was developed and validated.
2. The dose-response curves for five isothiocyanates against *G. pallida* J2 were determined.
3. Leaf tissues of 22 brassica cultivars belonging to 15 species as well as the non-brassica wheat were screened for activity against *G. pallida* J2. Several effective plants were identified.
4. A relatively inexpensive and rapid method for quantifying total glucosinolate was tested and validated.
5. The concentrations of glucosinolate in the leaf tissues screened for activity to *G. pallida* J2 were determined. Total glucosinolate was found to be a poor predictor of toxicity and reasons for this were discussed.

## Chapter 3: The effect of brassica green manures on encysted eggs of *Globodera pallida* in soil

### 3.1 Introduction

In order to control PCN in the field, biofumigation would have to target encysted eggs, rather than free-swimming second-stage juveniles (J2). This is because PCN invade potato roots for several weeks after potatoes start growing (Whitehead, 1992), the latest that a green manure could be incorporated is at the time of potato planting and isothiocyanates have half-lives of tens of hours at the very most (Borek *et al.*, 1995, Gimsing & Kirkegaard, 2006). The vast majority of J2 invading potato roots would hatch after isothiocyanate concentrations had become negligible. In addition to the cuticle, unhatched J2 within a cyst may be protected from xenobiotics by the cyst wall, the eggshell membrane and the perivitelline fluid.

As discussed in section 1.9.4, edaphic factors influence the effects of brassica green manures on soil-borne organisms and promising results *in vitro* have not always translated into pest or pathogen control in soil. Thus, an experiment was performed to determine the effects of brassica green manures on encysted eggs of *G. pallida* in soil.

#### 3.1.1 Available methods for quantifying viable PCN eggs

##### 3.1.1.1 Conventional methods for quantifying viable PCN eggs

Viability of PCN eggs can be assessed visually (Shepherd, 1970). This method underestimates mortality of fumigant-treated eggs since fumigants kill without causing visible deterioration of the egg (Whitehead, 1973). Nematode viability has also been determined using a number of vital and non-vital stains (Shepherd, 1962; Ogiga & Estey, 1974; Meyer *et al.*, 1988; Donald & Niblack, 1994). Non-vital stains, such as Meldola's Blue, stain the body contents of dead but not live nematodes. The stain only penetrates the body after deterioration of the eggshell membrane and nematode cuticle (Ogiga & Estey, 1974). Live individuals that have suffered damage and intact dead individuals can be falsely identified using the stain (Ogiga & Estey, 1974). Chemical treatment can kill without causing membrane damage and it can take several weeks after fumigation before non-vital stains are able to penetrate dead PCN eggs (Van der Laan & Bijloo, 1955 cited by Ogiga & Estey 1974). Presumably, sterilisation of the local environment by the

fumigant slows microbial degradation of the barriers to stain penetration. Thus, visual assessment may be unsuitable for evaluating the efficacy of biofumigation.

Egg viability can also be quantified by determining the proportion of the population that hatches on exposure to potato root diffusate or infects bioassay plants or by determining the final population after the test population is allowed to multiply on a host (Hague & Omidvar, 1962). Hatch, infectivity and multiplication rate are good measures of viability since they directly measure completion of one or more life-cycle stages. The major disadvantage is the time required to perform such assays, especially the infectivity and multiplication assays. Also, fumigant nematicides can delay hatch as well as kill encysted eggs (Hague & Omidvar, 1962).

### 3.1.1.2 Molecular methods for quantifying viable PCN eggs

Molecular methods for identifying and quantifying PCN have been developed (Atkinson & Ballantyne, 1977; Bates *et al.*, 2002, DEFRA, 2004), partly for research purposes and partly to meet the demand for rapid diagnostic tests to monitor these quarantine pests. Levels of adenosine triphosphate (ATP) have been used to quantify viable PCN eggs (Atkinson & Ballantyne, 1977) but PCR-based methods are now preferred. Bulman & Marshall (1997) developed a semi-quantitative PCR method and Bates *et al.* (2002) developed a quantitative real-time PCR method to diagnose the two species of PCN.

A potential problem with using DNA as a measure of viability is that DNA can persist for some time after death (DeSalle *et al.*, 1992). RNA is much more chemically labile than DNA (Lindahl, 1993), more prone to attack by endonucleases and persists for a much shorter duration after death (Aellen *et al.*, 2006). Reverse transcription (RT) of RNA to produce complementary DNA (cDNA), followed by PCR (RT-PCR) can be used to quantify mRNA. Researchers at Harper Adams University College developed a method of quantifying PCN egg viability by determining the ratio of mRNA to DNA (DEFRA, 2004). One primer pair amplified both complementary and genomic DNA in the same reaction. The genomic DNA product was separated from the 200 bp shorter cDNA product by gel electrophoresis and the intensity of the two bands was compared. In effect, the quantity of genomic DNA was used to normalise for variation in nucleic acid extraction efficiency with the assumption that the rate of DNA degradation will be negligible relative to the rate of RNA degradation. These researchers determined the rate of decline in the mRNA/DNA ratio after fumigation with 1,3-dichloropropene (DEFRA, 2004). In one population of PCN cysts, mRNA was undetectable by 28 days post treatment but in the

other three populations tested, there was no consistent decline in mRNA/DNA ratio. It was not determined whether this was because the fumigation treatment failed to kill the eggs or if mRNA persisted after death. Thus the duration of mRNA persistence after death of PCN eggs is still unknown. As with all conventional PCR, the method is at best semi-quantitative. None of the previous PCR-based methods of quantifying viable PCN are fully quantitative and all of them rely upon the untested assumption that the relevant nucleic acid degrades rapidly in the soil environment. In an attempt to improve upon these methods, an RT-qPCR method was developed. The method was tested for the ability to accurately quantify viable *G. pallida* eggs and the rate of mRNA degradation in soil was determined.

### 3.1.2 Aims

1. To develop an RT-qPCR method that can accurately quantify the abundance of viable PCN eggs.
2. To determine the duration of mRNA persistence in PCN eggs killed by isothiocyanate.
3. To evaluate the effects of biofumigation on viability of encysted eggs of *G. pallida* in open and plastic-covered soil.
4. To examine the relationship between toxicity of brassica green manures to encysted eggs of *G. pallida* in soil and glucosinolate content.



## 3.2 Methods

### 3.2.1 RT-qPCR for quantifying viable PCN eggs

#### 3.2.1.1 Designing primers for amplification of *Globodera* spp. actin cDNA

The *Globodera rostochiensis* actin 1 mRNA complete coding sequence (Genebank accession number AF539593, Kovaleva *et al.*, 2005) was downloaded from the National Centre for Biotechnology Information (NCBI) nucleotide database (<http://www.ncbi.nlm.nih.gov/>). Either the final 639 bp sequence at the 3' end of the sequence or the entire 1131 bp sequence were entered into Primer 3 (Version 0.4.0, <http://frodo.wi.mit.edu/primer3/>) and a product size of 80-180 bp was specified. All other parameters were set as default. Four pairs of primers were selected from those suggested by Primer3 (Table 3.1). These primers were selected because they had suitable annealing temperatures and because, except for the final pair, they had annealing sites toward the 3' end of the coding sequence. The primers were synthesised by Eurogentech, purified by the "Selected Precipitation Optimised Process" desalting method and delivered as a 100 µM aqueous solution.

#### 3.2.1.2 RNA extraction from *Globodera pallida* cysts

Duplicate samples of approximately 15 mg *G. pallida* Pa2/3 cysts were soaked overnight in 1 ml sterile distilled water in 1.5 ml micro-centrifuge tubes. RNA was extracted using the Qiagen RNeasy Plant Mini Kit (Qiagen, 74904). The protocol was a modified version of the manufacturer's instructions for extracting RNA from animal tissue and incorporated an on-column DNase treatment using RNase-free DNase I (Qiagen, 79254). Prior to RNA extraction, lyophilised DNase I (Qiagen, 79254) was dissolved in 550 µl RNase-free water. Aliquots of 10 µl were dispensed into 0.5 ml RNase-free tubes (Axygen, 321-05-051) and stored at -20 °C. RNase-free micro-pestles were prepared by soaking in "RNase-AWAY" (Molecular Bioproducts Inc., 732-2352) for at least one hour, rinsing three times in diethyl pyrocarbonate-treated water and drying in an oven at 65 °C. Immediately prior to performing RNA extraction, 10 µl aliquots of DNase I were thawed and 70 µl buffer RDD (provided with the DNase) was mixed into each. β-Mercaptoethanol was added to buffer RLT before use at a concentration of 10 µl per 1 ml buffer RLT. *Globodera* cysts in 1.5 ml micro-centrifuge tubes (Maxymum Recovery, Axgen) were dried by pipetting and wicking with strips of filter paper. The samples were frozen in liquid nitrogen and ground with a micro-pestle. 150 µl buffer RLT was

**Table 3.1:** Primer pairs designed and tested for amplification of *Globodera pallida* actin 1 cDNA.

Primer pair	Sequence	T <sub>m</sub> (°C)	Expected product size (bp)
GpACTF1 GpACTR1	5'-CCG CCG CCT CCT CCT CCT C-3' 5'-GCC GAC TCC ATG CCG ATG AAG-3'	68 68	130
GpACTF1b GpACTR1	5'-CGC TGG AGA AGA GCT ACG AG-3' 5'-GCC GAC TCC ATG CCG ATG AAG-3'	64 68	112
GpACTF2 GpACTR2	5'-AGA TCC TCA CTG AGC GTG GT-3' 5'-GCA GCT CGT AGC TCT TCT CC-3'	62 64	156
GpACTF3 GpACTR3	5'-CAT GGG ACA GAA GGA CTC GT-3' 5'-AGG TGT GGT GCC AGA TCT TC-3'	62 62	131

added and the micro-pestle was used to grind the frozen paste until it had melted. 300 µl buffer RLT was used to rinse the ground tissue from the micro-pestle into the tube. Samples were vortexed thoroughly and incubated for 10 min at room temperature then centrifuged at 8,000 *g* for 15 s to collect the contents at the bottom of the tubes. The lysate was resuspended by pipetting and transferred to a QIAshredder spin column placed in a 2 ml micro-centrifuge tube. The columns were centrifuged for 2 min at 14,000 *g* and 400 µl of the supernatant of the flow-through was carefully transferred to a clean, RNase-free 1.7 ml tube (Axygen, MCT-175-C) containing 200 µl ethanol and immediately mixed in by pipetting. The sample, including precipitate, was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 s at 8,000 *g*. The flow-through was discarded and 350 µl buffer RW1 was added to the RNeasy spin column. The column was centrifuged for 15 s at 8,000 *g* and the flow-through was discarded. 80 µl DNase I in buffer RDD was pipetted onto the RNeasy spin column membrane and incubated at room temperature for 15 min. 350 µl buffer RW1 was added to the RNeasy spin column. The column was centrifuged for 15 s at 8,000 *g* and the flow-through was discarded. 500 µl buffer RPE was added to the RNeasy spin column. The column was centrifuged for 15 s at 8,000 *g* and the flow-through was discarded. 500 µl buffer RPE was added to the RNeasy spin column. The column was centrifuged for 2 min at 8,000 *g*. The RNeasy spin column was placed into a new 2 ml collection tube and centrifuged at 14,000 *g* for 1 min. The RNeasy spin column was placed in a new RNase-free 1.5 ml collection tube (from the RNeasy kit). 30 µl RNase-

free water was added to the column membrane. The column was centrifuged for 1 min at 8,000 *g* to elute the RNA. The centrifugation step was repeated after adding the eluate from the previous step. The RNA was stored at -80 °C until use.

### 3.2.1.3 Reverse transcription of *Globodera pallida* RNA

cDNA was synthesised from RNA using SuperScript® II Reverse Transcriptase (Invitrogen, 18064-014) according to a modified version of the manufacturer's instructions. A master mix was prepared, containing the following components: 1.0 µl reaction<sup>-1</sup> CDS primer mixture (2.5 µM each primer), 1.0 µl reaction<sup>-1</sup> dNTP mixture (10 mM each dNTP), 7.5 µl sterile distilled water. 9.5 µl aliquots of this master mix were added to 0.5 ml RNase-free tubes (Axygen, 321-05-051) and 2.5 µl RNA sample was added to each tube. For each RNA sample, two reactions were prepared. One would go on to receive the RT enzyme while the other would be a control reaction with no RT enzyme. The tubes were vortexed gently for 10 s then briefly centrifuged to collect the contents. The mixtures were incubated at 65 °C for five min in a thermal cycler (PCR Express, Hybaid) then chilled on ice for three min. The contents of the tubes were collected by brief centrifugation. A second master mix had been prepared containing 3.8 µl reaction<sup>-1</sup> 5x First Strand Buffer and 2.0 µl reaction<sup>-1</sup> 0.1 M DTT (both supplied with the RT enzyme kit). 5.8 µl of this master mix was added to each tube and mixed in by gentle vortexing for 10 s. The tubes were incubated at 42 °C for two min in the thermal cycler. 1.0 µl (200 U) SuperScript II Reverse Transcriptase or water was pipetted onto the inner side of the tubes. The tubes were then simultaneously vortexed at 8,000 *g* for 15 s so that the RT enzyme was added to each sample at the same time. The enzyme was mixed in more thoroughly by vortexing gently for 10 s. The contents of the tubes were collected by brief centrifugation and the tubes were incubated at 42 °C for 50 min, followed by 70 °C for 15 min in the thermal cycler. The reaction products were stored at -20 °C until use.

### 3.2.1.4 Testing primers for amplification of *Globodera pallida* actin cDNA

The suitability of the four primer pairs (Table 3.1) in terms of amplification efficiency and product purity was tested in qPCR using *G. pallida* cDNA at a range of concentrations. A ten-fold dilution series of cDNA was prepared in sterile distilled water. Four PCR master-mixes were prepared, one for each of the four primer combinations. The master-mixes contained the following components: 1.0 µl reaction<sup>-1</sup>

7.5  $\mu\text{M}$  forward primer, 1.0  $\mu\text{l}$  reaction<sup>-1</sup> 7.5  $\mu\text{M}$  reverse primer, 12.5  $\mu\text{l}$  reaction<sup>-1</sup> Biorad iQ SYBR Green Supermix (Biorad, 170-8882) and 6.5  $\mu\text{l}$  reaction<sup>-1</sup> sterile distilled water. 20  $\mu\text{l}$  reaction<sup>-1</sup> master mix was added to the wells of a 96-well micro-titre plate (Agilent, 410088). For each primer combination, 5  $\mu\text{l}$  reaction<sup>-1</sup> of 1x, 10x, 100x or 1000x diluted cDNA or water alone was added to triplicate reactions. The plate was sealed with optical cap strips (Agilent, 401425) then briefly centrifuged in a manual centrifuge to collect the contents at the bottom of the wells. The plate was placed in a plate mixer (Eppendorf Mixmate) for two min at 600 rpm and briefly centrifuged once more. The plate was placed in a qPCR thermal cycler (Stratagene Mx3005P) and subjected to the following thermal profile: 95 °C for 3 min, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. This was followed by 95 °C for 60 s, 55 °C for 30 s and then gradually ramping to 95 °C over a period of approximately 5 min, to determine the dissociation curve of the reaction products. The thermal cycler was set to measure SYBR Green fluorescence and MxPro software was used to analyse fluorescence data. The software automatically set threshold fluorescence values and calculated the amplification efficiency.

#### 3.2.1.5 Determination of the accuracy of RT-qPCR for quantification of viable PCN eggs

In order to determine whether or not RT-qPCR could provide an accurate indication of the abundance of viable PCN eggs, qPCR was used to quantify actin transcripts in cDNA from samples containing varying proportions of viable eggs.

Two batches of approximately 100 mg *G. rostochiensis* cysts were added to 1.5 ml micro-centrifuge tubes (Sarstedt, D-51588) containing 1 ml sterile tap water. One tube was incubated at 65 °C overnight while the other was incubated at room temperature (~ 23 °C). Both batches of cysts were then stored at 4 °C for six months prior to use in the experiment. The *G. rostochiensis* cysts were from a laboratory stock that had been cultured on *Solanum tuberosum* ssp. *andigena* cv. Desiree and stored dry at 4 °C for several years.

Heat-killed and untreated cysts were picked with forceps and added to 1.5 ml tubes in the following proportions: 0:20, 5:15, 10:10, 15:5 and 20:0 heat-killed:untreated. There was a total of 20 cysts per tube and three replicates of each ratio. RNA was extracted from these cysts and used in reverse transcription reactions as described in sections 3.2.1.2 and 3.2.1.3. For each sample, one RT reaction and one control with no RT enzyme were performed. These controls were included to check for the presence of

contaminating genomic DNA. Quantitative PCR was carried out as described in section 3.2.1.7. This assay was repeated in its entirety once and then repeated again from the RT reaction stage, using the same RNA samples as on the second occasion. The second time the experiment was repeated, the duration of the reverse transcriptase incubation step was reduced to 10 min.

### 3.2.1.6 Determination of the duration of mRNA persistence in vitro and in soil

Cysts of *Globodera pallida* Pa2/3 were soaked in sterile distilled water for four days at room temperature in the dark. The hydrated cysts were divided into nine lots of 37 mg and transferred to 1.5 ml micro-centrifuge tubes containing 1 ml sterile tap water. Three lots of cysts were incubated at room temperature, three lots were incubated at 65 °C and three lots were incubated at room temperature with addition of 16.5 mg Basamid® granular soil fumigant (BASF, 634512, containing 99 % Dazomet, 1 % inerts). The tubes were incubated for 11 days.

After eleven days incubation, any undissolved Dazomet crystals that remained in the Dazomet treated samples were removed and all samples were incubated at 25 °C. After 2, 9, 17 and 36 days, twenty cysts were sampled from each tube and transferred to fresh tubes containing 1 ml sterile tap water. The water was removed and RNA was extracted as described in section 3.2.1.2. RNA was stored at -80 °C until the extractions from all time-points had been performed. RT reactions and qPCR were performed as described in sections 3.2.1.3 and 3.2.1.7, respectively. At 40 days post treatment, samples of cysts from the Dazomet and control treatments were transferred to sterile hatching jars containing 25 ml potato root diffusate and incubated at 20 °C in the dark. The number of hatched juveniles was counted after 20 days.

Next, the rate of RNA degradation in soil at 10 °C was determined. Five-milligram lots of dry *G. pallida* Pa1 cysts were mixed into separate lots of 7 g sand-loam, which consisted of one part sharp sand (J. Arthur Bowers) to one part Norfolk topsoil (Baileys of Norfolk), and transferred to 7 ml Bijou containers (VWR International, MARI3612000). The caps were screwed on loosely, allowing gaseous exchange and the containers were incubated at 10 °C for seven days for the cysts to hydrate. There were 20 such containers. After the seven days, 1.2 ml of a solution containing 3.6 % dimethyl sulphoxide (DMSO, Sigma, D-5879), 18% 2-propanol (Fisher Scientific, P/7503/15) and 14 mg ml<sup>-1</sup> Basamid® (BASF, 634512, containing 99 % Dazomet, 1 % inerts) was added to 12 of the containers. The caps were screwed on tightly and the containers were

incubated at 10 °C in the dark. 1.2 ml sterile tap water was added to the other 24 containers. Twelve of these were incubated at 10 °C while the other twelve were incubated at 65 °C overnight before being transferred to the 10 °C incubator.

At 0, 4, 9 and 21 days post-treatment, three samples per treatment were collected for extraction of cysts. At each time-point, the contents of the Bijou containers were emptied into plastic weigh boats and allowed to dry for six hours in a fume hood with airflow. Cysts were extracted by the Fenwick can method, retained on folded filter papers (240 mm  $\phi$ , Whatman, 1573 1/2) and immediately transferred with forceps to 1.5 ml micro-centrifuge tubes containing 1 ml sterile tap water. Water was removed by pipette and residual water was removed by adding three strips of filter paper (125 mm  $\phi$ , Whatman, 1001 125) and leaving to dry overnight at 4 °C. RNA was extracted the following day as described in section 2.2.6. Thus, the timepoints were actually 1, 5, 10 and 22 days post treatment. RNA was stored at -80 °C until the RNA extractions from all time-points had been performed. RT reactions were carried out as described in section 3.2.1.3 and qPCR was carried out as described in section 3.2.1.7.

### *3.2.1.7 Real-time quantitative PCR for quantification of viable PCN eggs*

cDNA samples and no RT control samples (DNase-treated RNA samples that were used in mock reverse transcription reactions lacking reverse transcriptase) were diluted 20x in sterile distilled water. A master mix was prepared containing 1.0  $\mu$ l reaction<sup>-1</sup> 7.5  $\mu$ M forward primer (GpACTF1), 1.0  $\mu$ l reaction<sup>-1</sup> 7.5  $\mu$ M reverse primer (GpACTR1), 12.5  $\mu$ l reaction<sup>-1</sup> Biorad iQ SYBR Green Supermix (Biorad, 170-8882) and 6.5  $\mu$ l reaction<sup>-1</sup> sterile distilled water. 20  $\mu$ l reaction<sup>-1</sup> master mix was added to the wells of a 96-well micro-titre plate (Agilent, 410088). 5  $\mu$ l reaction<sup>-1</sup> diluted cDNA or no RT control sample was added to triplicate wells. In addition to the test samples, a 10-fold dilution series of cDNA of known concentration was included. This cDNA had been prepared using RNA extracted from untreated cysts and its concentration had been determined spectrophotometrically using a Nanodrop ND-1000 (Labtech International). Controls with water instead of cDNA were also included to test for contamination of PCR reagents.

The plate was sealed with optical cap strips (Agilent, 401425) then briefly centrifuged in a manual centrifuge to collect the contents at the bottom of the wells. Reaction components were mixed by placing the plate in a plate mixer (Eppendorf Mixmate) for two min at 600 rpm and then collected with another brief centrifugation. The plate was

placed in a qPCR thermal cycler (Stratagene Mx3005P) and subjected to the following thermal profile: 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 60 s and finally, in order to determine the dissociation curve of the reaction products, 90 °C for 60 s, 50 °C for 60 s then ramping to 95 °C over a period of approximately 5 min. The thermal cycler was set to measure SYBR Green fluorescence and MxPro software was used to analyse fluorescence data. Threshold fluorescence values were set automatically by the software. Ct values obtained from the cDNA dilution series of known concentration were plotted against the log<sub>10</sub>-transformed cDNA concentration in order to generate a standard curve. This curve was used to determine the initial concentration of cDNA in test samples from their Ct values. Means of three technical replicates were taken as the cDNA concentration for each biological replicate.

### 3.2.2 Quantification of the effects of brassica green manures on viability of encysted eggs of *Globodera pallida* in soil

Two glasshouse trials were carried out to determine the toxicity of brassica green manures to encysted eggs in soil. In the first trial, green manures of *B. juncea* cv. Nemfix, *R. sativus* cv. Weedcheck, *N. officinale*, *B. juncea* cv. Arid, *T. aestivum* (Table 3) and *B. juncea* cv. ISCI99 were tested. The former three were selected as they had shown a high degree of activity toward J2 *in vitro* (Figure 10). *B. juncea* cv. ISCI99 was also selected because it is marketed as a biofumigant crop in the UK and was recommended by Dr. Andy Barker. The low glucosinolate content *B. juncea* cultivar Arid and the non-brassica wheat were included as controls. These plants were all tested in soil in open containers while *R. sativus* cv. Weedcheck and *N. officinale* were also tested in soil covered with polyethylene.

The second glasshouse trial was much more extensive with green manures of 12 plants tested in open and covered pots. In addition to the six plants tested in the initial glasshouse trial, *E. sativa* cv. Nemat, *B. rapa* #132, *M. moricandioides*, *B. juncea* cv. Fumus, *E. sativa* #8, and *Barbarea vulgaris* were tested. These plants are listed in Table 2.2. The first four of these plants were selected because they had shown a high degree of activity toward J2 *in vitro* (Figure 10). *E. sativa* #8 was included as a control since it had previously appeared to contain a low concentration of glucosinolate (Figure 12) and *B. vulgaris* was included as it had shown very low activity *in vitro* despite containing a high concentration of glucosinolate. Material from each of the brassicas

tested in the second glasshouse trial was sent to the Research Centre for Industrial Crops (ISCI), Bologna, Italy, for quantification of individual glucosinolates by HPLC.

### 3.2.2.1 Initial glasshouse trial

The plants were grown as described in section 2.2.4.2. When the plants were harvested, the *B. juncea* cultivars were at the initiation of flowering stage while *R. sativus* and *N. officinale* were still in the vegetative stage. *G. pallida* cysts were from the culture prepared as described in section 2.2.1.1. The mean number of eggs per milligram of cysts was determined and 60 kg sand-loam (one part J. Arthur Bowers sharp sand to one part Norfolk topsoil, Baileys of Norfolk) was infected at a rate of 50 eggs g<sup>-1</sup> soil. The infested soil was stored in 40 liter plastic buckets at 20 °C for three days prior to addition of green manures. 255 g leaf and shoot material of each of the plants was homogenised in a high power commercial blender (Waring). This material was added to a 40 liter plastic bucket containing 5.1 kg nematode-infested sand-loam together with 830 ml tap water. Green manures were thoroughly mixed in and the containers were left open. For *R. sativus* and *N. officinale*, the process was repeated but this time the containers were sealed. Two control batches of 5.1 kg nematode-infested sand-loam received only 830 ml tap water. One was left open and the other was sealed. The containers of soil were kept at ~ 20 °C underneath a glasshouse bench for three days.

3.5 " diameter plant pots were lined with polyethylene (500 gauge black refuse sacks). 275 g treated soil was placed into each pot (18 replicates per treatment). The surface of the soil in each pot was covered with a layer of horticultural grit to prevent evaporation. For covered treatments, a double layer of polyethylene was used to seal the tops of the pots. Pots were randomly arranged in trays containing two pots from each treatment. Trays were placed on benches in a glasshouse maintained at 18 °C. Once every three to four days, 30 ml water was added to each of the uncovered pots. Glasshouse temperature was monitored using two Tinytag Plus 2 data-loggers (RS Components Ltd.).

After 60 days, the contents of the pots were air emptied into trays and allowed to air dry for 12 days. By this stage, it had been determined that the RNA extraction protocol is quantitative with a greater quantity of cysts than originally anticipated. Hence, duplicate samples were combined and stored at 4 °C for 20 to 40 days until cyst extraction. Samples were processed in five blocks, each containing one replicate from each treatment. Cysts were extracted with a Fenwick can as described in paragraphs 78, 79



and 82 of Southey (1970). Cysts were transferred to micro-centrifuge tubes and immediately dried by pipetting followed by overnight incubation at 4 °C with three strips of filter paper (125 mm  $\phi$ , Whatman, 1001 125) placed in the open tubes to aid evaporation. For each block of samples, RNA extraction (section 3.2.1.2) was performed on the day following cyst extraction. RNA samples were stored at -80 °C until all of the samples had been prepared. RT reactions were performed as described in section 3.2.1.3 except that the duration of the RT enzyme incubation step was reduced to 20 min. Quantitative PCR was carried out as described in section 3.2.1.7 except that Biorad SYBR Green was replaced with Stratagene SYBR Green (Brilliant II SYBR Green, Agilent, 600828) and consequently the thermal profile was changed to 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s. Data were analysed with MxPro software. The fluorescence threshold was set automatically and concentrations of cDNA in test samples were calculated from Ct values by means of a standard curve. The mean cDNA concentration of triplicate qPCR was taken for each biological replicate. The mean of five biological replicates was calculated for each treatment and differences between means were analysed by ANOVA with a post-hoc test.

### 3.2.2.2 Full glasshouse trial

The plants listed above (section 3.2.2) were grown under the same conditions as described in section 2.2.4.2 except for the following differences. They were grown in a glasshouse during the summer (6<sup>th</sup> May to 18<sup>th</sup> June). Natural light was supplemented between 04:00 and 08:00 and between 16:00 and 20:00 with additional light from 250 W metal halide bulbs (HPI-T, Phillips) 1.5 m above the bench. Two fans (Hotbox, Levant) per glasshouse section were used to circulate air. The minimum temperature was 14 °C and the maximum was 28 °C.

At the time of incorporation, the *Brassica juncea* cultivars were all at the full flowering stage. All of the other brassicas were at the initiation of flowering except for *Barbarea vulgaris*, which was in the vegetative stage. At the time of incorporation, 100 g leaf material identical to that incorporated was frozen in liquid nitrogen and stored at -80 °C. This material was ground and lyophilised as described in section 2.2.4.3. The dry material was weighed. Approximately 7 g dry leaf tissue of each brassica was sent to ISCI for quantification of individual glucosinolates by HPLC. Remaining material was

used for extraction of glucosinolates and quantification of total glucosinolate as described in section 2.2.5.

*G. pallida* cysts that passed through a 600  $\mu\text{m}$  sieve and were retained on a 500  $\mu\text{m}$  sieve were used in this experiment. The number of eggs per milligram was determined and 54 kg sand-loam (one part J. Arthur Bowers sharp sand to one part Norfolk topsoil, Baileys of Norfolk) was infested at a density of 182 eggs  $\text{g}^{-1}$  soil. The infested soil was stored in 40 liter plastic buckets at 20 °C for three days prior to addition of green manures. 135 g leaf material of each of the plants was homogenised in a high power commercial blender (Waring). This material was added to a 40 liter plastic bucket containing 2.6 kg nematode-infested sand-loam together with 320 ml tap water. Control batches of soil received water alone. The green manures and water were thoroughly mixed into the soil. Sixteen replicate samples of 168 g treated soil were immediately dispensed into 3½" diameter plant pots lined with polyethylene (500 gauge black refuse sacks). The surface of the soil in each pot was covered with a layer of horticultural grit to prevent evaporation. Eight of the pots were left open and the other eight were covered with a double layer of polyethylene. The pots were placed in a glasshouse with a temperature of  $17.1 \pm 3.2$  (SD) °C. One day after incorporation, four open and four covered pots were randomly selected and weighed. These pots were weighed every week to monitor evaporation and water was added to all of the pots to compensate for this loss. Glasshouse temperature was monitored using two Tinytag Plus 2 data-loggers (RS Components Ltd.).

After 74 days, the contents of the pots were emptied into trays and allowed to dry for 14 days in the same glasshouse as the experiment. The soil was stored at 4 °C for 79 to 99 days until cyst extraction. Five blocks were processed, with one replicate of each treatment per block. Cysts were extracted, dried and stored as in the initial glasshouse trial (section 3.2.2.1), except that the samples were allowed to dry at 4 °C for one week and stored at 4 °C for a further two weeks until RNA extraction. RNA extraction, RT reactions and qPCR were carried out as in the initial glasshouse trial (section 3.2.2.1). The mean cDNA concentration in mock-treated samples from open and covered containers were taken to be 100 % relative viability for samples from open and covered containers, respectively. For each sample, the relative viability of eggs after treatment was estimated as the cDNA concentration in the sample divided by the mean cDNA concentration in the corresponding mock treatment. Proportion viability data were arcsin square-root transformed and analysed by one-way analysis of variance with S-N-K post-hoc test using SPSS 16.0 (IBM, Somers, NY, USA).

### 3.2.3 HPLC analysis of individual glucosinolates

At the time of incorporation, 100 g leaf material was frozen in liquid nitrogen and stored at -80 °C. This material was ground to a fine powder in liquid nitrogen and freeze-dried. Material was weighed before and after drying in order to calculate tissue moisture content. Approximately 7 g dry leaf tissue of each brassica was sent for quantification of individual glucosinolates by HPLC. Glucosinolate content was determined by the ISO 9167 (1992) procedure with some minor modifications (Wathelet *et al.*, 2004) using a Hewlett Packard chromatograph 1090 L equipped with a diode array detector and a 200 x 4.6 mm column (HP ODS Hypersil C18, 5µm). The amount of glucosinolate incorporated into soil as part of brassica tissues was calculated by multiplying the concentration in dry leaf tissue by the mass of dry matter incorporated.

## 3.3 Results

### 3.3.1 Development of an RT-qPCR method to quantify viable *G. pallida* eggs

#### 3.3.1.1 Testing primers for amplification of *G. pallida* actin cDNA

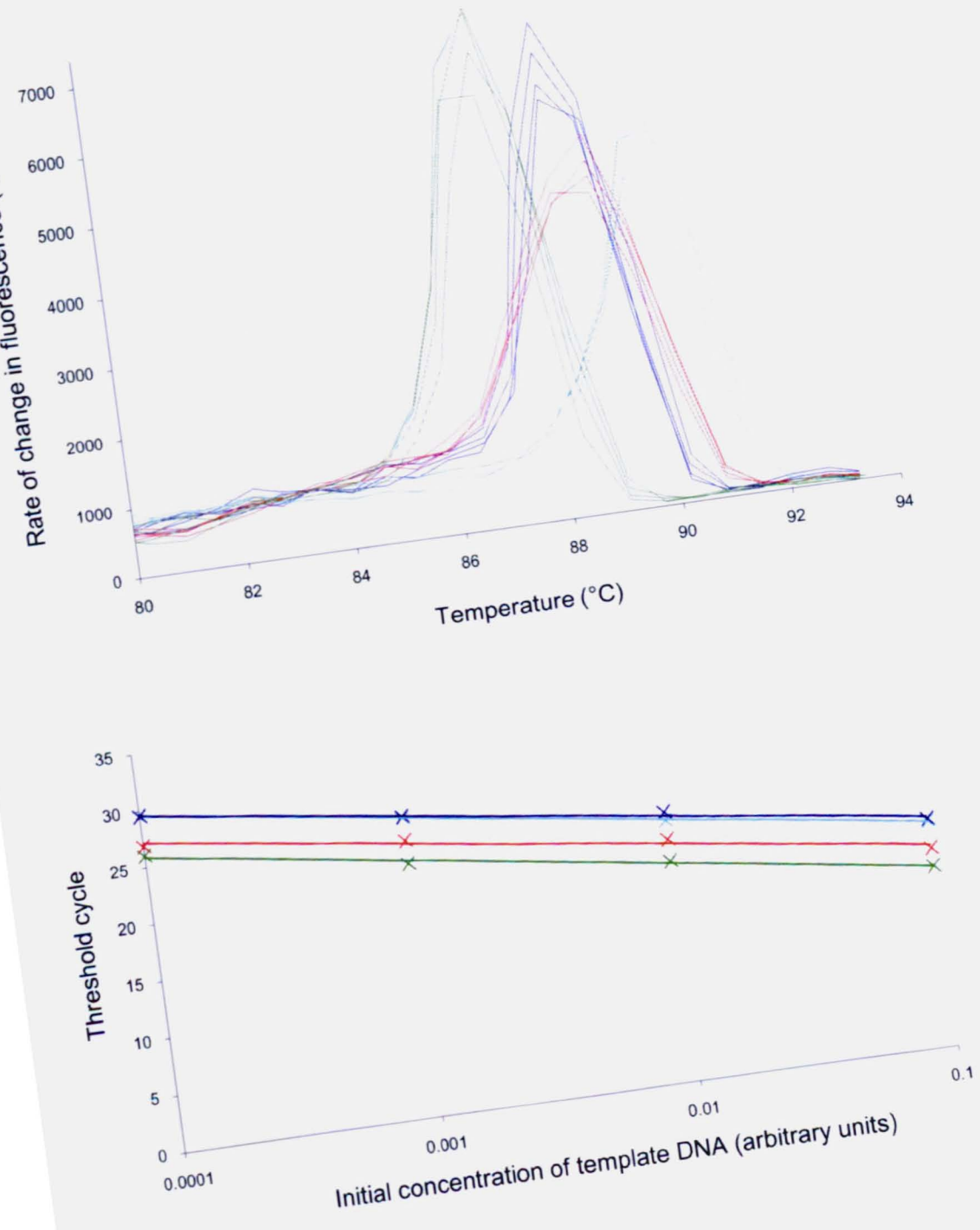
All four primer pairs tested (Table 3.1) successfully amplified *G. pallida* cDNA (Figure 3.1). Dissociation curves with single peaks were observed with all four primer sets, indicating the presence of pure PCR products with little contamination by primer dimers or non-target DNA amplification. Differences in melting temperature could be explained by differences in amplicon size and GC content. The amplification efficiencies of primer pairs one to four were close to 100 %. Primer pair 1 was selected for use in further experiments since it produced a clean product, had an ideal amplification efficiency, its amplicon is near the 3' end of the actin 1 gene coding sequence and the annealing temperatures of the forward and reverse primers are both predicted to be identical.

#### 3.3.1.2 Determination of the accuracy of RT-qPCR for quantification of viable PCN eggs

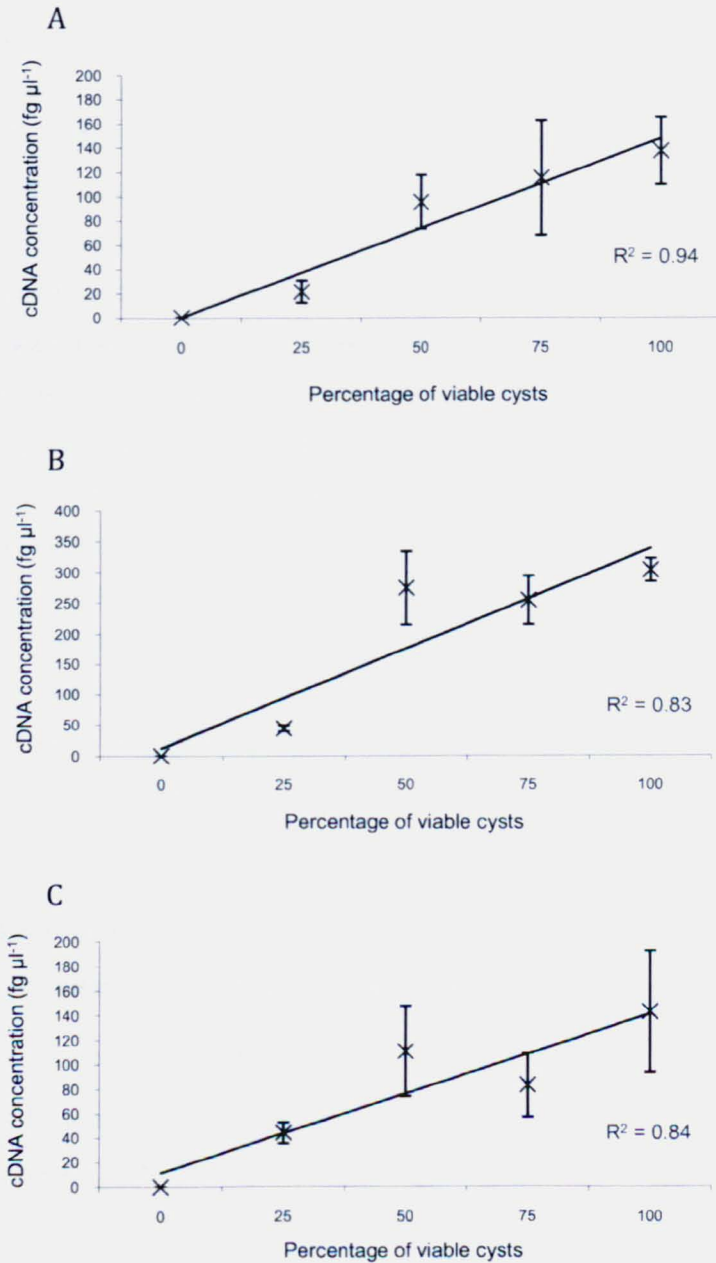
A significant relationship was observed between the proportion of viable cysts in a sample and the concentration of cDNA prepared from that sample (Figure 3.2a). Similar results were obtained when the experiment was repeated in its entirety with new biological samples (Figure 3.2b) and when RT-qPCR was repeated using the same RNA samples as on the second occasion (Figure 3.2c). On this third occasion, the duration of the reverse transcription reaction was reduced to 10 min. This did not increase the correlation between the concentration of cDNA and the proportion of viable cysts, although it did reduce the absolute cDNA yield. The maximum difference between the true percentage of viable cysts in a sample and the percentage predicted by the regression line was 15, 30 and 27 % for experiments 1, 2 and 3, respectively and the mean difference was 7, 12 and 11 %, respectively.

#### 3.3.1.3 Determination of the duration of mRNA persistence *in vitro* and *in soil*

Experiments were conducted to ascertain the duration of persistence of mRNA in *Globodera* eggs after death. Exposure of cysts to saturated solutions of Dazomet or heating to 65 °C were presumed to cause 100 % mortality. Sub-samples of treated and untreated cysts were allowed to hatch in potato root diffusate and treated cysts showed a complete lack of hatching (data not shown).



**Figure 3.1:** Comparison of four primer sets (detailed in Table 3.1) tested for amplification of *G. pallida* cDNA in qPCR (light blue = GpACTF1 + GpACTR1; red = GpACTF1b + GpACTR1; dark blue = GpACTF2 + GpACTR2; green = GpACTF3 + GpACTR3). A) Dissociation curves of qPCR products B) Standard curves used to calculate amplification efficiency.



**Figure 3.2:** Relationship between the proportion of viable cysts in mixtures of viable and non-viable, heat-killed *G. rostochiensis* cysts and the concentration of cDNA preparations made from those cysts. A) Initial assay, in which RNA was extracted from triplicate samples of 20 cysts per treatment and cDNA was produced in reverse transcription (RT) reactions of 50 min duration ( $F = 49.0$ ,  $P = 0.006$ ,  $R^2 = 0.92$ ,  $y = 1.477x + 0.331$ ). B) Repetition of the assay, in which new RNA samples were extracted from another three replicate samples of 20 cysts per treatment and cDNA was produced in RT reactions of 50 min duration ( $F = 14.7$ ,  $P = 0.031$ ,  $R^2 = 0.83$ ,  $y = 3.274x + 11.70$ ). C) Second repetition of the assay, in which new cDNA samples were prepared in RT reactions of 10 min duration using the same RNA samples as prepared during the first repetition of the assay ( $F = 16.0$ ,  $P = 0.028$ ,  $R^2 = 0.84$ ,  $y = 1.297x + 11.21$ ). Error bars show SEM.

In the first experiment, *in vitro* at 20 °C, no mRNA was detected at any time point in cysts that had been exposed to Dazomet or heat (Figure 3.3a). The earliest timepoint was two days post treatment (dpt), although the treatment itself lasted for 11 days. Thus, it is possible to say that mRNA did not persist for longer than 13 days after death. The cDNA concentration in preparations from control cysts was 23.7 ng  $\mu\text{l}^{-1}$  at two dpt and declined exponentially until 36 dpt, when levels were approaching zero.

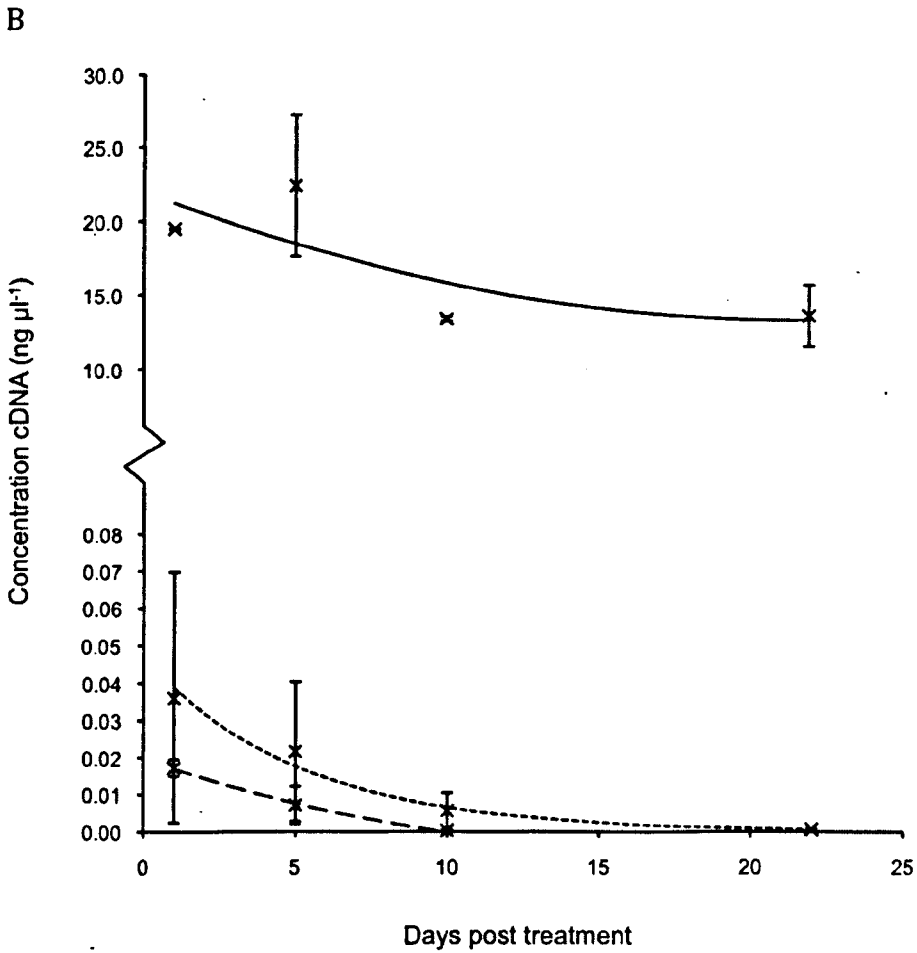
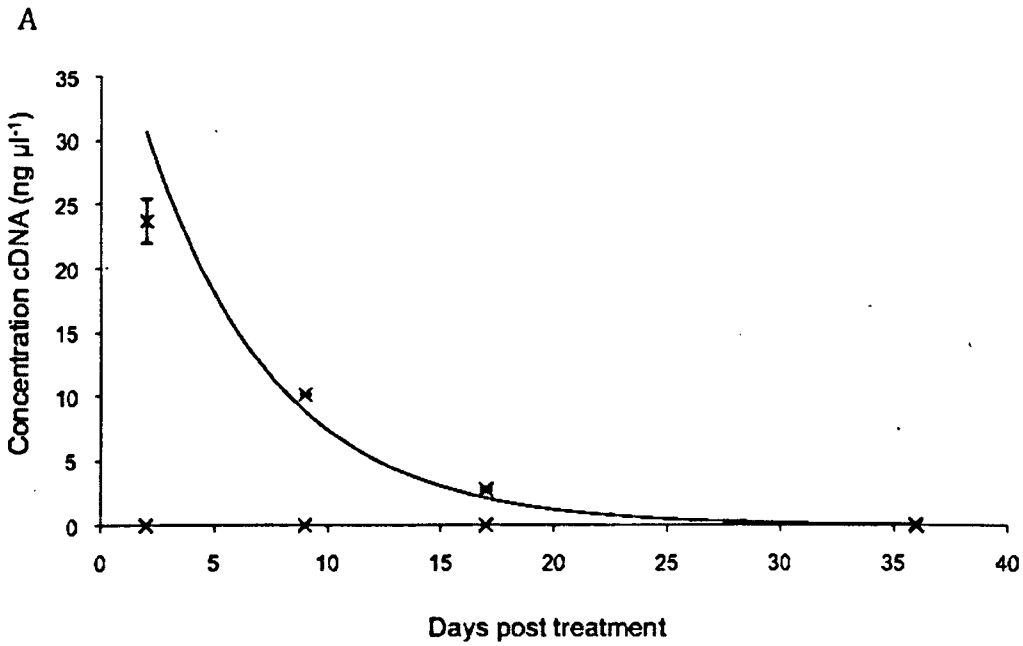
In the second experiment, in soil at 10 °C, the duration of chemical or heat treatment was less than one day and RNA was extracted two days after the beginning of the treatment. By this time, mRNA levels in treated cysts were three orders of magnitude lower than in untreated viable cysts (Figure 3.3b). RNA abundance continued to decline exponentially after treatment with Dazomet or heat. RNA decay appears to have been less rapid in heat-treated than Dazomet-treated cysts. By 10 days post treatment with Dazomet and 22 days post treatment with heat, RNA was effectively undetectable. The concentration of cDNA detected was similar to that detected when water was used instead of cDNA in control PCR, less than 1 fg  $\mu\text{l}^{-1}$ . Abundance of RNA in control cysts declined by 32 % from two to ten dpt then remained constant until the final timepoint at 22 dpt. Concentrations of cDNA from controls cysts at 2 dpt were similar to those in the previous experiment.

### **3.3.2 Glucosinolate content and effects of brassica green manures on viability of *Globodera pallida* encysted eggs in soil**

#### *3.3.2.1 Initial glasshouse trial*

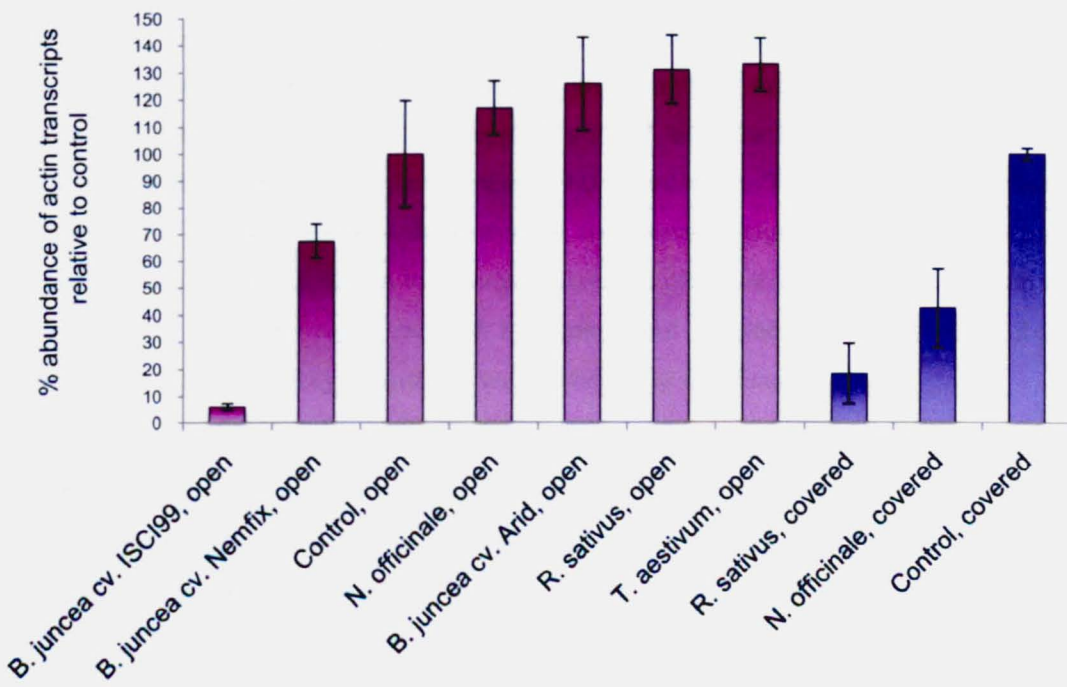
The brassica lines *B. juncea* cv. Nemfix, *N. officinale* and *R. sativus* cv. Weedcheck were selected as promising candidate biofumigant crops on the basis of their performance in the *in vitro* screen (Chapter 2). *B. juncea* cv. ISCI99 was added as it is known to contain very high levels of glucosinolate and *B. juncea* cv. Arid was included as a control with low levels of glucosinolate. Leaf tissue of these plants was incorporated into PCN-infested soil at 5 % (w/w) and the viability of treated PCN eggs was measured by quantifying abundance of *Globodera* actin mRNA.

In open pots, incorporation of *B. juncea* cv. Nemfix and *B. juncea* cv. ISCI99 caused 32.5 and 93.9 % reduction in viability, respectively (Figure 3.4). None of the other brassicas or wheat affected viability in open containers. Two plants that had been ineffective in open pots caused a reduction in viability in polyethylene-covered pots. These were *N.*



**Figure 3.3:** Rate of RNA degradation in encysted *G. pallida* eggs following treatment with Dazomet (long dashed line), heat (short dashed line) or nothing (solid line) A) *in vitro* at 20 °C or B) in soil at 10 °C Note the scale change in B. Error bars show SEM. N = 3.





**Figure 3.4:** Results of an initial glasshouse trial to determine the effects of green manures on encysted eggs of *G. pallida*. Columns indicate the percentage reduction in viability of *G. pallida* eggs in soil after treatment with green manures (5 % w/w) in polyethylene-covered (blue columns) and open (purple columns) soil relative to untreated controls. Viability was measured by quantifying abundance of actin 1 mRNA. Error bars show SEM. N = 5.

*officinale* and *R. sativus* cv. Weedcheck, which caused 57.6 and 81.7 % reduction in viability, respectively. The *B. juncea* cultivars were not tested in covered pots in this experiment. The mean cDNA concentrations in control cysts in open and closed pots were  $11.0 \pm 2.16$  (SEM)  $\text{ng } \mu\text{l}^{-1}$  and  $15.5 \pm 0.36$  (SEM)  $\text{ng } \mu\text{l}^{-1}$ .

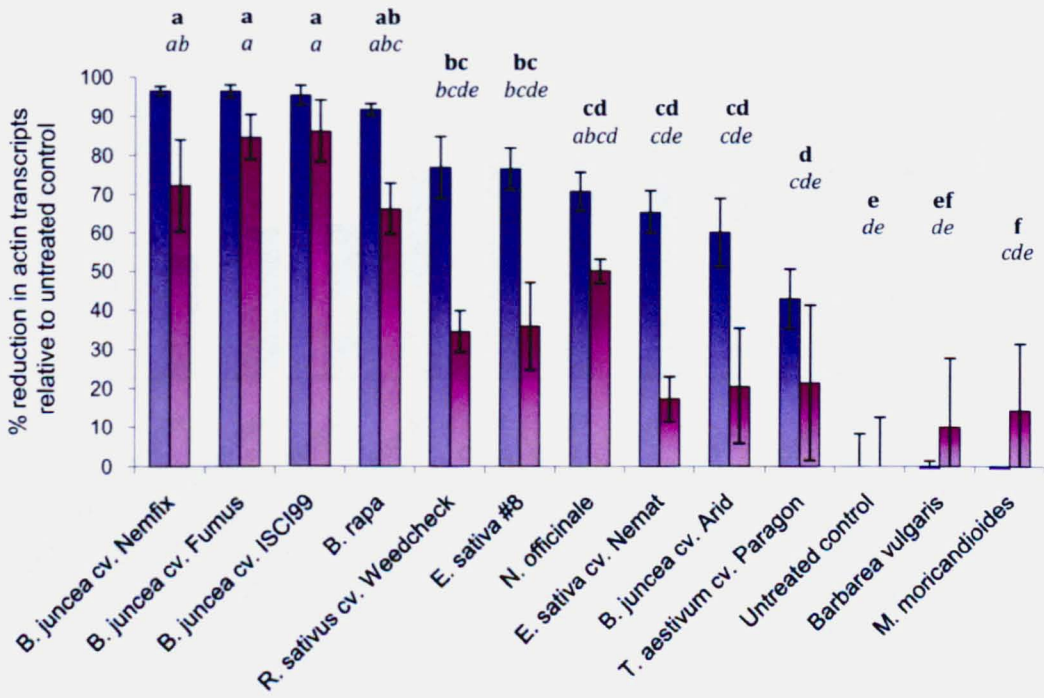
### 3.3.2.2 Full glasshouse trial

A more extensive glasshouse trial was carried out using 11 brassica lines all tested in open and polyethylene-covered soil. In covered soil, all of the green manures besides *B. vulgaris* and *M. moricandioides* caused a significant reduction in the abundance of mRNA (Figure 3.5). The high-glucosinolate *B. juncea* cultivars had the strongest effect, each causing >95 % reduction in abundance of actin 1 transcripts. *B. rapa* was almost as effective, causing a 92 % decline. *R. sativus* cv. Weedcheck, *E. sativa* #8, *N. officinale* and *E. sativa* cv. Nemat each caused between 65 and 77 % reduction in mRNA. The low-glucosinolate *B. juncea* cultivar, Arid, caused a 60 % reduction. The non-brassica, wheat, while not as effective as most of the brassicas, still caused a significant 43 % reduction in mRNA levels. Levels of mRNA in *B. vulgaris* treated cysts were 12.4 % higher than in controls, which was not significant. *M. moricandioides* treated cysts contained 52 % more mRNA than control cysts and this was statistically significant.

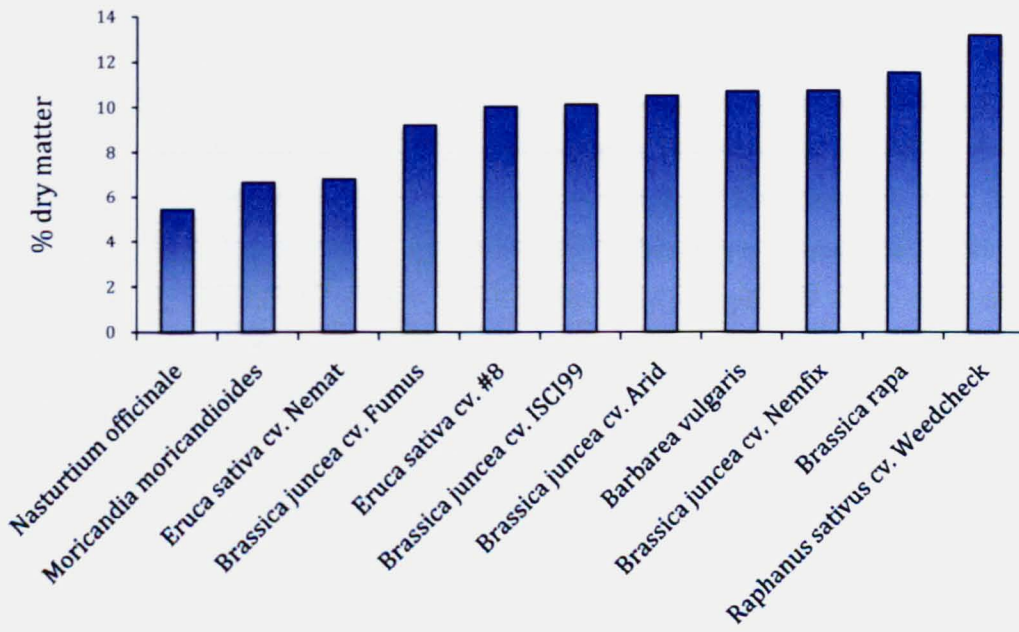
In open soil, the high glucosinolate *B. juncea* cultivars were still the most effective treatments, although the order of efficacy was slightly different from that with covered pots (Figure 3.5). As in covered soil, *B. rapa* was the fourth most effective green manure, causing a 66 % decline in mRNA abundance. None of the other treatments had a statistically significant effect in open containers, partly due to the greater variability in the data than in covered containers. Although not statistically significant, *N. officinale* caused a 50 % reduction in actin transcripts. This was more effective than expected from its performance in covered containers.

The mean cDNA concentrations in untreated control cysts in open and covered pots were  $130 \pm 16$  (SEM)  $\text{ng } \mu\text{l}^{-1}$  and  $69 \pm 5.6$  (SEM)  $\text{ng } \mu\text{l}^{-1}$ , respectively. As in the initial glasshouse trial, variation in *G. pallida* actin 1 mRNA was greater in the untreated open pots controls than in the untreated covered pots. The mean number of cysts per sample was  $63.6 \pm 0.232$  (SEM) and this did not differ significantly between treatments. Thus, differences in mRNA abundance were due to variation in concentration per cyst.

Water content at the time of incorporation varied between brassicas (Figure 3.6). Most of the plants had similar dry matter contents between 9 and 11 % dry matter. Three



**Figure 3.5:** Results of a glasshouse trial to determine the effects of green manures on encysted eggs of *G. pallida*. Columns indicate the percentage reduction in viability of *G. pallida* eggs in soil after treatment with green manures (5 % w/w) in polyethylene-covered (blue columns) and open (purple columns) soil relative to untreated controls. Viability was measured by quantifying abundance of actin 1 mRNA. Error bars show SEM. N = 5. Separate ANOVA with REGWQ post-hoc tests were performed on data for open and covered soil treatments. Bold letters indicate homogenous subsets within covered soil treatments and italicised letters indicate homogenous subsets within the open soil treatments. Error bars show SEM. N = 5.



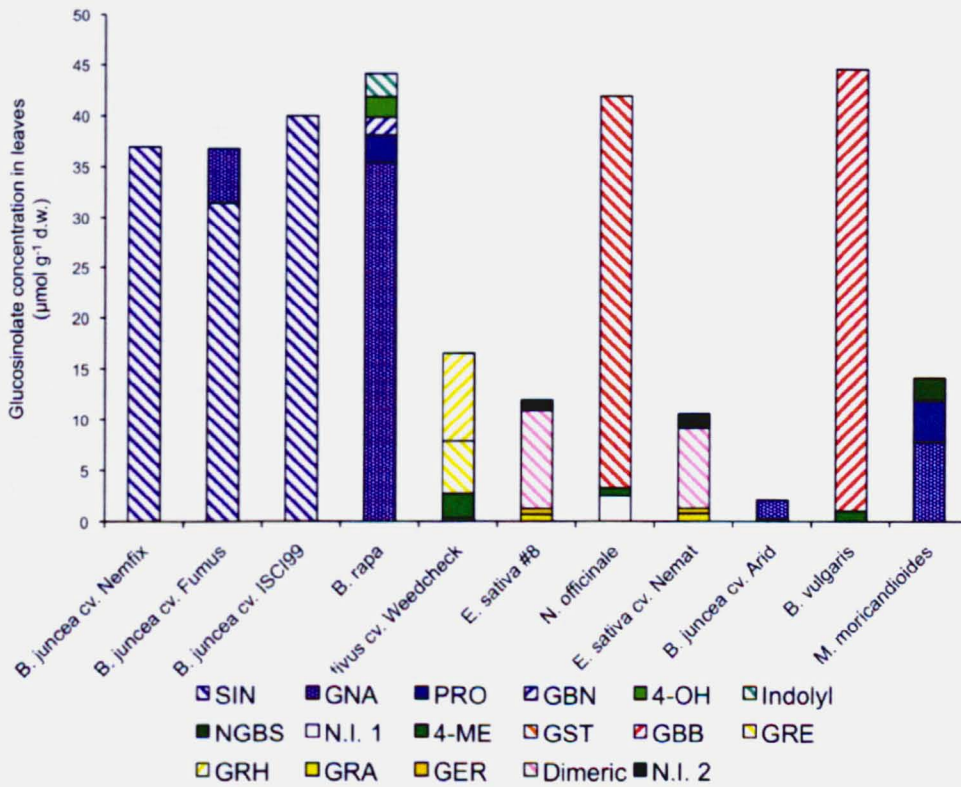
**Figure 3.6:** Dry matter content of brassicas at the time of incorporation into soil in the full glasshouse trial.

plants, *N. officinale*, *M. moricandioides* and *E. sativa* cv. Nemat had high moisture content, each with less than 7 % dry matter while two plants, *B. rapa* and *R. sativus*, contained relatively low levels of moisture, with 11.5 and 13.2 % dry matter, respectively. This probably explains part of the variation in efficacy among brassica lines.

### 3.3.2.3 Plant glucosinolate profiles

The glucosinolate profiles of brassicas tested in the glasshouse trial were determined by HPLC. In terms of total glucosinolate content, the plants could be divided into groups; six high glucosinolate plants containing more than 35  $\mu\text{mol g}^{-1}$  d.w. and five low glucosinolate plants containing less than 17  $\mu\text{mol g}^{-1}$  d.w. (Figure 3.7). Among the high glucosinolate plants were the three *B. juncea* cultivars that were highly effective against PCN eggs (Figure 3.5). These cultivars shared similar glucosinolate profiles. *B. juncea* cv. ISCI99 and *B. juncea* cv. Nemfix each contained between 36 and 40  $\mu\text{mol g}^{-1}$  d.w. 2-propenyl glucosinolate as the sole glucosinolate (Figure 3.7). *B. juncea* cv. Fumus contained 31.4  $\mu\text{mol g}^{-1}$  d.w. 2-propenyl, as well as 5.4  $\mu\text{mol g}^{-1}$  d.w. 3-butenyl glucosinolate. *B. rapa* contained predominantly 3-butenyl glucosinolate (80 % of total) as well as roughly equal quantities of 4-pentenyl, (*R*)-2-hydroxy-3-butenyl, indolyl and 4-hydroxyindol-3-ylmethyl glucosinolate. The other two high-glucosinolate plants were *Nasturtium officinale* and *Barbarea vulgaris*, which contained 41.9 and 44.6  $\mu\text{mol g}^{-1}$  d.w. total glucosinolates. *N. officinale* contained predominantly 2-phenylethyl glucosinolate (92 %) with a small proportion of an unidentified glucosinolate (6 %) and 4-methoxyindol-3-ylmethyl glucosinolate (2 %). *B. vulgaris* contained almost exclusively 2-(*R*)-hydroxy-2-phenylethyl glucosinolate (98 %) with a small proportion of 4-methoxyindol-3-ylmethyl glucosinolate (2 %).

The brassicas with lower levels of total glucosinolate were *Raphanus sativus* cv. Weedcheck, the two *Eruca sativa* cultivars, *B. juncea* cv. Arid and *Moricandia moricandioides*. *R. sativus* cv. Weedcheck contained a total of 16.5  $\mu\text{mol glucosinolate g}^{-1}$  d.w., of which 53 % was 4-methylthio-3-butenyl, 31 % was 4-methylsulphinyl-3-butenyl, 15 % was 4-methoxyindol-3-ylmethyl and 2 % was 4-hydroxyindol-3-ylmethyl glucosinolate. The two *E. sativa* cultivars had similar profiles. *E. sativa* #8 contained 11.9  $\mu\text{mol g}^{-1}$  d.w. total glucosinolate, 12 % more than *E. sativa* cv. Nemat. This difference could be accounted for by the lower concentration of the predominant glucosinolate in Nemat. This glucosinolate formed a dimer during HPLC and has been putatively identified as 4-mercaptobutyl glucosinolate (Bennett *et al.*, 2002). *E. sativa* #8 and *E. sativa* cv. Nemat contained, respectively, 82 % and 75 % mercaptobutyl



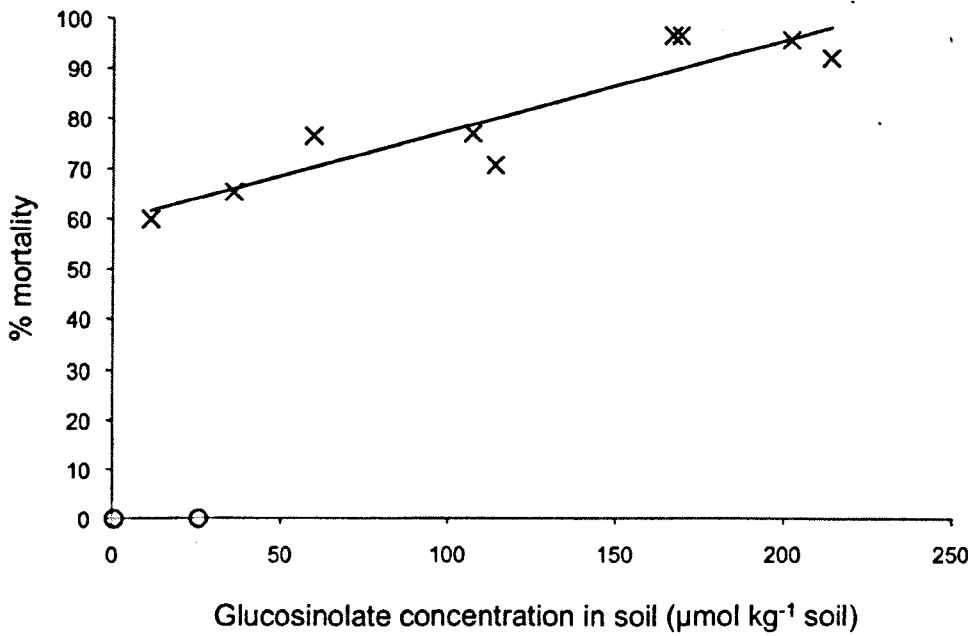
**Figure 3.7:** Glucosinolate profiles of brassicas at the time of incorporation into soil in the full glasshouse trial. The key to the glucosinolates is as follows: SIN = 2-propenyl, GNA = 3-butenyl, PRO = (*R*)-2-hydroxy-3-butenyl, GBN = 4-pentenyl, 4-OH = 4-hydroxyindol-3-ylmethyl, Indolyl = indolyl, NGBS = 1-methoxyindol-3-ylmethyl, N.I. 1 = not identified 1, 4-ME = 4-methoxy-indol-3-ylmethyl, GST = 2-phenylethyl, GBB = 2(*R*)-hydroxy-2-phenylethyl, GRE = 4-methylsulphinyl-3-butenyl, GRH = 4-methylthio-3-butenyl, GRA = 4-(methylsulphinyl)butyl, GER = 4-(methylthio)butyl, dimeric = putatively identified as sativin, N.I. 2 = not identified 2. Aliphatic glucosinolates are shown in blue, indolyl in green, thiofunctionalised in yellow and aromatic in red.

glucosinolate, 8 % and 13 % of an unidentified glucosinolate different to that found in *N. officinale*, 6 % and 7 % 4-(methylsulphinyl)butyl and 5 % each 4-(methylthio)butyl glucosinolate. *B. juncea* cv. Arid contained 2.1  $\mu\text{mol g}^{-1}$  d.w. total glucosinolate, of which 89 % was 3-butenyl and 11 % was 2-propenyl glucosinolate. *M. moricandioides* contained 14.1  $\mu\text{mol g}^{-1}$  d.w. total glucosinolate, of which 55 % was 3-butenyl, 29 % was (*R*)-2-hydroxy-3-butenyl and 16 % was 1-methoxyindol-3-ylmethyl glucosinolate.

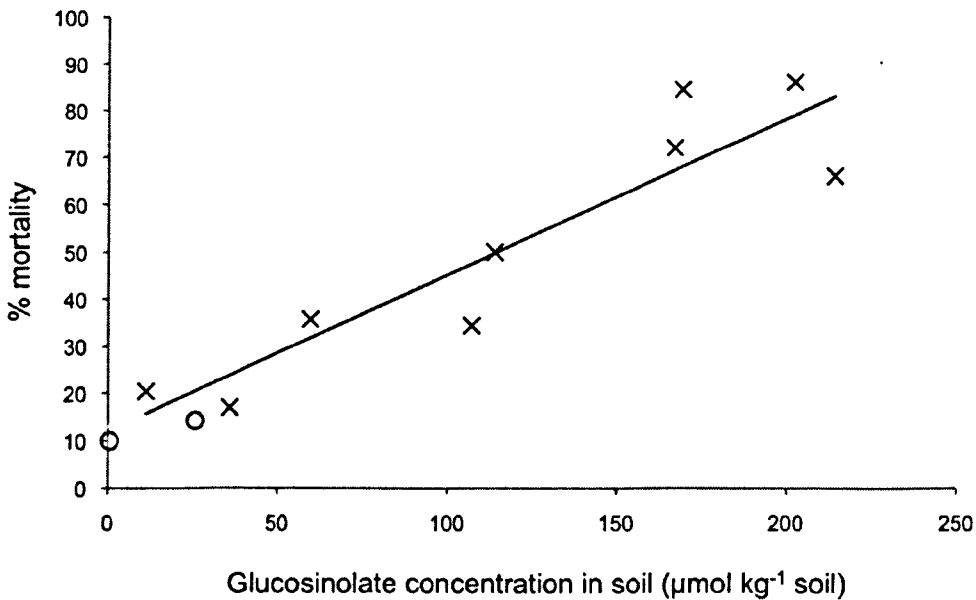
#### 3.3.2.4 Relationship between glucosinolate content and efficacy in soil

There were significant correlations between the isothiocyanate-producing glucosinolate content of the brassica green manures and mortality in both the covered ( $R^2 = 0.839$ ,  $P = 0.001$ ) and open containers ( $R^2 = 0.843$ ,  $P < 0.001$ ) (Figure 3.8). In open containers the high  $R^2$  value and a predicted intercept for the regression line close to the origin suggest that isothiocyanates were the sole cause of the observed mortality. In contrast, with covered containers, the intercept is much higher, around 60 % when the two ineffective green manures (*B. vulgaris* and *M. moricandioides*) are excluded from the analysis. This suggests that some of the mortality caused by the effective green manures in covered containers was independent of isothiocyanate production. In contrast to the above results, there was no significant correlation between the total glucosinolate content of brassica green manures and mortality of *G. pallida* eggs in either open or polyethylene-covered soil.

A



B



**Figure 3.8:** Correlations between total isothiocyanate-producing glucosinolate in effective plants (crosses) and percentage mortality of *G. pallida* eggs after incorporation of brassica green manures into infested soil in (A) polyethylene-covered and (B) open containers. The ineffective plants *Barbarea vulgaris* and *Moricandia moricandioides* (circles) have been excluded from the analysis. There were significant correlations with mortality in covered containers ( $R^2 = 0.839$ ,  $P = 0.001$ ) and open containers ( $R^2 = 0.843$ ,  $P < 0.001$ ).



## 3.4 Discussion

### 3.4.1 Reverse transcription quantitative PCR to quantify viable PCN eggs

RT-qPCR was found to be a reliable means of quantifying viable PCN eggs. Within two days of death, the concentration of actin 1 mRNA transcripts in encysted eggs killed by heat or by Dazomet was three orders of magnitude lower than that in viable untreated cysts (Figure 3.3). In this preliminary experiment, there was a close relationship between the proportion of viable eggs in a sample and the estimated abundance of mRNA (Figure 3.2). Further improvements were made to the experimental design in the full glasshouse trial in order to minimise biological variation; only cysts with a diameter between 500 and 600  $\mu\text{m}$  were used, the number of cysts per RNA sample was more than doubled and the number of replicates was increased from three to five. The final experimental design was powerful enough to detect significant differences between cysts that differed in mRNA abundance by a factor of 1.25 (compare treatments with *B. juncea* ISCI99 and *R. sativus* cv. Weedcheck in covered pots, Figure 3.5).

DNA-based qPCR methods simultaneously developed at other laboratories are similarly accurate. Toyota *et al.* (2008) found a significant relationship between the amount of viable *Globodera* eggs in a sample and the cycle threshold determined in qPCR with SYBR Green. The mean difference between minimum and maximum Ct values in triplicate PCR was 0.5 cycles, corresponding to a 30 % difference in the number of viable eggs. Adams *et al.* (2009) spiked 2-200 eggs into 250 g soil, carried out direct DNA extraction from soil and quantified the abundance of PCN DNA using TaqMan qPCR. There was a very close relationship between the threshold cycle and the number of eggs in the sample ( $R^2 = 0.92$  for *G. pallida* and 0.98 for *G. rostochiensis*).

The methods of Toyota *et al.* (2008) and Adams *et al.* (2009) rely on DNA being a good indicator of viability. Adams *et al.* (2009) state, "in the harsh environment of soil, DNA is unlikely to survive in a detectable form for very long". This has not been tested but DNA persisted in fumigated cysts *in vitro* for at least one month after treatment (DEFRA, 2004) and DNA persisted for longer than RNA in bacterial cells killed with antibiotics (Aellen *et al.*, 2006). The present study has shown that RNA degrades in encysted PCN eggs in soil within two days of death. In this regard, an RT-qPCR method may be advantageous. However, changes in the abundance of mRNA are likely to occur in living eggs, especially in the transition from diapause to hatching, which can occur upon hydration (Oostenbrink, 1950 cited by Turner & Evans, 1998). Precautions were taken to prevent variation in mRNA within viable cysts. The mRNA target was actin 1, which is

constitutively expressed (Kovalena *et al.*, 2005). Cysts were hydrated in soil prior to the experiment and extreme care was taken to treat all samples identically. Separate control treatments were used for the open and covered pot treatments in case environmental differences between these treatments caused variation in gene expression.

### 3.4.2 Glucosinolate profiles and effects of brassica green manures on viability of *Globodera pallida* encysted eggs in soil

Pot trials showed that brassica green manures can have very substantial effects on the viability of *G. pallida* eggs in soil. Incorporated into infected soil at a rate of 5 % (w/w), three *B. juncea* cultivars caused over 70 % mortality. When the soil was covered with polyethylene, mortality was increased to over 95 %. This compares favorably with levels of control provided by synthetic fumigants. For instance, in fields in northern England, metham sodium or dichloropropene fumigants caused mean mortality rates of 60 and 64 %, respectively (Storey, 1982). Fumigation with 1,3-dichloropropene in clay soils in the Netherlands caused from 48 to 72 % mortality (Been & Schomaker, 1999).

Aires *et al.* (2009) added sufficient powdered brassica leaf tissues to provide 0.2  $\mu\text{mol}$  glucosinolate per 100 g soil to the soil around potatoes growing in *G. rostochiensis* infected soil. The rate of multiplication of *G. rostochiensis* was reduced by as much as 97 % compared to control. In the present study, the amount of material incorporated was sufficient to provide a glucosinolate concentration at least two orders of magnitude greater yet percentage reduction in viable PCN was less. These observations are difficult to reconcile and it seems likely that Aires *et al.* (2009) were mistaken in the concentration of glucosinolate added to soil. They state that they incorporated sufficient *N. officinale* leaf material containing 34.4  $\mu\text{mol}$  glucosinolate  $\text{g}^{-1}$  d.w. to achieve a concentration of 0.2  $\mu\text{mol}$  glucosinolate  $100 \text{ g}^{-1}$  soil. This would mean incorporating the material at a rate of 0.0058 % (w/w), which seems unlikely.

A close correlation was observed between the toxicity of brassica green manures to *G. pallida* and their content of isothiocyanate-producing glucosinolate (Figure 3.8), implicating isothiocyanates as the main cause of toxicity. Both *B. vulgaris* and *M. moricandioides* lacked efficacy against *G. pallida* (Figure 3.5). These plants contained large quantities of indole glucosinolate or glucosinolates containing a hydroxyl group at the C-2 (Figure 3.6), neither of which form stable isothiocyanates (Halkier & Gershenzon, 2006). In open soil, the concentration of isothiocyanate-liberating glucosinolate was the principal determinant of the nematicidal activity of brassica green

manures (Figure 3.8). In covered soil, other factors may have been influential since there was a predicted baseline of around 60 % mortality independent of glucosinolate content. These findings are similar to those of Potter *et al.* (1998), who found a weak, non-significant correlation between leaf glucosinolate content and suppression of *Pratylenchus neglectus* ( $R^2 = 0.48$ ,  $P > 0.05$ ,  $N = 6$ ) with an intercept at approximately 50 %. In contrast, McLeod & Steele (1999) found suppression of *Meloidogyne javanica* to be unrelated to glucosinolate content, although in their study brassica leaf tissues were only coarsely chopped, rather than thoroughly pulverized, which would have impeded glucosinolate hydrolysis and thus isothiocyanate production.

*B. juncea* cv. Arid caused 60 % mortality in covered soil, despite containing only 1.9  $\mu\text{mol g}^{-1}$  d.w. 3-butenyl glucosinolate and 0.2  $\mu\text{mol g}^{-1}$  d.w. 2-propenyl glucosinolate. *M. moricandioides* contained four times this concentration of 3-butenyl glucosinolate yet caused no significant mortality. This suggests that other products besides glucosinolate catabolites also contributed to the nematicidal activity of *B. juncea* in covered soil. In addition to the glucosinolate hydrolysis products, decomposing brassica tissues produce other volatile sulphur-containing toxins, including dimethyl sulphide, dimethyl disulphide, carbon disulphide and methanethiol (Lewis & Papavizas, 1970; Bending & Lincoln, 1999; Wang *et al.*, 2009). These compounds are toxic to nematodes (Chapman & Parker, 1929; Roskopf *et al.*, 2006; Gu *et al.*, 2007) and carbon disulphide acts synergistically with methyl isothiocyanate in toxicity to fungi (Canesa & Morrell, 1995). Amounts of methyl sulphide and dimethyl disulphide emitted by different brassica green manures has been found to vary over several orders of magnitude and to be inversely proportional to the density of *Verticillium dahliae* in bioassay potato plants (Wang *et al.*, 2009). Emission of these compounds from decomposing brassica tissues is favoured under anaerobic conditions (Forney *et al.*, 1991; Gamliel & Stapleton, 1993). Such conditions can develop following incorporation of green manures into soil subsequently covered with plastic (Blok *et al.*, 2000) and may have occurred in the covered soil microcosms in the present study due to the high moisture content of the soil in the covered containers. Despite attempts to control soil moisture content, the open soil did dry out between weekly watering events, while the covered soil remained between field capacity and completely waterlogged. When the coverings were removed for addition of water, unpleasant odours characteristic of anaerobic conditions were observed. A hypothesis to explain the observations in the present study is that covering the soil led to anaerobic conditions, under which volatile sulphur containing compounds besides glucosinolate catabolites were produced in varying quantities by decomposing brassica tissues and influenced the mortality of PCN eggs. The fact that Potter *et al.*

(1998) also observed a baseline of 50 % nematode suppression when brassica tissues were added to sealed soil microcosms is consistent with this hypothesis. Trapping of isothiocyanates by the polyethylene is another potential explanation but polyethylene is permeable to methyl isothiocyanate (Austerweil *et al.*, 2006). Larger isothiocyanates would be expected to permeate less rapidly but not to be trapped altogether.

The most effective plants in the present study were the *B. juncea* cultivars, which produced low volatility 2-propenyl isothiocyanate. *R. sativus* cv. Weedcheck and *E. sativa* contained less than half the total glucosinolate of the *B. juncea* cultivars. As discussed in section 2.4.3, it is unlikely that the isothiocyanates produced by *R. sativus* and *E. sativa* are especially potent against PCN. It seems more likely that these plants produce high levels of other toxins such as those mentioned above. It was noted that *R. sativus* leaf extracts possess a strong odour similar to that of boiled cabbage. Such odours from tend to be caused by dimethyl sulphide and methanethiol (Forney *et al.*, 1991).

### 3.5 Summary

The main outcomes from this chapter are as follows:

1. An RT-qPCR method was developed that is capable of accurately quantifying viable PCN eggs in mixtures of viable and non-viable cysts.
2. Actin 1 mRNA was shown to degrade within two days of encysted eggs being killed by heat or the soil fumigant Dazomet.
3. Green manures of brassica cultivars incorporated into *G. pallida*-infected soil at 5 % (w/w) were found to reduce the population density by up to 95 %.
4. Leaf glucosinolate profiles of the brassica cultivars were determined by HPLC. The concentration isothiocyanate-producing glucosinolate was the principal determinant of the toxicity of these brassica tissues towards *G. pallida*.
5. Other compounds besides the glucosinolate hydrolysis products appear to have contributed to the toxicity of brassica green manures in covered soil, possibly due microbial activity under anaerobic conditions.

## Chapter 4: Activity of isothiocyanates and brassica green manures toward *Rhizoctonia solani*

### 4.1 Introduction

#### 4.1.1 Toxicity of brassica-derived volatiles to microorganisms

*In vitro* studies have shown that the growth of a wide range of microorganisms is inhibited when exposed to volatiles from brassica tissues (Angus *et al.*, 1994; Sarwar *et al.*, 1998; Olivier *et al.*, 1999; Yulianti *et al.*, 2006; Larkin & Griffin, 2007; Mattner *et al.*, 2008; Fan *et al.*, 2008). These volatile mixtures include isothiocyanates, thiocyanates and nitriles derived from glucosinolates, non-glucosinolate-derived sulphur compounds, such as dimethyl disulfide, terpenes, aromatic compounds, such as benzaldehyde, and various straight chain hydrocarbons from the lipoxygenase pathway (Rohloff & Bones, 2005). Many of these compounds possess fungistatic activity, including isothiocyanates (Drobnica *et al.*, 1967; Angus *et al.*, 1994; Vaughn *et al.*, 1997; Olivier *et al.*, 1999; Sarwar *et al.*, 1998; Manici *et al.*, 1997, 2000; Smith & Kirkegaard, 2002; Yulianti *et al.*, 2006), thiocyanates (Angus *et al.*, 1994), dimethyl disulfide (Xu *et al.*, 2004), benzaldehyde (Xu *et al.*, 2004) and several C<sub>6</sub> and C<sub>9</sub> aldehydes of the lipoxygenase pathway (Hamilton-Kemp *et al.*, 1992; Vaughn & Gardner, 1993). Of these, the most active compounds towards fungi appear to be the isothiocyanates since the concentration of isothiocyanate emitted by various brassica homogenates correlates with *in vitro* fungal suppression (Olivier *et al.*, 1999). When the fungistatic activity of pure compounds has been tested, isothiocyanates have been found to be more toxic than other brassica-derived volatiles (Angus *et al.*, 1994; Manici *et al.*, 2000). For instance, butyl thiocyanate and 2-phenylethyl isothiocyanate are emitted from chopped roots of *B. juncea* (Angus *et al.*, 1994). Both are toxic to the fungal wheat pathogen *Gaeumannomyces graminis* but the ED<sub>50</sub> of 2-phenylethyl isothiocyanate is approximately one order of magnitude lower than that of butyl thiocyanate (Angus *et al.*, 1994).

There is considerable variation in sensitivity to isothiocyanates amongst soil-borne microorganisms (Drobnica *et al.*, 1967; Manici *et al.*, 1997; Smith & Kirkegaard, 2002). Smith & Kirkegaard (2002) carried out an extensive study of the sensitivity of various pathogenic and non-pathogenic soil-borne microorganisms to 2-phenylethyl isothiocyanate. ED<sub>50</sub> values ranged from around 5 - 20 µM for sensitive species such as *G. graminis* and *Pythium sulcatum* to as much as 2 - 3 mM for tolerant species such as a

*Trichoderma* sp. (Smith & Kirkegaard, 2002). *R. solani* was intermediately sensitive to 2-phenylethyl isothiocyanate with ED<sub>50</sub> values ranging from 53 to 900 µM (Smith & Kirkegaard, 2002).

#### 4.1.2 Different anastomosis groups of *R. solani* may differ in sensitivity to isothiocyanates

The degree of intra-species variation in sensitivity to isothiocyanates shown by *R. solani* is unusually high compared with other species (Smith & Kirkegaard, 2002). This is perhaps unsurprising given that different anastomosis groups (AG) of *R. solani* are genetically isolated from one another and differ in physiology and host range (Cubeta & Vilgalys, 1997). It would be valuable to know if AG differ in susceptibility to isothiocyanates since different AG are problematic in different cropping systems. For instance, over 90 % of isolates from UK potato crops belong to AG3 (Woodhall *et al.*, 2007). From the data of Smith & Kirkegaard (2002), it was not possible to test the hypothesis that AG differ in sensitivity to isothiocyanates, although AG2-1, which is pathogenic on brassicas (Budge *et al.*, 2009), did tend to be more tolerant (Smith & Kirkegaard, 2002).

#### 4.1.3 Aims

1. Evaluate the toxicity of a range of isothiocyanates with diverse variable group structures to *R. solani*.
2. Determine if anastomosis groups of *R. solani* differ in sensitivity to isothiocyanates.
3. Quantify the effects of brassica green manures on *R. solani* inoculum density and pathogenic activity.
4. Determine if any suppression of *R. solani* by brassica green manures is due to glucosinolate content.

## 4.2 Methods

### 4.2.1 Toxicity of isothiocyanates to *Rhizoctonia solani*

#### 4.2.1.1 Isolation of a new *R. solani* isolate and determination of anastomosis group

A seed tuber (*Solanum tuberosum* L. var. Desiree, grade E2, Reg. No. UK/S 1409, Crop ID No. 05 1409 03 01) was rinsed in water and sclerotia of *R. solani* were observed. Several pieces of sclerotium were transferred to potato dextrose agar (PDA) (39.0 g l<sup>-1</sup>, Oxoid, CM0139) containing 0.6 % penicillin G sulphate (Sigma, P3032) and 2.0 % streptomycin sulphate (Sigma, S6501) and incubated at 18 °C in the dark. After three days growth, sections of media containing uncontaminated hyphae of *R. solani* were transferred to fresh unamended PDA to obtain pure cultures. These cultures were incubated at 18 °C in the dark and after three weeks, two samples of sclerotia were removed and used for DNA extraction.

DNA was extracted using a modified version of the protocol accompanying Invitrogen's ChargeSwitch gDNA Plant Kit (Invitrogen, CS18000). Samples were ground in 1.5 ml lysis buffer, 100 µl 10 % SDS was added, samples were vortexed and incubated at room temperature (23 °C) for five minutes before 400 µl cold (4 °C) precipitation buffer was added and mixed in by vortexing. The precipitate was removed by vortexing and the supernatant was transferred to a new tube containing 40 µl CST magnetic beads (from the Invitrogen kit). A magnetic Pickpen (Bio-Nobile, 1M) was used to transfer the DNA, bound to the magnetic beads, through the following solutions: 100 µl detergent, two separate 1 ml volumes of wash buffer (from the Invitrogen kit) and finally 150 µl elution buffer (TE). Each time the beads were transferred, they were released from the pickpen and thoroughly resuspended. Purity and concentration of DNA samples was checked using a spectrophotometer (Nanodrop ND-1000, Labtech International).

The DNA from the two samples of the new isolate, as well as DNA from isolates 1936 (AG3, positive control) and Y3 (AG2-1, negative control) were used in TaqMan PCR with primers and probe specific to AG3 β-tubulin (Table 4.2). PCR master mix was prepared as follows: 2.5 µl reaction<sup>-1</sup> 25 mM buffer; 5.5 µl reaction<sup>-1</sup> 10 mM MgCl<sub>2</sub>; 2.0 µl reaction<sup>-1</sup> dNTPs (7.5 µM each), 1.0 µl reaction<sup>-1</sup> of 7.5 µM forward primer, 1.0 µl reaction<sup>-1</sup> of 7.5 µM reverse primer, 0.5 µl reaction<sup>-1</sup> 5 µM probe; 0.125 µl reaction<sup>-1</sup> Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, 104AP04-02) and 11.4 µl reaction<sup>-1</sup> water (molecular biology grade, BDH Biomedical, 443847D). One microlitre of each DNA sample (containing ~30 ng DNA) and a water negative control were added to 24 µl of

master mix in wells of a PCR plate (96 well half skirt PCR microplate, clear, Axygen Scientific, 321-63-051) in duplicate. Plates were sealed with sealing strips (PCR strip caps, Axygen Scientific, 321-11-071), micro-centrifuged to remove air bubbles and placed in a thermal cycler (ABI Prism 7900HT Sequence Detection System) and subjected to the following thermal profile: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Wavelengths used for fluorophore excitation and fluorescence detection were appropriate for FAM and TAMRA fluorophore and quencher and Ct values were calculated automatically using SDS 2.2.2 software.

#### 4.2.1.2 Culture of isolates

*R. solani* isolates (Table 4.1) were selected in order to give good coverage of the AG capable of causing disease on potato, especially AG2-1 and AG3, the principal groups infecting potato in the UK (Woodhall *et al.*, 2007). Sufficient isolates of AG2-1, AG3 and AG4 were tested to allow statistical comparison of their susceptibility to isothiocyanate toxicity. Aside from the newly isolated "Rs08JL", the isolates were from existing laboratory cultures maintained on potato dextrose agar (PDA) or stored as infected barley seed at -20 °C. All isolates were maintained on PDA plates (39.0 g l<sup>-1</sup>, Oxoid, CM0139) incubated at 18 °C.

#### 4.2.1.3 Addition of test compounds to culture medium

The isothiocyanates tested were the same as those tested against *G. pallida* (Table 2.1). In addition, three fungicides were tested. These were Azoxystrobin (proprietary name Amistar, Bayer Crop Science), Pencycuron (proprietary name Monceren, Bayer Crop Science) and metham sodium (Sigma, 45570), a methyl isothiocyanate-producing synthetic fumigant. Two-fold dilution series of ITC or fungicide were prepared in ethanol at 800x final concentration. Bottles containing 800 ml molten PDA (39.0 g l<sup>-1</sup>, Oxoid, CM0139) were prepared and allowed to cool to 50 °C in a water bath. Then, in a laminar flow hood, 1 ml ITC/ethanol mixture was added and mixed in by inversion for 10 min or until dissolved and the chemically-amended agar was poured into Petri dishes (Sterilin, 101VR20), 20 ml per plate, taking care to keep any undissolved ITC well dispersed. There were three replicates of each of ten concentrations of ITC or fungicide and six replicates of the ethanol control. Once solidified, plates were sealed with a strip of tape and stored in the dark at 18 °C overnight.



**Table 4.1:** *Rhizoctonia solani* isolates tested.

<b>Code<sup>1</sup></b>	<b>AG</b>	<b>Original host</b>	<b>Geographical origin</b>	<b>Source<sup>2</sup></b>	<b>Reference</b>
<b>Y3</b>	2-1	Potato	Unknown	1	
<b>Y25</b>	2-1	Potato	Unknown	1	Woodhall <i>et al.</i> (2008)
[ <b>2015</b> ]	2-1	Brassica	UK	6	
<b>UN (R112)</b>	3	Potato	Shropshire, UK	1	Woodhall <i>et al.</i> (2007)
<b>Rs08JL</b>	3	Potato	Scotland	New isolate	
(R8) [ <b>1900</b> ]	3	Potato	Scotland	3	
<b>HARC3</b>	3	Potato	Shropshire, UK	1	
R3 ( <b>R51</b> )	4	Unknown	USA	2	Woodhall <i>et al.</i> (2007)
[ <b>1873</b> ]	4 HG-I	Rose	Japan	4	
[ <b>1938</b> ]	4 HG-II	Iris	Netherlands	5	
[ <b>1968</b> ]	4 HG-II	Brassica	UK	6	
<b>Y55</b>	5	Potato	Unknown	1	Woodhall <i>et al.</i> (2008)
SH51 (R28) [ <b>1911</b> ]	8	Barley	UK	3	Woodhall <i>et al.</i> (2007)

<sup>1</sup> Original codes used in referenced papers are shown without parentheses. Scottish Crop Research Institute (SCRI) codes are shown in round parentheses and Central Science Laboratory (CSL) codes are shown in square parentheses. Codes used hereafter in this thesis are shown in bold.

<sup>2</sup> Source: 1 = Harper Adams University College; 2 = Colorado State University; 3 = Dr. Alison Lees, SCRI; 4 = Prof. Hyakumachi, Gifu University, Japan; 5 = Dr. Paul van den Boogert, Plant Research International, Wageningen; 6 = Giles Budge, Central Science Laboratories, York.

#### 4.2.1.4 Inoculation with *R. solani* mycelium and measurement of colony growth

The day after the plates were poured, within a laminar flow cabinet, the tape was removed and the plates were inoculated in the centre with a 10 mm diameter plug of PDA from the margins of actively growing colonies of the *R. solani* isolates (Table 4.1). The plates were resealed, stacked in a randomised pattern and incubated at 18 °C in the dark for three to four days before the colonies were measured. The diameter of each colony was measured along two axes, one perpendicular to the other. Plates in which colony growth was zero were retained and the following day, the agar plugs containing the inoculum were transferred to fresh PDA plates free from toxins. After a further seven to nine days incubation, the colonies were recorded as growing or not growing.

#### 4.2.1.5 Analysis of in vitro toxicity data

For each colony, the diameter of the original agar plug was subtracted from the mean of the two measurements. The reduction in colony growth as a proportion of control growth was calculated and arcsine-square-root-transformed. For each compound x isolate combination, these transformed data were plotted against the log-transformed concentration of toxin. Straight-line relationships were observed and linear regression was used to fit dose-response lines to the data.

In order to test for differences in toxicity between compounds and for differences in susceptibility between AG, a two-way ANOVA was performed with isothiocyanate and AG as fixed factors and log-transformed ED<sub>50</sub> value as the dependent variable. ED<sub>50</sub> values for each isolate x isothiocyanate combination were log-transformed. The data were normally distributed and homoscedastic with respect to anastomosis group but heteroscedastic with respect to isothiocyanate. Two-way ANOVA was used with REGWQ post-hoc test for anastomosis group and Games-Howell test for isothiocyanate.

### 4.2.2 Development of a method to quantify viable *Rhizoctonia solani* in soil

#### 4.2.2.1 Beetroot seed pouches

Pouches containing ten *Beta vulgaris* cv. Bultardy seeds were prepared. The pouches were made from pieces of nylon net curtain material (Arran 183 cm white Filigree, Filigree) 120 by 40 mm, folded lengthways and sewn together down the side. Staples were inserted at both ends and in the middle so that there were two compartments with five seeds in each. Pouches and seed were autoclaved (20 min at 121 °C) before use.

#### 4.2.2.2 Preparation of inoculated compost

Compost infected with *R. solani* at 0x, 1x and 2x mg hyphae per gram soil was prepared as follows. 21 day old cultures of *R. solani* isolate Rs08JL (AG3) growing on PDA plates were cut into small pieces (~5 mm  $\varnothing$ ) with a scalpel and mixed into 3 kg John Innes No. 3 compost. Two kg of this inoculated compost was divided into three equal portions in 5" diameter plant pots. The other 1 kg inoculated compost was diluted with 1 kg uninoculated compost. This compost, as well as 2 kg uninoculated compost, was divided into three equal portions in 5" diameter plant pots. Approximately 250 ml water was added to each pot. The pots were incubated at room temperature for three days.

#### 4.2.2.3 Seed-baiting time-course and quantification of *Rhizoctonia solani* inoculum

Ten holes were bored into the compost of each pot using a wooden skewer and one pouch was inserted into each hole. Every day for five days, two pouches were removed from each pot and stored at -20 °C. DNA was extracted from the bait seed samples and used in TaqMan qPCR. Each DNA sample was used in two sets of duplicate reactions, one with each of two sets of primer and probe (Table 4.2). One set was specific to *R. solani* AG3  $\beta$ -tubulin. The other set was designed to amplify the potato cytochrome oxidase gene (Weller *et al.*, 2000) but was tested and found to also amplify *B. vulgaris* DNA. The latter was used to normalise the data for differences in DNA extraction efficiency, assuming that the amount of plant DNA was constant. The seeds from the two pouches from each replicate x timepoint combination were combined in a sterile Petri dish (Sterilin, Cat. No. 101VR20) and shaken to remove adhered soil. The seeds or roots were transferred to an extraction bag (Bioreba, 470100) containing 4 ml CTAB buffer and 40  $\mu$ l antifoam B emulsion (Sigma, A6707) and placed on ice. The samples were thoroughly beaten with a wooden mallet, homogenised further by 30 s grinding with a sample homogeniser (Bioreba AG, CH-4153) and returned to ice. From each bag, two 2 ml aliquots were transferred to 2 ml micro-centrifuge tubes (Star Lab, E1420 2700) and micro-centrifuged for two min at 2,000 g. Two 500  $\mu$ l aliquots of supernatant (one from each tube) were combined in a fresh 2 ml tube containing 250  $\mu$ l Buffer B (from the Wizard Magnetic DNA Purification System for Food, Promega, FF3751). 750  $\mu$ l of precipitation solution (from the Promega kit) was added and the sample was vortexed thoroughly. The samples were micro-centrifuged at 13,000 rpm for 10 min in an Eppendorf Minispin micro-centrifuge and 750  $\mu$ l of supernatant was transferred to a 2 ml tube containing 50  $\mu$ l Magnesil bead suspension (from the Promega kit). 600  $\mu$ l isopropanol was added to each tube, which was then vortexed. The tubes were

incubated at room temperature (~23 °C) for 5 min and occasionally inverted to maintain the Magnesil beads in suspension. A Kingfisher 96 automated magnetic bead handling robot (Thermo Labsystems) was used to carry out the final steps of the DNA isolation, in which the magnetic beads with the DNA bound were passed through first 1 ml buffer B (Promega kit), then two consecutive 1 ml volumes of 70% ethanol, before being heated to 65 °C for 5 min to evaporate remaining ethanol. DNA was eluted in 200 µl TE. DNA samples were stored at -20 °C. The DNA samples were used as template in TaqMan qPCR as described above.

**Table 4.2:** Primers and probes used in TaqMan qPCR.

Oligonucleotide <sup>1</sup>	Sequence	Dye	Target	Reference
AG3-F	TCT ACA GGG ATT CCA GAT TAC GC	FAM / TAMRA	<i>R. solani</i> AG3 β-tubulin gene	Budge <i>et al.</i> (2009b)
AG3-P	AGG AAC CGG TGC TGG TAT GGG GAC TC			
AG3-R	TCA CGG ATC TTG GAA ATC AAC A			
COX-F	CGT CGC ATT CCA GAT TAT CCA	VIC / TAMRA	Plant cytochrome oxidase gene	Weller <i>et al.</i> (2000)
COX-P	ACC GCA TTC CAT CCA GCG TAA GCA			
COX-R	CAA CTA CGG ATA TAT AAG AGC CAA AAC TG			

<sup>1</sup>F = forward primer, R = reverse primer, P = probe.

## 4.2.3 Toxicity of brassica green manures to *Rhizoctonia solani* in soil

### 4.2.3.1 Plants

*Raphanus sativus* cv. Weedcheck, *Raphanus sativus* cv. "Low", *Brassica juncea* cv. Nemfix, *Brassica juncea* cv. Arid, *Nasturtium officinale* and *Triticum aestivum* cv. Paragon were grown in John Innes No. 3 compost in 3.5 " diameter pots in a glasshouse (25 ± 3 °C) under natural light (May to June, 53 °N).

### 4.2.3.2 Preparation of inoculated compost

Inoculum was prepared according to a modified version of the method of Papavizas & Davey (1962). Within a laminar flow cabinet, eight mycelial plugs from the growing margins of a 12 day old culture of *R. solani* isolate Rs08JL (AG3) were used to inoculate each of 20 autoclave bags containing a mixture of 2.55 kg silver sand, 150 g oatmeal (Tesco) and 450 ml deionised water that had previously been autoclaved twice on two

consecutive days at 121 °C for 30 min. The bags were tied loosely in a “swan’s neck” to allow gaseous exchange and stored at room temperature (~23 °C) out of direct sunlight. After seven and 21 days, the bags were thoroughly kneaded so that aggregates of hyphae were broken down and contents mixed. After 41 days, the bags were opened and inspected for contamination. The contents of five bags that were free from obvious contamination (15 kg) were thoroughly mixed into ~130 kg John Innes No. 3 compost and the mixture was divided into eight  $17.1 \pm 0.2$  kg portions in polythene bags. 1.8 kg sharp sand was mixed into 15.3 kg of JI No. 3 compost to form the uninoculated, *R. solani*-free control and this was placed in a polythene bag.

#### 4.2.3.3. Amendment of inoculated compost with green manures

When the plants were 42 days old, the above ground tissues were harvested. At this age, the *R. sativus* and *N. officinale* plants were at the early flowering stage, while the *Brassica juncea* were at the full flowering stage. The stems below the first set of leaves were removed and 900 g of the remaining material (30 to 50 plants) was thoroughly homogenised for at least ten minutes in a commercial blender (Waring) with gradual addition of 400 ml water. The macerated tissue was added to one 17.1 kg bag of inoculated compost with another 600 ml water and thoroughly mixed in. The amended compost was divided into six pots (3 kg per pot). Twelve 2.85 kg portions of inoculated compost were placed into pots. 670 ml water containing 20.8 µl azoxystrobin was applied over the surface of the compost in six of these pots and 670 ml water alone was applied to the other six. Six 2.85 kg portions of uninoculated compost were placed into pots for the uninoculated control and 670 ml water was applied. The pots were placed on benches in a glasshouse ( $25 \pm 3$  °C) in a randomised pattern and the compost was kept moist by regular watering.

#### 4.2.3.4 Quantification of fungal inoculum and disease severity on potato plants

Beetroot seed pouches were prepared as described above. At 2, 13 and 37 days post addition of plant material, two holes were bored into the compost of each pot using a wooden skewer and one pouch was inserted into each hole. The pouches were allowed to remain in the soil for three days before being removed and stored at -20 °C.

Seed tubers (*Solanum tuberosum* L. var. Estima, grade EC3, class A, Reg. No. UK/S 0044, Crop ID No. 01 0044 27 02) were inspected and found to be free from sclerotia. Pieces

containing the bud end, weighing  $18.9 \pm 4.5$  (SD) g, were excised and allowed to suberise at room temperature ( $\sim 20$  °C) for 48 h. Five days after addition of green manures, the suberised tuber pieces were planted, two per pot, bud end up, 6-8 cm deep in the treated compost. Thirty-five days after addition of green manures, the potato plants were removed washed of compost and stems and roots were inspected for disease according to the method of Carling & Leiner (1990). The stem disease severity index was as follows: 0 = no damage or lesions present, 1 = one or more lesions less than 5 mm in diameter, 2 = one or more lesions greater than 5 mm in diameter and/or some girdling, 3 = several lesions greater than 5 mm in diameter and girdling or death on most stems, 4 = all stems killed. The root disease index was as follows: 0 = no visible damage, 1 = lesions on 1 - 10% of the root area, 2 = lesions on 11 - 25% of the root area, 3 = lesions on 26 - 50% of the root area, 4 = lesions on more than 50% of the root area. In addition, the number of killed, infected and uninfected stems and stolons was counted.

Samples of stem, stolon and tuber tissue containing lesions were removed from several plants in order to re-isolate the pathogen and verify its identity as *R. solani* AG3. These samples were cut into pieces, each containing the margin of a lesion, surface sterilised in 10 % sodium hypochlorite for 30-60 s, rinsed in water and plated out on PDA containing 0.6 % penicillin G sulphate (Sigma, P3032) and 2.0 % streptomycin sulphate (Sigma, S6501). Pure cultures of *R. solani* were isolated, DNA was extracted and used in qPCR with primers and probe specific to *R. solani* AG3  $\beta$ -tubulin, as described above. Roots were freed from remaining compost, weighed and stored at  $-20$  °C. DNA was extracted from bait seed and root samples as described above.

DNA samples from potato roots were diluted 20x and those from bait seed 200x in TE before use in TaqMan PCR. PCR on root DNA samples was carried out in duplicate in 96-well plates using the same PCR mixture and conditions as described above. PCR on bait seed DNA samples was carried out in duplicate in 384-well plates (ABA, 430 98 49) sealed with optically clear heat seals (VWRI, AB-0812). The final reaction mixture was identical to the PCR carried out in 96-well plates but instead of adding  $11.375 \mu\text{l}$  reaction<sup>-1</sup> water to the master mix, only  $2.375 \mu\text{l}$  reaction<sup>-1</sup> was added.  $10 \mu\text{l}$  of DNA sample (which had been diluted 10x more than the root DNA samples) was added to each reaction, instead of  $1 \mu\text{l}$ . This was in order to ensure accurate loading of the PCR plates by the liquid handling robot (Micro Lab Star, Hamilton) used to load 384-well plates. The thermal cycler was set up for a 384-well plate. Otherwise, the PCR conditions were identical to those used for root DNA samples. In addition to the test samples, each

PCR plate contained duplicate negative controls with DNA from isolates of AG2-1 and AG5, several water negative controls and two duplicate ten-fold dilution series ranging from  $1 \times 10^0$  to  $1 \times 10^{-6}$  ng  $\mu\text{l}^{-1}$  of pure *R. solani* DNA extracted from sclerotia of isolate Rs08JL (as described above). Ct values were automatically calculated by SDS 2.2.2 software. Concentrations of DNA were calculated from Ct values by means of standard curves. The mean DNA concentration of duplicate qPCRs was taken for each biological replicate.

## 4.3 Results

### 4.3.1 Toxicity of isothiocyanates and fungicides to *Rhizoctonia solani*

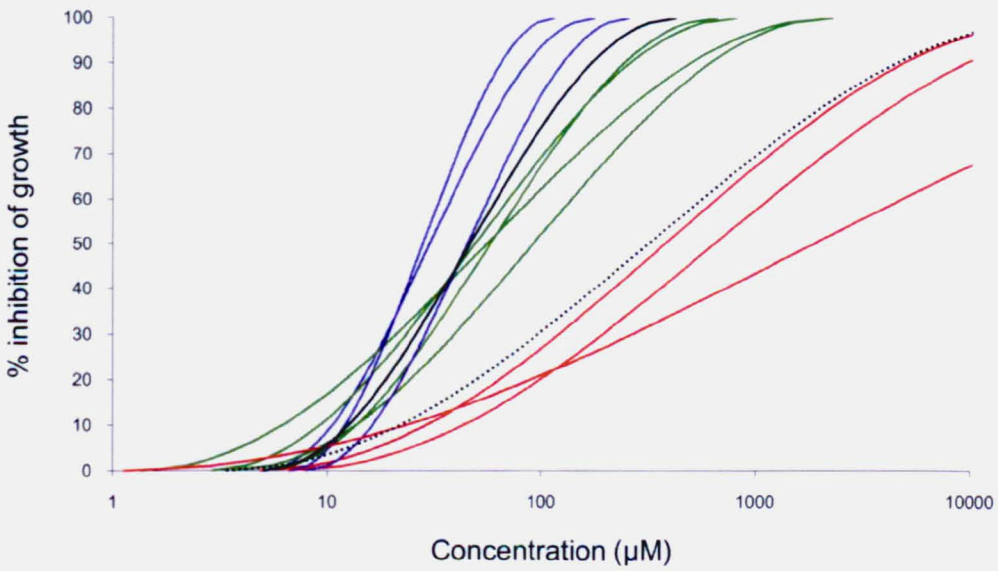
Twelve isolates of *R. solani* were exposed to a range of concentrations of six isothiocyanates, metham sodium, azoxystrobin or penicuron. All of the compounds inhibited growth of all isolates but the dose-response relationship depended on the compound and the isolate (Figures 4.1 – 4.9).

There were significant differences in toxicity between isothiocyanates. The descending rank order of toxicity of isothiocyanates to *R. solani* was 3-(methylthio)propyl > benzyl = 2-phenylethyl > propyl = 2-propenyl > 2-methylbutyl isothiocyanate (Table 4.3). The mean ED<sub>50</sub> for 3-(methylthio)propyl isothiocyanate was 64 µM. This was significantly lower than the mean ED<sub>50</sub> values of benzyl and 2-phenylethyl isothiocyanate, 150 and 300 µM, respectively. Propyl and 2-propenyl isothiocyanate were significantly less toxic with mean ED<sub>50</sub> values of approximately 600 µM. 2-methylbutyl isothiocyanate was the least toxic of all, with a mean ED<sub>50</sub> value of 1700 µM. The methyl isothiocyanate-liberating fumigant metham sodium was equally toxic to the most toxic isothiocyanate (Table 4.3). ED<sub>100</sub> values were an order of magnitude greater than ED<sub>50</sub> values (Tables 4.3 and 4.4) and lethal concentrations were typically four times greater than ED<sub>100</sub> values (Table 4.4).

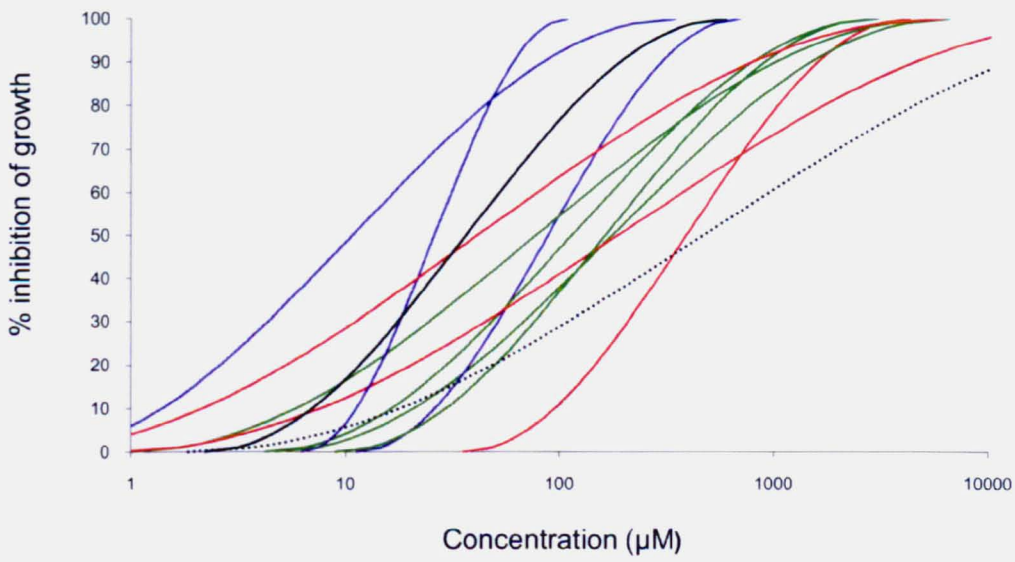
Anastomosis groups differed in sensitivity to isothiocyanates (Figure 4.10). Isolates of AG2-1 and AG8 were significantly more tolerant than isolates of other AG while isolates of AG3 were significantly more sensitive. AG3 isolates were also relatively sensitive to metham sodium, three of the four most sensitive isolates belonging to AG3 (Table 4.3). Isolates of AG4 and AG5 were intermediately sensitive. Since there was only one isolate belonging to AG5 and one belonging to AG8, it is not possible to draw firm conclusions as to the sensitivity of these AG, only the individual isolates.

There was a binomial distribution of ED<sub>50</sub> values for penicuron, isolates either being sensitive (ED<sub>50</sub> < 5 µg l<sup>-1</sup>) or insensitive (ED<sub>50</sub> > 2 mg l<sup>-1</sup>) (Table 4.3). Sensitivity to azoxystrobin varied to an even greater extent than to penicuron, ED<sub>50</sub> values ranging over eight orders of magnitude but with a more continuous spread of sensitivities among isolates than with penicuron. There was little evidence that sensitivity to azoxystrobin or penicuron was dependent on AG or that sensitivity to the two fungicides was related (Table 4.3).

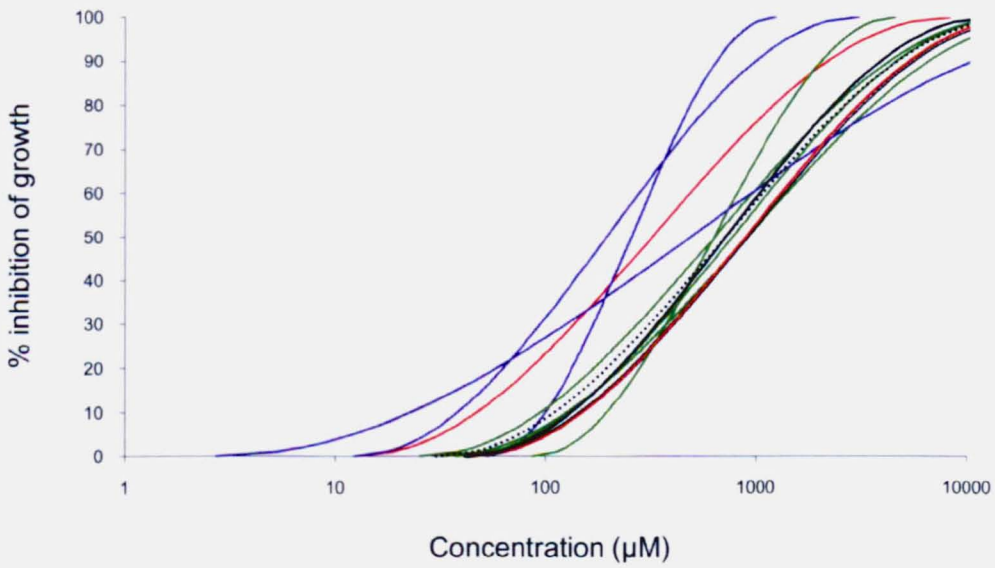




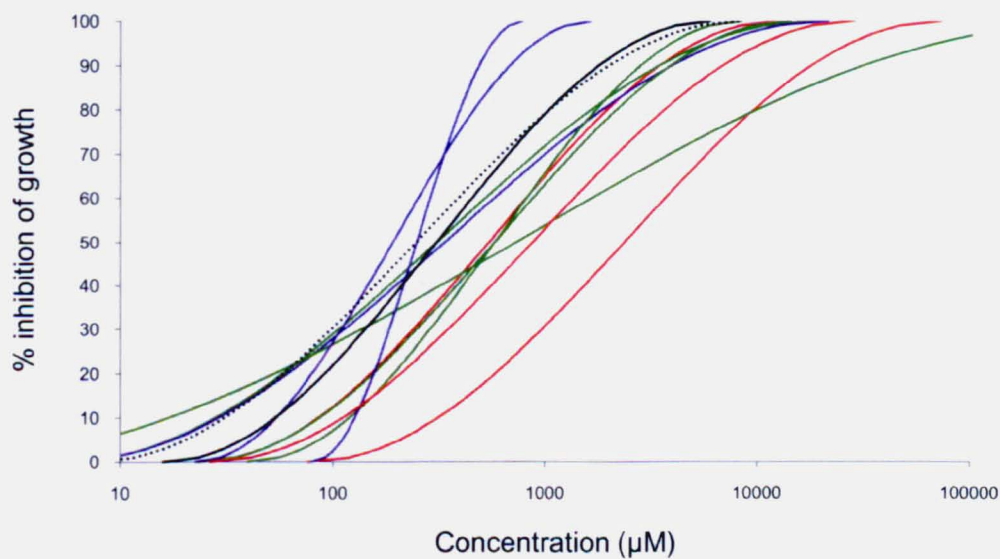
**Figure 4.1:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of 2-phenylethyl isothiocyanate in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.



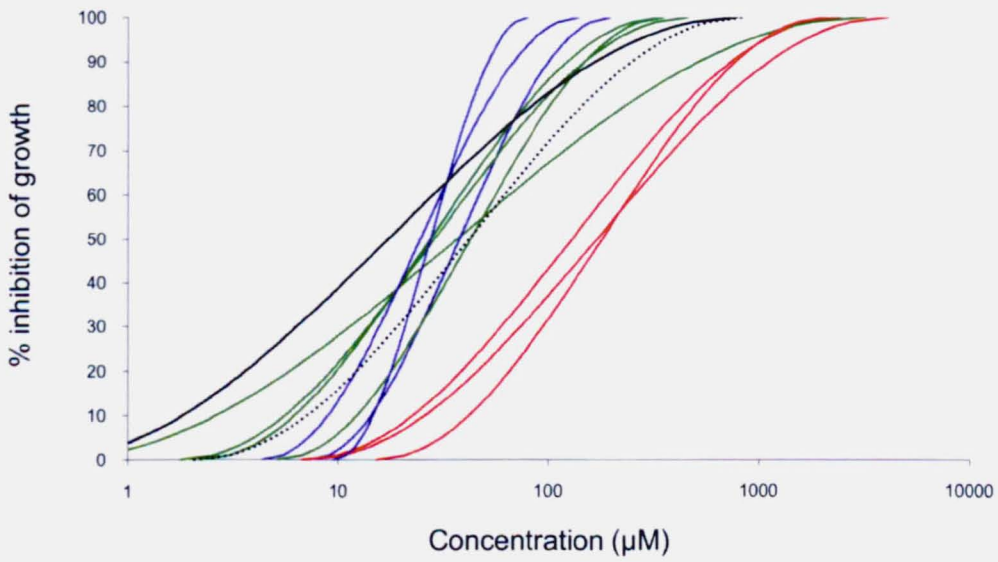
**Figure 4.2:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of benzyl isothiocyanate in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.



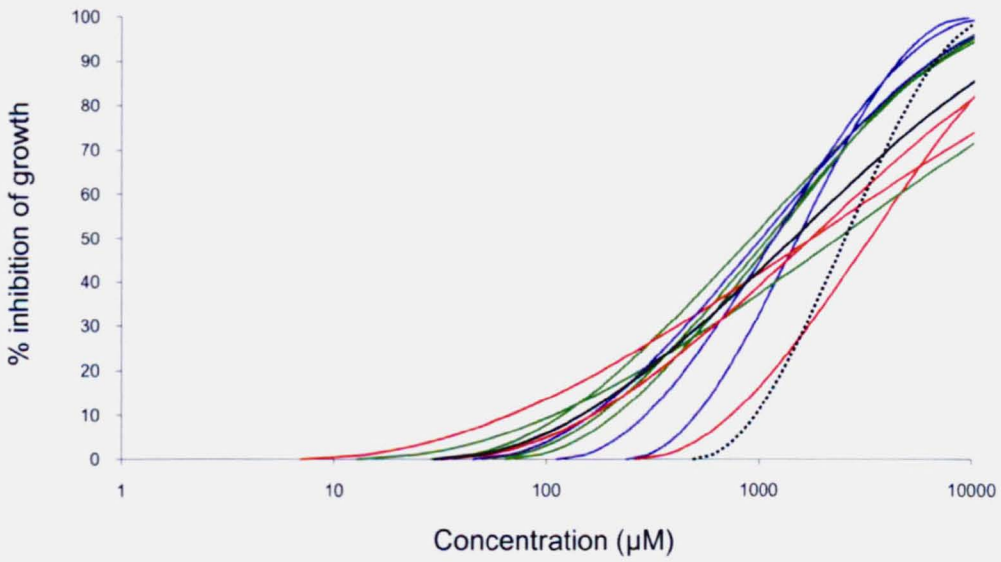
**Figure 4.3:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of 2-propenyl isothiocyanate in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.



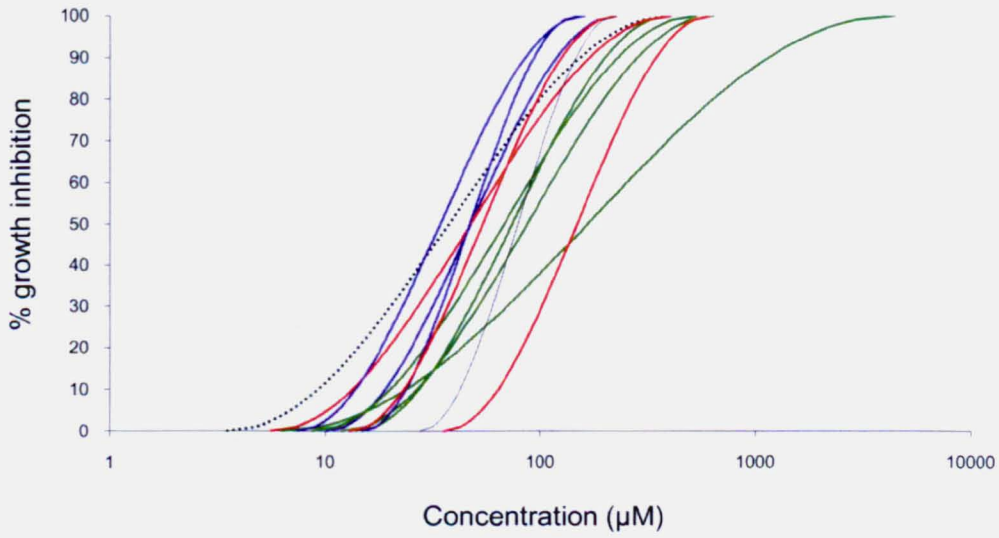
**Figure 4.4:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of propyl isothiocyanate in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.



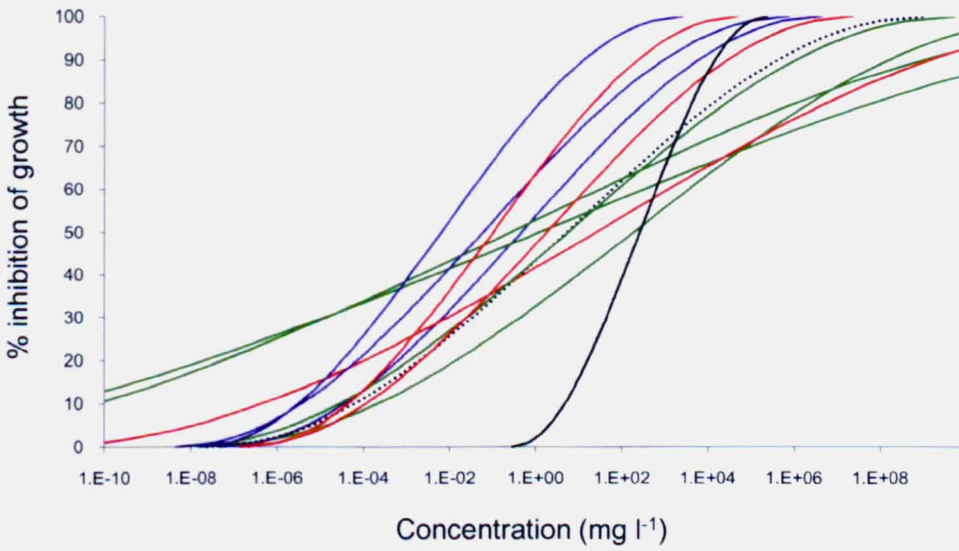
**Figure 4.5:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of 3-(methylthio)propyl isothiocyanate in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.



**Figure 4.6:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of 2-methylbutyl isothiocyanate in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.

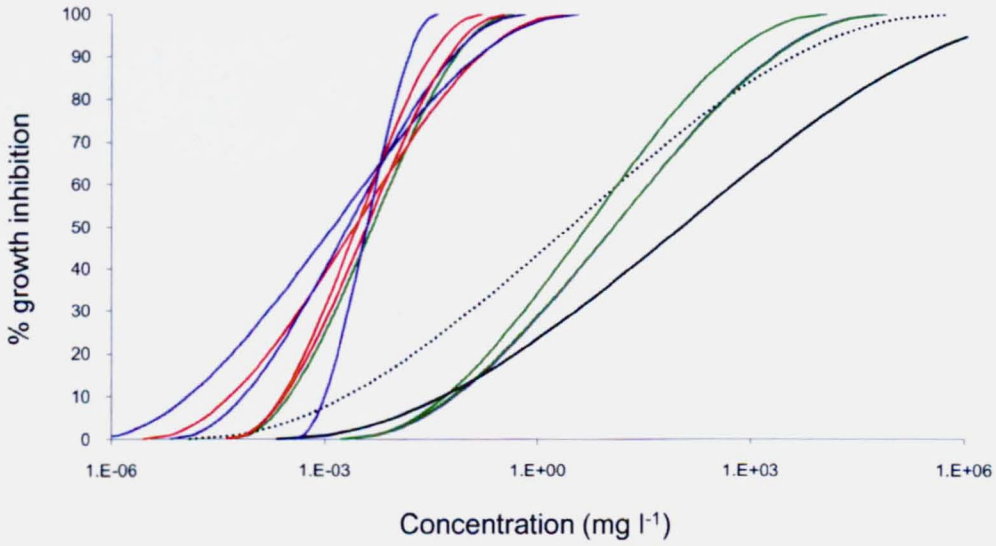


**Figure 4.7:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of metham sodium in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.



**Figure 4.8:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of azoxystrobin in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data.





**Figure 4.9:** Inhibition of growth of 12 isolates of *R. solani* by a range of concentrations of penicycuron in the agar substrate. The isolates belonged to the following anastomosis groups: AG2-1 (red), AG3 (blue), AG4 (green), AG5 (solid black), AG8 (dotted black). Curves are back-transformed linear regressors through transformed experimental data. NB: The curve with the second highest ED<sub>50</sub> is actually two curves that are very similar.

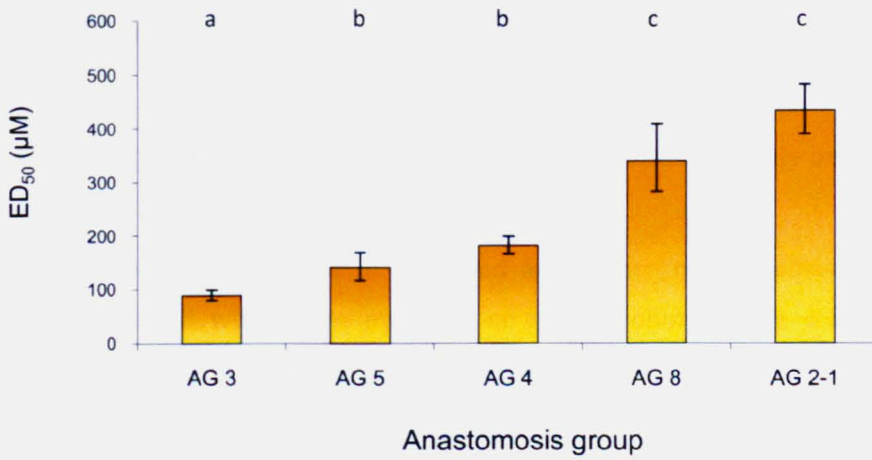
**Table 4.3:** ED<sub>50</sub> values for twelve isolates of *Rhizoctonia solani* exposed to a range of concentrations of six isothiocyanates (ITC) and three fungicides. 3-MPT ITC = 3-(methylthio)propyl ITC, B ITC = benzyl ITC, 2-Ph ITC = 2-phenylethyl ITC, 2-P ITC = 2-propenyl ITC, P ITC = propyl ITC, 2-M ITC = 2-methylbutyl ITC.

Isolate	AG	ED <sub>50</sub> values (µM)						ED <sub>50</sub> values (µg l <sup>-1</sup> )		
		3-MTP ITC	B ITC	2-Ph ITC	P ITC	2-P ITC	2-M ITC	Metham sodium	Pencycuron	Azoxystrobin
Rs08JL	3	24.6	10.8	45.1	191	194	1176	34.3	3.85	6.15
UN	3	27.5	26.0	28.0	248	486	1023	47.0	2.09	56.9
1900	3	37.8	88.0	30.1	336	254	1515	47.1	11,600	446
1873	4	36.8	176	57.6	309	631	1169	86.9	4.60	179,000
Y55	5	17.3	36.4	46.3	308	715	1450	78.5	104,000	252,000
1968	4	27.5	77.2	48.9	577	771	1103	67.7	11,500	1140
1938	4	42.6	114	55.5	577	907	917	74.0	4,850	244
R51	4	28.7	163	91.3	727	634	2339	166	1.28*	5440
1911	8	41.9	466	314	247	713	2540	37.5	2,740	4,930
Y3	2-1	129	389	640	540	319	3320	148	2.76	1,730
Y25	2-1	179	41.1	1849	868	886	1748	53.9	3.80	97.6
2015	2-1	169	186	376	2359	897	1763	47.4	2.60	23,800
<b>Mean</b>		<b>63.5</b>	<b>148</b>	<b>298</b>	<b>607</b>	<b>617</b>	<b>1672</b>	<b>67.3</b>		

\* Isolate HARC3 (AG3) was used instead of isolate R51 in the experiment with pencycuron.

**Table 4.4:** Minimum concentrations of isothiocyanates and metham sodium required to completely inhibit mycelial growth of twelve isolates of *Rhizoctonia solani*. Mycelium that did not grow at all after four days incubation on PDA amended with a toxin was transferred to fresh toxin-free PDA and incubated for a further seven days. The minimum concentrations after exposure to which mycelium failed to resume growth on toxin-free media are shown in parentheses. See Table 4 legend for key to isothiocyanate codes. n.m = not measured.

Isolate	AG	ED <sub>100</sub> (lethal concentration in parentheses) (mM)						
		3-MTP ITC	B ITC	2-Ph ITC	P ITC	2-P ITC	2-M ITC	Metham sodium
Rs08JL	3	0.3 (1.0)	0.3 (>4.1)	0.5 (>4.1)	2.0 (n.m)	2.0 (>8.2)	8.2 (>8.2)	0.16 (0.64)
UN	3	0.1 (0.3)	0.1 (2.0)	0.1 (0.3)	1.0 (n.m)	0.5 (2.0)	4.1 (>8.2)	0.16 (0.64)
1900	3	0.1 (n.m)	0.3 (4.1)	0.3 (1.0)	2.0 (8.2)	2.0 (4.1)	4.1 (>8.2)	0.32 (1.3)
1873	4	0.5 (1.0)	2.0 (4.1)	1.0 (4.1)	2.0 (8.2)	4.1 (4.1)	>8.2 (>8.2)	0.64 (0.64)
Y55	5	0.3 (2.0)	0.5 (2.0)	0.5 (2.0)	4.1 (n.m)	4.1 (4.1)	8.2 (>8.2)	0.32 (1.3)
1968	4	1.0 (1.0)	2.0 (>4.1)	0.3 (>4.1)	4.1 (n.m)	4.1 (4.1)	>8.2 (>8.2)	0.64 (0.64)
1938	4	1.0 (1.0)	4.1 (>4.1)	2.0 (n.m)	8.2 (>8.2)	4.1 (8.2)	>8.2 (>8.2)	0.32 (0.64)
R51	4	0.5 (1.0)	4.1 (>4.1)	2.0 (>4.1)	2.0 (>8.2)	4.1 (>8.2)	8.2 (8.2)	0.32 (n.m)
1911	8	0.5 (8.2)	>4.1 (>4.1)	>4.1 (>4.1)	2.0 (n.m)	4.1 (>8.2)	>8.2 (>8.2)	0.32 (0.64)
Y3	2-1	1.0 (4.1)	2.0 (>4.1)	>4.1 (>4.1)	> 8.2 (>8.2)	4.1 (>8.2)	>8.2 (>8.2)	0.32 (n.m)
Y25	2-1	0.5 (1.0)	0.5 (4.1)	>4.1 (>4.1)	4.1 (n.m)	4.1 (8.2)	>8.2 (>8.2)	0.32 (0.64)
2015	2-1	1.0 (4.1)	2.0 (>4.1)	>4.1 (>4.1)	>8.2 (>8.2)	8.2 (>8.2)	>8.2 (>8.2)	0.64 (1.3)



**Figure 4.10:** Sensitivity of *R. solani* anastomosis groups to isothiocyanates. Data presented are back-transformed means of log-transformed ED<sub>50</sub> values for six isothiocyanates against one or more isolates of *R. solani* (four AG4 isolates, three AG3 isolates, three AG2-1 isolates, one AG5 isolate and one AG8 isolate). 2-way ANOVA, carried out on log-transformed data, revealed significant differences in sensitivity between anastomosis groups ( $P < 0.01$ ) and REGWQ homogenous subsets are represented by letters. Error bars show SEM.

### 4.3.2 Effect of brassica green manures on *Rhizoctonia solani* inoculum density and disease on potato

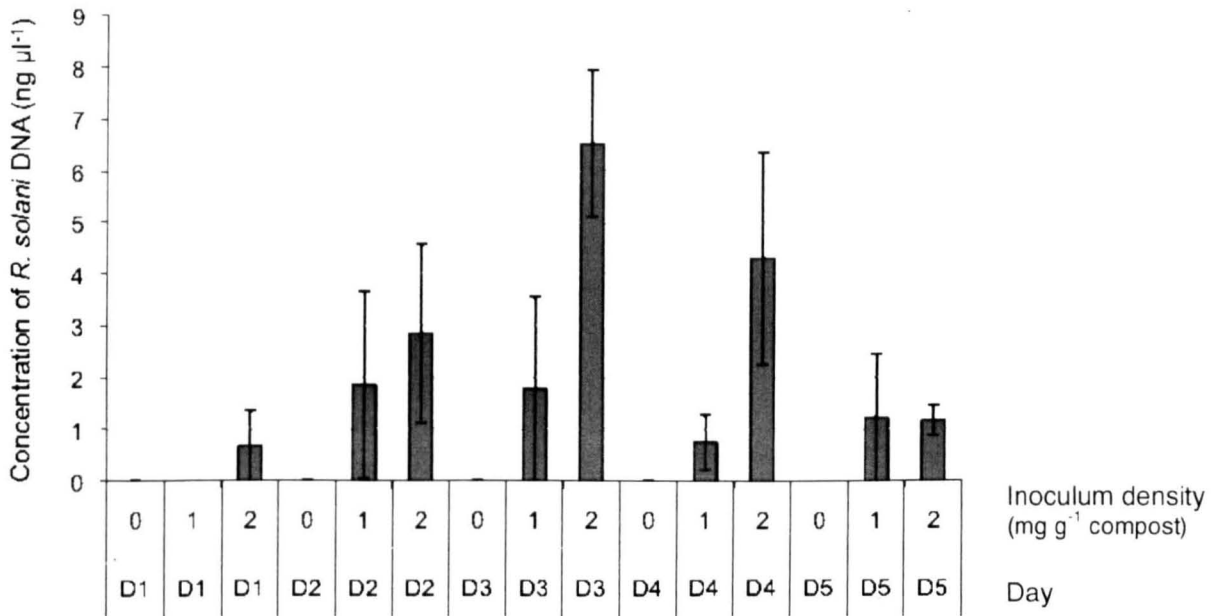
#### 4.3.2.1 Development of a method for quantifying viable *R. solani* inoculum in soil

A seed baiting method was developed for the quantification of viable *R. solani* inoculum in soil. Autoclaved *Beta vulgaris* (beet root) seed were buried in soil to allow colonisation by the fungus, DNA was extracted from the seed and *R. solani* DNA was quantified by qPCR. A test was performed to ascertain whether or not the method was quantitative and to determine the optimum period of time to allow for fungal colonisation of the seed. After two to four days in infected compost, the quantity of *R. solani* DNA detected in bait seed was proportional to the inoculum density in the compost (Figure 4.11).

#### 4.3.2.2 Effect of brassica green manures on *R. solani* inoculum density and disease on potato

*R. solani* DNA was detected in bait seed and potato roots recovered from infected compost treated with green manures or azoxystrobin (Figure 4.12) but not from uninfected compost (data not shown). The only treatment to cause a consistent reduction in fungal inoculum density relative to control was azoxystrobin (Figure 4.12a-4.12c). Green manure of *R. sativus* cv. Weedcheck appeared to exert a strong suppressive effect on soil inoculum density but only by 37 days post amendment. *R. sativus* and *N. officinale* both reduced root infection but azoxystrobin did not (Figure 4.12d).

All green manures and azoxystrobin reduced the incidence of disease on potato plants to some extent, although in almost all cases this was not statistically significant. (Figure 4.13). No brassica green manure was consistently more effective than the others and surprisingly, the non-brassica, *T. aestivum* (wheat), had by far the strongest effect. *T. aestivum* green manure reduced the incidence of stem and stolon infection and the severity of stem infection by a similar amount as the commercial fungicide. No other green manure was consistently more effective than the others. Despite the relatively low amounts of *R. solani* DNA detected in potato roots after treatment with *R. sativus* and *N. officinale* (Figure 4.12d), the severity of root infection was not reduced by these treatments (Figure 4.13c).



**Figure 4.11:** Timecourse of *R. solani* colonisation of *Beta vulgaris* bait seed. Compost was inoculated with *R. solani* at three different densities (0, 1 or 2 mg fungal mycelium per gram compost), bait seed pouches were buried in the compost and removed after one to five days (D1-D5), DNA was extracted from the bait seed and *R. solani* DNA was quantified by qPCR. Error bars show SEM. N = 3.



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#### 5.2.4 Photography and morphological identification of nematodes

Randomly selected slides were removed from -80 °C and placed on the stage of a Leica DMRB microscope. The specimens were examined under bright field illumination at 40x magnification with the condenser adjusted for parallel light. A camera (Olympus C-5050) was attached to the microscope via a connecting ring (Olympus c3030-ADU) and 1x lens and the specimens were photographed. Photographs were used to putatively identify nematodes on the basis of morphology by comparison with reference photographs. One hundred individuals were photographed and transferred to sodium hydroxide for DNA extraction.

#### 5.2.5 Extraction of DNA from individual nematodes

Once a specimen had been photographed, the slide was transferred to a Leica MZ16 microscope, 40-100 µl water was added to the edge of the cover slip and a hooked wire was used to gently transfer the specimen to a 500 µl tube containing 20 µl 0.25 M NaOH. DNA was extracted from individual nematodes according Floyd *et al.* (2002). In detail, samples were incubated at -80 °C for two weeks, incubated overnight at 25 °C, incubated at 99 °C for three min then allowed to cool to room temperature. Tubes were micro-centrifuged briefly at 13,000 rpm before the following reagents were added: 4 µl 1 M HCl; 10 µl 0.5 M Tris-HCl (pH 8.0); 5µl 2% triton X-100. The contents were mixed briefly by vortexing and collected by brief micro-centrifugation. Samples were heated for 3 min at 99 °C and then cooled to room temperature. The lysate was stored at -80 °C.

#### 5.2.6 PCR amplification of 18S rRNA gene with nematode-universal primers

In order to generate sufficient DNA for gene sequencing, DNA from single nematode digests was amplified using the primers SSU18A (AAAGATTAAGCCATGCATG) and SSU26R (CATTCTTGCAAATGCTTTCG). These primers were designed by Blaxter *et al.* (1998) to amplify 18S rDNA gene regions from annealing sites universally conserved among nematodes.

The following PCR master mix was prepared: 5 µl reaction<sup>-1</sup> dNTP mixture (2 mM each nucleotide), 5 µl reaction<sup>-1</sup> 10x Biotaq Red buffer (Bioline, BIO-21041), 1.5 µl reaction<sup>-1</sup> 50 mM MgCl<sub>2</sub>, 0.5 µl reaction<sup>-1</sup> 100 µM forward primer (SSU18A), 0.5 µl reaction<sup>-1</sup> 100 µM reverse primer (SSU26R), 1.5 µl reaction<sup>-1</sup> Biotaq Red enzyme (Bioline, BIO-21041) and 31 µl reaction<sup>-1</sup> ddH<sub>2</sub>O. In the wells of a 96-well PCR plate (Agilent, 410088), 5 µl



single nematode digest (see section 5.2.5) or water was combined with 45 µl master mix. The PCR plate was sealed with strip caps (Agilent, 401425), vortexed for two minutes at 600 rpm on a plate mixer (Eppendorf Mixmate) and micro-centrifuged briefly in a manual centrifuge. PCR was carried out in a Hybaid PCR Express thermal cycler using the following thermal profile: 94 °C for five min, 35 cycles of 94 °C for 60 s, 52 °C for 90 s and 68 °C for 120 s, and finally 68 °C for a further 10 min. Once the reactions were complete, strip caps were carefully removed and reaction products were transferred to 500 µl micro-centrifuge tubes and stored at -80 °C.

Products of PCR with universal primers were separated by electrophoresis through 1.2 % agarose (Melford, MB1200) gels containing 5 ppm ethidium bromide in TAE buffer at 75 v for one hour. 15 µl PCR product or 4 µl 1 kb DNA ladder (Fermentas Generuler 1 kb Ladder, SM0318) was loaded into each well. After 30, 45 and 60 min, DNA bands were photographed on a u.v. transilluminator.

### *5.2.7 Sequencing 18S rDNA genes of individual nematodes*

In order to identify the 100 individuals sampled from the Shropshire field soil, a region of their 18S rDNA genes was sequenced. The products of PCR with the universal primers (see section 5.2.6) were purified using a QIAquick PCR Purification Kit (Qiagen, 28106) according to the manufacturer's instructions (p. 19-20 Qiaquick Spin Handbook). DNA was eluted in 30 µl elution buffer. DNA concentrations were determined by measuring the absorbance of light at 260 nm in duplicate 1 µl samples. DNA samples were diluted to 1.65 ng µl<sup>-1</sup> and sent for sequencing with primer SSU9R. DNA sequencing was performed at the University of Leeds Genetic Analysis Facility using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) followed by analysis on an ABI377 autosequencer (Applied Biosystems). The resulting trace files were viewed and analysed using Chromas 2.3 software. Mis-called bases were corrected and terminal sequences were removed where base identity was unclear.

Homologous sequences were searched for using the BLAST provided by NCBI ([www.ncbi.nlm.nih.gov/blast/Blast.cgi](http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), June 2009). The non-redundant database (including GenBank, EMBL, DDBJ and PDB sequences) was searched for nucleotide sequences (blastn). Values for "expect" and "word size" were set at 10 and 11, respectively, and the filter was "low complexity". The closest matching sequences from the database and their percentage identity to the query sequences were recorded.

### 5.2.8 Calculation of structure and enrichment indices and prioritisation of genera for which to design primers

Identification of nematodes from their 18S rDNA gene sequences allowed the nematode community to be characterised and values for the structure and enrichment indices to be determined. Structure and enrichment index scores were calculated according to Ferris *et al.* (2001) (see Section 5.1.4). Functional guilds assigned to each nematode family are given in Table 5.2. The family Mylonchulidae has not previously been assigned a c-p value and was assigned a score of four based upon its predaceous feeding habit, large body size, small gonads (Jairajpuri, 1970) and its relatedness to the Mononchidae (c-p 4) (Griffiths *et al.*, 2006). The structure and enrichment indices were re-calculated several times, each time excluding one genus from the analysis. Thus, the contribution of each genus to the two indices was determined. Genera having the greatest effect on the two indices were prioritised for primer design.

**Table 5.2:** Functional guilds of selected nematode families.

Family	Functional guild <sup>1</sup>
<i>Rhabditidae</i>	Ba <sub>1</sub>
<i>Cephalobidae</i>	Ba <sub>2</sub>
<i>Plectidae</i>	Ba <sub>2</sub>
<i>Monohysteridae</i>	Ba <sub>2</sub>
<i>Bastianiidae</i>	Ba <sub>3</sub>
<i>Achromadoridae</i>	Ba <sub>3</sub>
<i>Alaimidae</i>	Ba <sub>4</sub>
<i>Aphelenchoididae</i>	Fu <sub>2</sub>
<i>Anatonchidae</i>	Ca <sub>4</sub>
<i>Mononchidae</i>	Ca <sub>4</sub>
<i>Aporcelaimidae</i>	Om <sub>5</sub>

<sup>1</sup> Trophic guilds are assigned according to Yeates *et al.* (1993): Ba, bacteriovore; Fu, fungivore; Ca, carnivore; Om, omnivore. Subscript numbers indicate coloniser-persister scores as defined by Bongers (1990, 1999) and Bongers & Bongers (1998).

### 5.2.9 Design of family-specific and genus-specific primers

GenBank sequences with the greatest percentage identity to the 18S rRNA gene sequences of the specimens sampled from the field soil were aligned by the Clustal W method using DNASTAR MegAlign software (version 4.05). This alignment was used to map regions of similarity and divergence among the 51 sequences and is referred to hereafter as the "overall alignment". A phylogenetic tree was constructed in DNASTAR MegAlign Version 4.05.

When primers were designed for a particular taxon, several GenBank sequences corresponding to that taxon were entered in a new MegAlign file and aligned by the Clustal W method. These sequences included all of the closest matches to specimens from North Lynn farm as well as sequences corresponding to specimens sampled from Leeds University farm, Headley Hall, Yorkshire by Wang (2009). Regions conserved within the taxon were mapped and compared with regions of divergence and conservation among all of the taxa, revealed in the overall sequence alignment. A sequence corresponding to the taxon of interest was searched for potential primers using DNASTAR PrimerSelect Version 4.05. Primer parameters were set as follows: length = 17-24 bp; 3' pentamer stability = -8.5 kc/M; unique 3' sequence of 7 bp; accepted dimer duplexing of 2 bp; accepted hairpin duplexing of 2 bp; ignored duplexing of 8 bp from 3' end; ambiguous residues = 0 bp. Primers lying within regions that were conserved within the target taxon but divergent from other taxa were located. Primer pairs with predicted annealing temperatures of  $60 \pm 2$  °C and amplicon lengths between 90 and 400 bp were identified. These primers were checked for predicted formation of secondary structure, self-dimerisation and primer pair dimerisation using PrimerSelect and for specificity to the target taxon by alignment with all non-target sequences in the overall alignment. Primers predicted to specifically amplify the members of each target taxon were synthesised by Eurogentech.

### 5.2.10 Testing primer specificity

Each primer pair was tested for amplification of DNA from each of the genera present in the initial sample of 100 nematodes. Separate master mixes were prepared for each primer pair. Master mixes contained the following: 12.5  $\mu\text{l}$  reaction<sup>-1</sup> SYBR Green Supermix (Stratagene), 5.5  $\mu\text{l}$  reaction<sup>-1</sup> ddH<sub>2</sub>O, 1.0  $\mu\text{l}$  reaction<sup>-1</sup> forward primer (7.5  $\mu\text{M}$ ) and 1.0  $\mu\text{l}$  reaction<sup>-1</sup> reverse primer (7.5  $\mu\text{M}$ ). Aliquots of DNA digests from individual nematodes were diluted eight-fold in sterile distilled water. In a laminar flow hood, 5  $\mu\text{l}$

aliquots of diluted DNA solution were loaded into the wells of a 96-well PCR plate (Agilent, 410088) and 20 µl master mix was added to each sample. PCR plates were sealed with strip caps (Agilent, 401425), vortexed for two minutes at 600 rpm in a plate shaker (Eppendorf Mixmate) and micro-centrifuged briefly in a manual centrifuge. The plates were placed in a qPCR thermal cycler (Stratagene Mx3005P) and subjected to the following thermal profile: 95 °C for three min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by 95 °C for 60 s, 55 °C for 30 s, ramping to 95 °C and remaining at 95 °C for 30 s. Data were analysed using MxPro software, with the fluorescence threshold set at 200 fluorescence units. Reactions showing an exponential rise in fluorescence and giving a dissociation curve with one or more distinct peaks were judged to have amplified the template DNA. Primer pairs that amplified DNA of the intended target taxon whilst failing to amplify DNA from non-target taxa were selected for use in qPCR of pooled nematode samples from experimentally treated soil. In three cases, primer pairs were accepted despite some amplification of non-target genera. In each case, the Ct value of the reactions with non-target template DNA was at least four cycles higher than with target DNA.

#### *5.2.11 Determination of the effects of fumigation and biofumigation on the free-living nematode community*

The soil collected on 26<sup>th</sup> August 2009 (see section 5.2.1) was thoroughly mixed and 5 kg portions were dispensed into 12 " diameter clay pots, which were then plunged into the ground on an outdoor plot at Grove House Gardens Experimental Station, Leeds. The pots were sunk so that the surface of the soil in the pots was level with that around. On 10<sup>th</sup> September 2009, the pots and contents were removed from the ground. The contents of each pot were transferred to a 40 litre plastic bucket and treated. The soil from five pots received 250 g homogenised fresh leaf tissue of *B. juncea* ISCI99 plus 200 ml water, five received 350 mg Basamid® granular soil fumigant (BASF, 634512, containing 99 % Dazomet, 1 % inerts) plus 200 ml water and five received 200 ml water alone. The leaf tissue, Dazomet or water were thoroughly mixed into the soil, which was then replaced into the pots. The pots were covered with a double layer of polyethylene (500 gauge black refuse sack) and replaced in the outdoor plot in five blocks of three treatments. At 10 days post treatment the polythene was removed. Just prior to treatment (10<sup>th</sup> September 2009), at 10 days post treatment (20<sup>th</sup> September 2009) and 190 days post treatment (19<sup>th</sup> March 2010), 100 g soil was sampled from each pot using a soil corer. This soil was immediately taken to the lab and used in nematode extraction,

as described in section 5.2.2. Leaf tissue samples were collected at the time of incorporation, flash-frozen in liquid nitrogen and stored at -80 °C for subsequent quantification of glucosinolate, as described in section 2.2.5.

Extracted nematodes were transferred to a small Petri dish. Under a microscope (Leica MZ16), nematodes were randomly selected and transferred to 20 µl 0.25 M NaOH using a hooked wire. As many as possible to a maximum of 100 individuals were transferred from each sample into one aliquot of sodium hydroxide. In some cases, fewer than 100 individuals were present in a sample. For each sample, the number of individuals collected and the proportion of the sample searched were recorded for determination of total nematode abundance. These pooled nematode samples were stored at -80 °C until all samples had been collected. DNA was then extracted from the samples using the same method as for the individual nematodes (see section 5.2.5).

In order to quantify the abundance of each nematode taxon of interest, each DNA sample was used in quantitative PCR, as described in section 5.2.10, in combination with each of the primer sets listed in Table 5.4. DNA samples from individual nematodes of known genus were used as positive controls and water was used instead of DNA in negative control reactions. In order to prevent any differences in conditions between PCR runs from biasing results, each run contained one DNA sample from each treatment x timepoint combination. The abundance of nematodes was calculated as  $2^{\Delta C_t}$ , where  $\Delta C_t$  is the mean threshold cycle in reactions containing DNA from a single nematode of the target genus minus the threshold cycle of the test sample. Values were rounded up to the nearest integer. The threshold was set at a constant 200 fluorescence units.

Abundance data were used to calculate EI and SI scores, as described in section 5.2.8. Data from reactions with primer sets Isolai\_F12 + Isolai\_R15 and Het\_4d + Het\_8f were not included in calculation of the ecological indices. This is because: a) the identity of specimens putatively identified as *Isolaimium* and *Heterorhabditis* is uncertain due to low sequence homology toward GenBank sequences (Table 5.3); b) neither *Heterorhabditis* nor the other genera matching specimens 53 and 173 (Table 5.3) are free-living nematodes.

The effects of fumigation with Dazomet and biofumigation with *B. juncea* on the abundance of particular nematode taxa and on the structure and enrichment indices were analysed by ANOVA with S-N-K post-hoc test when the data met the criteria of normality and homoscedasticity. Otherwise, the non-parametric Kruskal-Wallis test was used.

## 5.3 Results

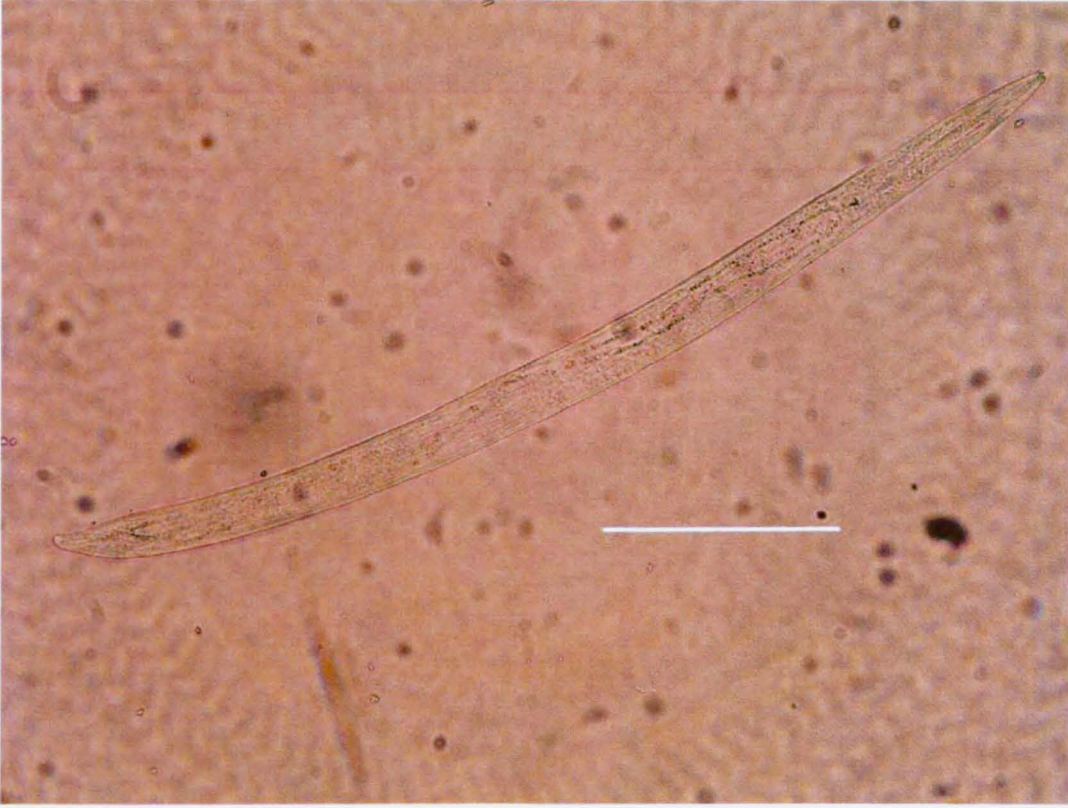
### 5.3.1 Nematode identification by 18S rRNA gene sequence homology

One hundred nematodes extracted from soil from North Lynn farm, Shropshire, were putatively identified to family level by morphology. Good quality 18S ribosomal RNA gene sequences were obtained for 81 of these specimens, which were conclusively identified based on 18S rRNA gene sequence homology to identified sequences on the NCBI GenBank database. Representative specimens of several of the genera are shown in Figure 5.1. Alignment between the 18S rRNA gene sequence from one specimen and the closest matching GenBank sequences is presented in Figure 5.2.

In 65 out of 81 cases, sequence identity between the query sequence and the closest GenBank sequence(s) was  $\geq 98\%$  (Table 5.3). In some cases, two GenBank sequences showed equal identity to a query sequence. In nearly all cases when this occurred, the alternative genera belonged to the same family. For instance, sequences of five specimens had equal homology to GenBank sequences belonging to *Rhabditis* and *Pellioiditis*, both members of the Rhabditidae family. Four specimens were identified as either *Acrobeloides* or *Acrobeles* and one specimen as *Acrobeloides* or *Chiloplacus*, all members of the Cephalobidae family. Two specimens were identified as *Filenchus* or *Tylenchus*, both Tylenchidae, and one specimen was identified as *Diplogaster* or *Pristionchus*, both Diplogasteridae. From here on, these alternatives will be referred to by the name of the more commonly identified alternative. For example, specimens identified as *Rhabditis* or *Pellioiditis* will be referred to as *Rhabditis*. In two cases, the 18S rRNA gene sequence showed equal identity to GenBank sequences belonging to nematodes of different families (Table 5.3). The sequence of specimen 173 showed 82% identity to sequences from *Heterorhabditis* (Heterorhabditidae), *Nippostrongylus* (Heligmonellidae) and *Oscheius* (Rhabditidae). The sequence of specimen 53 was 100% identical to that of specimen 173 but was three base pairs shorter.

### 5.3.2 Nematode community composition

Of the 81 nematodes identified, 42 (52%) were bacteriovores, 34 (42%) were plant-parasites, three (4%) were predators and two (2%) were animal parasites (Figure 5.3). There were no obligate fungivores present but *Filenchus* can feed on plant roots (Yeates *et al.*, 1993) or fungal hyphae (Okada & Harada, 2005). All of the genera showing identity to specimens 53 and 173 are parasitic or necromenic towards animals and spend part of their life-cycle in the soil environment and part within a host's body.

A) *Acrobeles* or *Acrobeloides* \*

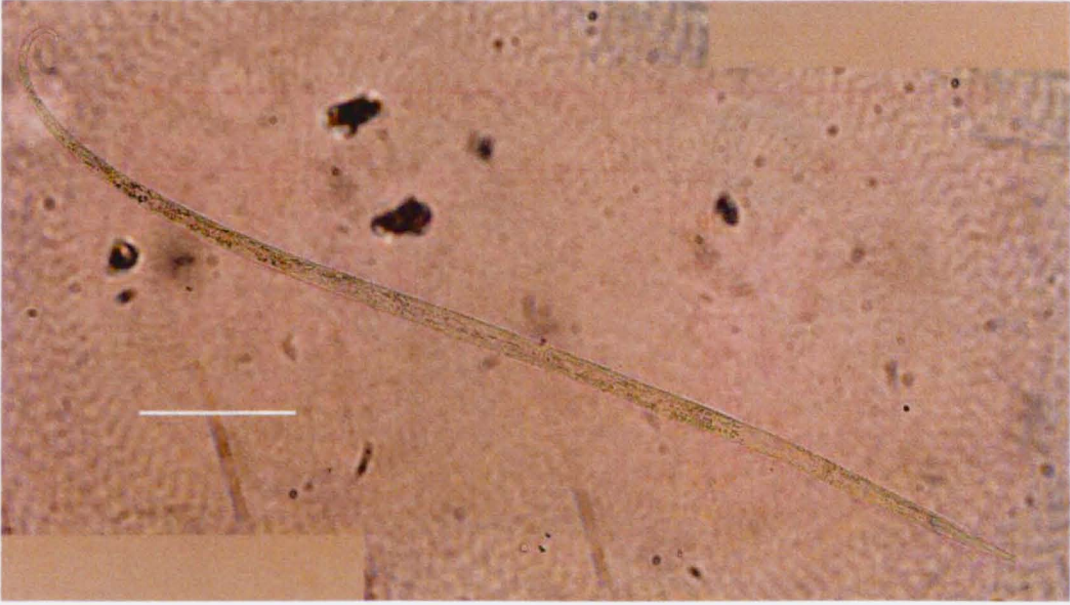
**Figure 5.1:** See page 160 for figure legend.



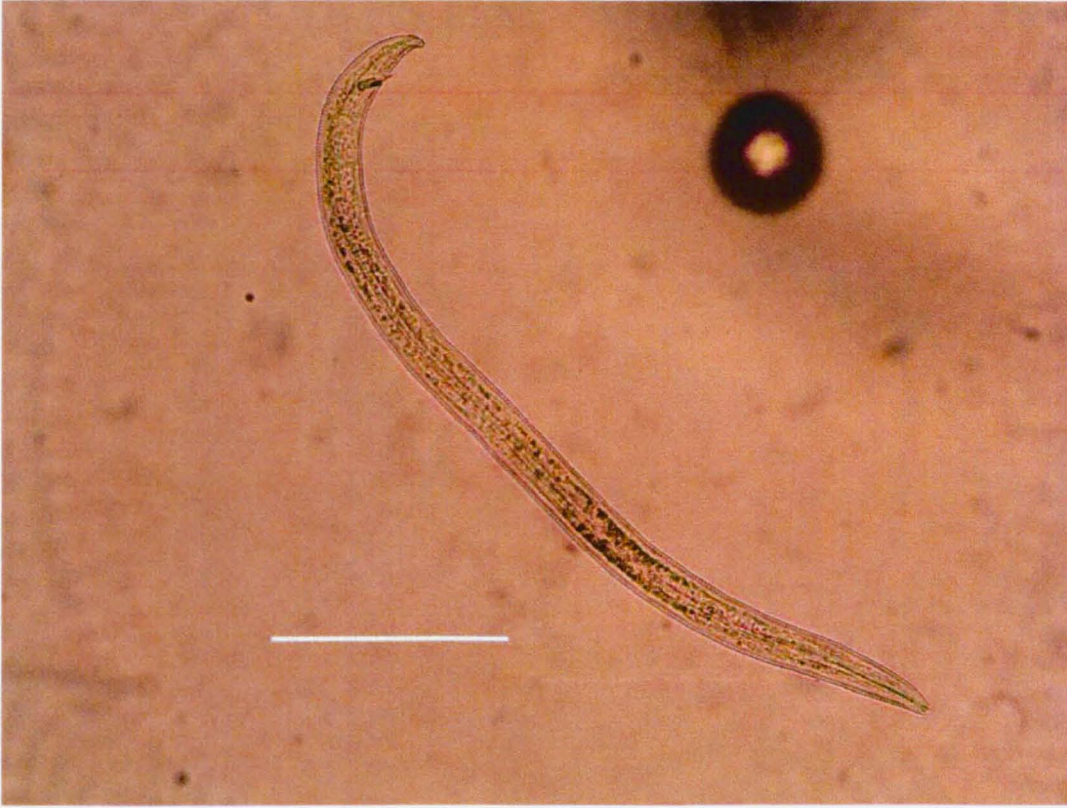
B) *Aglenchus*, *Coslenchus* or *Miculenchus* \*

**Figure 5.1:** See page 160 for figure legend.



C) *Alaimus*

**Figure 5.1:** See page 160 for figure legend.

D) *Chiloplacus* or *Acrobeloides* \*

**Figure 5.1:** See page 160 for figure legend.

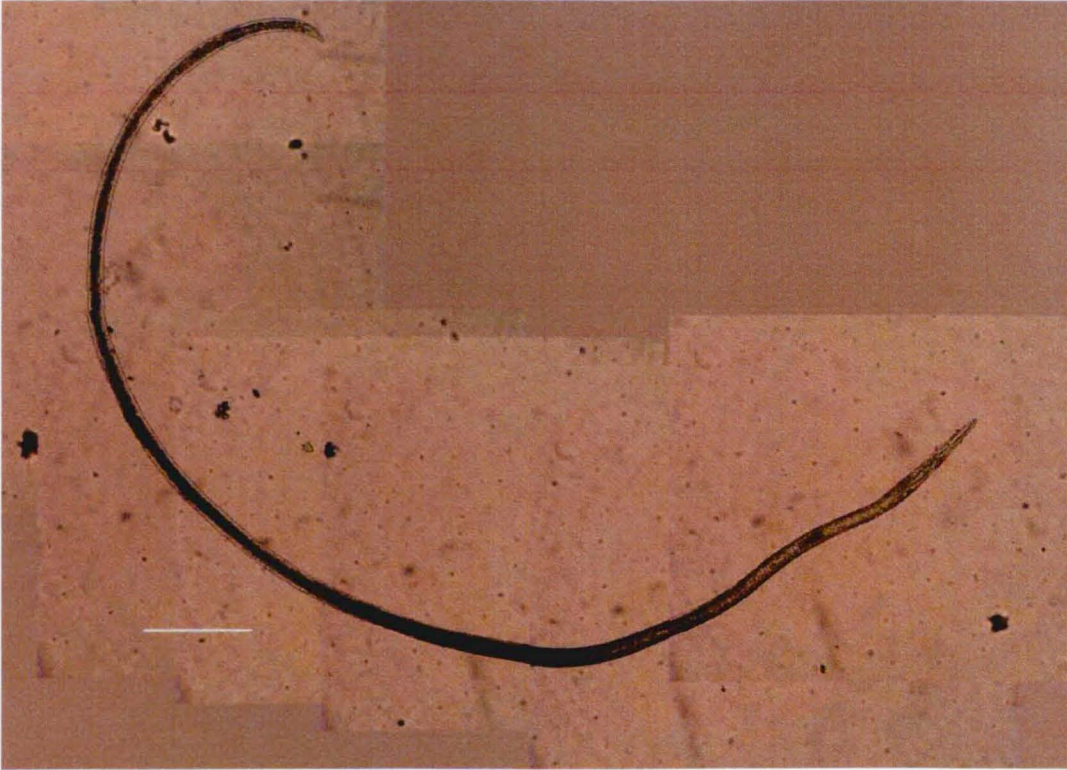
E) *Clarkus*

**Figure 5.1:** See page 160 for figure legend.



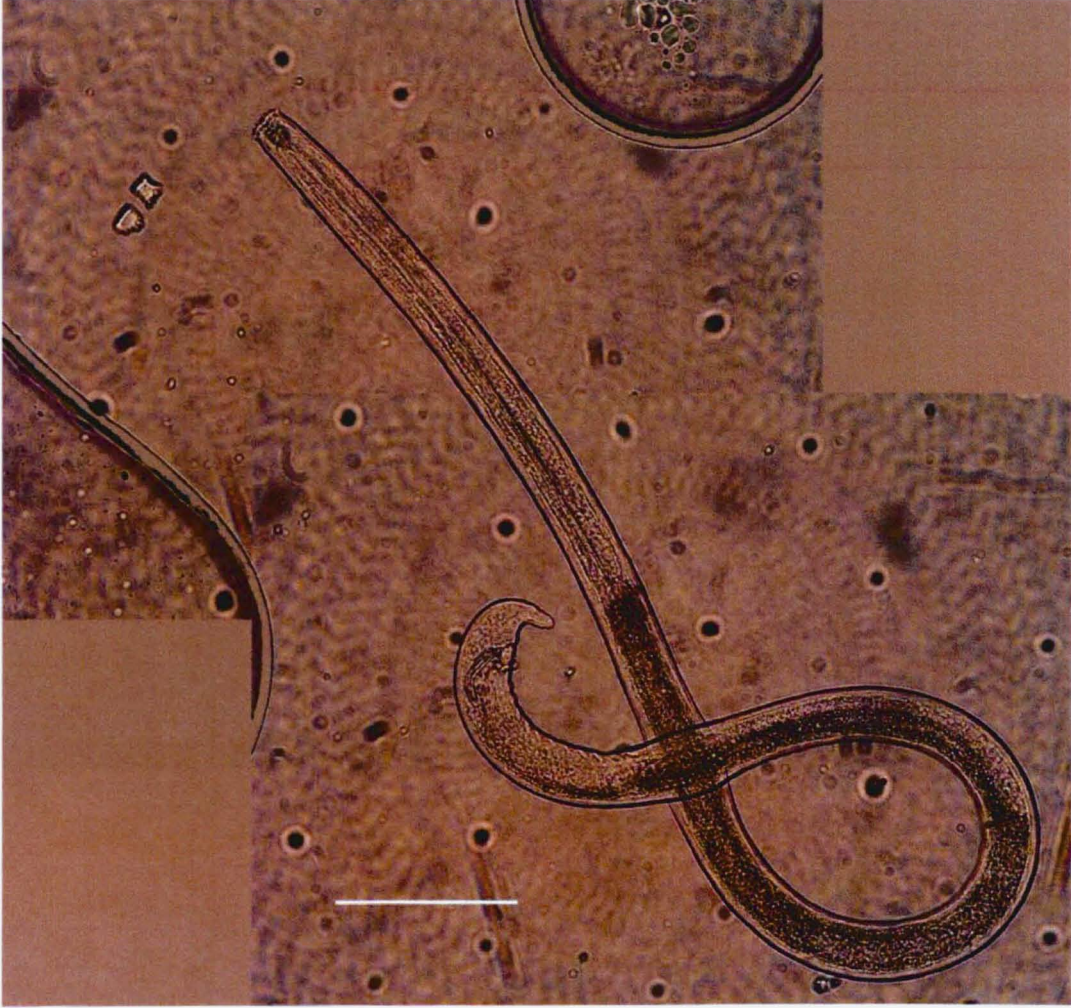
F) *Eucephalobus*

**Figure 5.1:** See page 160 for figure legend.

G) *Longidorus*

**Figure 5.1:** See page 160 for figure legend.



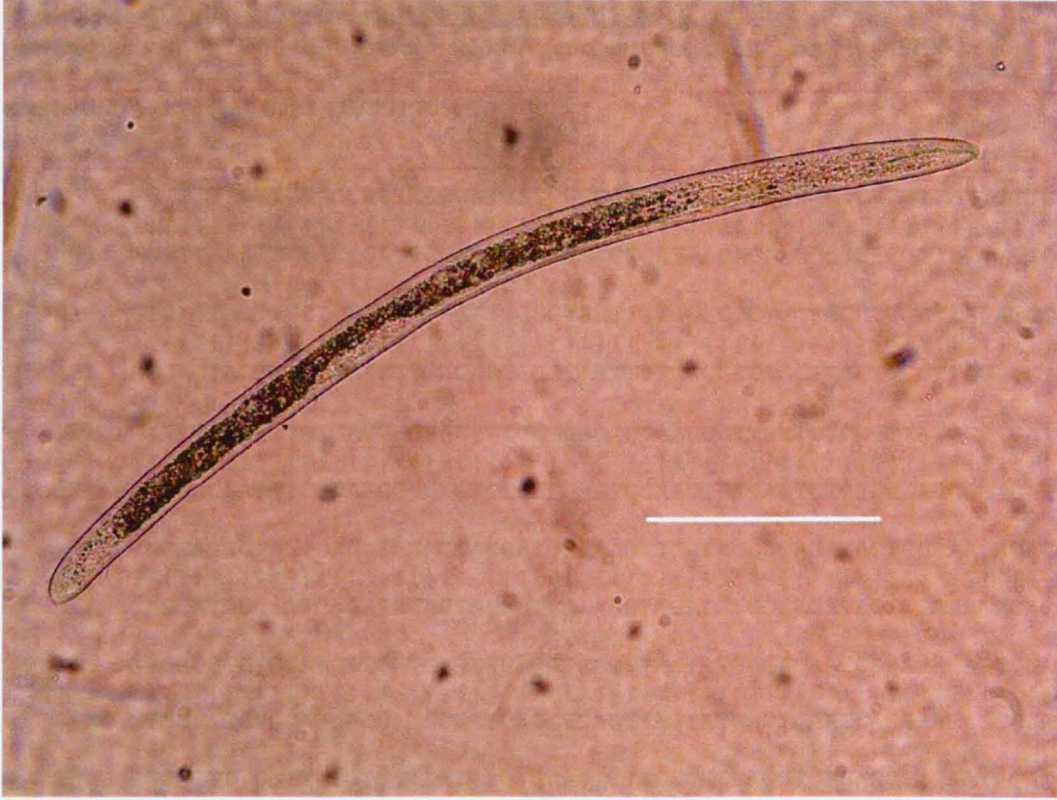
H) *Mylonchulus*

**Figure 5.1:** See page 160 for figure legend.

I) *Pratylenchus*

**Figure 5.1:** See page 160 for figure legend.



J) *Trichodorus*

**Figure 5.1:** Photographs of individual nematodes extracted from soil from North Lynn farm, Shropshire: A) specimen #185 *Acrobeles* or *Acrobeloides*\*; B) specimen #146 *Aglenchus*, *Coslenchus* or *Miculenchus*\*; C) specimen #181 *Alaimus*; D) specimen #155 *Chiloplacus* or *Acrobeloides*\*; E) specimen #136 *Clarkus*; F) specimen #215 *Eucephalobus*; G) specimen #182 *Longidorus*; H) specimen #37 *Mylonchulus*; I) specimen #83 *Pratylenchus*; J) specimen #201 *Trichodorus*. Images were captured with an Olympus C-5050 camera attached to a Leica DMRB microscope at 40x magnification with bright field illumination. Images of specimens that did not fit on one photograph were tiled using the Fourier Correlation method in Image-Pro Analyser software. Scale bars represent 100  $\mu\text{m}$ .

\* Identification is uncertain since multiple NCBI database sequences belonging to different genera have equal identity to the sequence belonging to the test specimen (see Figure 5.5.2).



Query sequence = Specimen 185.

Subject sequence = GenBank accession [gi|158827550|gb|EU196016.1|](https://www.ncbi.nlm.nih.gov/nucl/158827550),

*Acroboles maximus* strain DF5048 18S ribosomal RNA gene, partial sequence (length = 1700 bp).

Identities = 482/491 (98%), Gaps = 0/491 (0%).

```

Query 1      GGTTTAAATTGTACCCATTCCGATGACCGGCCTCATAAGAGAACGGTCTCGTTATTTTTC 60
|
Sbjct 491    GGTTTAAATTGTACCCATTCCGATGACCGGCCTCATAAGAGAACGGTCTCGTTATTTTTC 432

Query 61     GTCACTACCTCCTCGTGCTGAGAGTGGGTAATTTTCGCGCCTGCTGCCTTCCTTAGATGT 120
|
Sbjct 431    GTCACTACCTCCTCGTGCTGAGAGTGGGTAATTTTCGCGCCTGCTGCCTTCCTTAGATGT 372

Query 121    GTTCGCGATTCTCAGGCGAACTCTCCGAGCCGAACCCCTTATTCTCCGTTACGCGTGAT 180
|
Sbjct 371    GTTCGCGATTCTCAGGCGAACTCTCCGAGTCGAACCCCTTATTCTCCGTTACGCGTGAT 312

Query 181    CACCATGGTAGTCAGATAACTACCATCGAAAGTTGATACGGCAGATACTCGAAAGACAC 240
|
Sbjct 311    CACCATGGTAGTCAGATAACTACCATCGAAAGTTGATACGGCAGATACTTGAAGACAC 252

Query 241    GTCGCCGGTGCAAGACCGTGCGATCAGCTCAGTTACTATGAGTCAACCAGAAACAGCCGA 300
|
Sbjct 251    GTCGCCGGTGCAAGACCATGCGATCAGCTCAGTTACTATGAGTCAACCAGAAACAGCCGA 192

Query 301    AGCCGCTTGGTTTTGTTCTAATAAGTGCACCTCTCCCGTGAGGTCAGAGCTTTAATGCAT 360
|
Sbjct 191    AGCCGCTTGGTTTTGTTCTAATAAATGCACCTCTCCCGTGAGGTCAGAGCTTTAATGCAT 132

Query 361    GTATTAGCTCTAGAATTACCACAGTTATCCAAGTAGGATTGTGTCAAGATCAAGTAGATT 420
|
Sbjct 131    GTATTAGCTCTAGAATTACCACAGTTATCCAAGTAGGATAGTGTCAAGATCAAGTAAATT 72

Query 421    TTAGCTGTTATAATGAGCCATTCGCGGTTTCACGATTAGANCGTTTATACTTAGACATGC 480
|
Sbjct 71     ATAGCTGTTATAATGAGCCATTCGCGGTTTCACGATTAAATCGTTTATACTTAGACATGC 12

Query 481    ATGGCTTAATC 491
|
Sbjct 11     ATGGCTTAATC 1

```

**Figure 5.2:** See following page for figure legend.

Query sequence = Specimen 185.

Subject sequence = GenBank accession [gi|170783755|gb|EU543175.1](https://www.ncbi.nlm.nih.gov/nuclseq/gi/170783755|gb|EU543175.1),

*Acroboloides thornei* strain DWF1109 small subunit ribosomal RNA gene, partial sequence (length = 1771 bp).

Identities = 486/493 (98%), Gaps = 0/493 (0%).

```

Query 1      GGTTTAAATTGTACCCATTCCGATGACCGGCCTCATAAGAGAACGGTCTCGTTATTTTC 60
          |||
Sbjct 504     GGTTTAAATTGTACCCATTCCGATGACCGGCCTCATAAGAGAACGGTCTCGTTATTTTC 445

Query 61     GTCACTACCTCCTCGTGCTGAGAGTGGGTAATTTTCGCGCCTGCTGCCTTCCTTAGATGT 120
          |||
Sbjct 444     GTCACTACCTCCTCGTGCTGAGAGTGGGTAATTTTCGCGCCTGCTGCCTTCCTTAGATGT 385

Query 121    GTTCGCGATTTCTCAGGCGAACTCTCCGGAGCCGAACCCTTATTCTCCGTTACGCGTGAT 180
          |||
Sbjct 384     GTTCGCGATTTCTCAGGCGAACTCTCCGGAGCCGAACCCTTATTCTCCGTTACGCGTGAT 325

Query 181    CACCATGGTAGTCAGATACACTACCATCGAAAGTTGATACGGCAGATACTCGAAAGACAC 240
          |||
Sbjct 324     CACCATGGTAGTCAGATACACTACCATCGAAAGTTGATACGGCAGATACTTGAAGACAC 265

Query 241    GTCGCCGGTGCAAGACCGTGCATCAGCTCAGTTACTATGAGTCAACCAGAAACAGCCGA 300
          |||
Sbjct 264     GTCGCCGGTGCAAGACCGTGCATCAGCTCAGTTACTATGAGTCAACCAGAAACAGCCGA 205

Query 301    AGCCGCTTGGTTTTGTTCTAATAAGTGCCTCTTCCCGTGAGGTCAGAGCTTTAATGCAT 360
          |||
Sbjct 204     AGCCGCTTGGTTTTGTTCTAATAAATGCCTCTTCCCGTGAGGTCAGAGCTTTAATGCAT 145

Query 361    GTATTAGCTCTAGAATTACCACAGTTATCCAAGTAGGATTGTGTCAAGATCAAGTAGATT 420
          |||
Sbjct 144     GTATTAGCTCTAGAATTACCACAGTTATCCAAGTAGGATTGTGTCAAGATCAAGTAAATT 85

Query 421    TTAGCTGTTATAATGAGCCATTCGCGGTTTCACGATTAGANCGTTTATACTTAGACATGC 480
          |||
Sbjct 84      ATAGCTGTTATAATGAGCCATTCGCGGTTTCACGATTAAATCGTTTATACTTAGACATGC 25

Query 481    ATGGCTTAATCTT 493
          |||
Sbjct 24     ATGGCTTAATCTT 12

```

**Figure 5.2:** Sequence alignments between the partial 18S ribosomal RNA gene sequence of one nematode extracted from soil from North Lynn farm (query sequence) and homologous sequences from the National Centre for Biotechnology Information (NCBI) database (subject sequences). The former was determined by amplifying a region of the 18S ribosomal RNA gene by PCR with the primers SSU18A and SSU26R (Blaxter *et al.*, 1998) and sequencing the purified PCR product using the SSU18A primer. The resulting sequence was searched against the NCBI database using BLAST. In this case, two NCBI sequences share equal identity to the query sequence.

**Table 5.3:** Identity of nematode specimens extracted from soil from North Lynn farm, Shropshire, based on sequence homology of 18S rRNA gene sequences.

Specimen	Closest matching GenBank sequence(s)		% Identity
	Accession number	Taxon	
14	<a href="#">gi 51094025 gb AY593940.1 </a>	<i>Achromadora</i>	93
34	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	98
34	<a href="#">gi 158827550 gb EU196016.1 </a>	<i>Acrobeles</i>	98
135	<a href="#">gi 16519092 gb AF430517.1 </a>	<i>Acrobeloides</i>	98
211	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	98
211	<a href="#">gi 158827550 gb EU196016.1 </a>	<i>Acrobeles</i>	98
199	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	98
199	<a href="#">gi 34105867 gb AY284671.1 </a>	<i>Acrobeles</i>	98
155	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	98
155	<a href="#">gi 34105873 gb AY284677.1 </a>	<i>Chiloplacus</i>	98
185	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	98
185	<a href="#">gi 158827550 gb EU196016.1 </a>	<i>Acrobeles</i>	98
210	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	98
91	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	97
91	<a href="#">gi 34105862 gb AY284666.1 </a>	<i>Eucephalobus</i>	97
21	<a href="#">gi 170783755 gb EU543175.1 </a>	<i>Acrobeloides</i>	97
57	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
77	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
152	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
205	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
181	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
86	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
93	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
99	<a href="#">gi 90968520 emb AJ966514.1 </a>	<i>Alaimus</i>	99
212	<a href="#">gi 90968479 emb AJ966473.1 </a>	<i>Anaplectus</i>	99
119	<a href="#">gi 34105921 gb AY284725.1 </a>	<i>Bastiania</i>	94
136	<a href="#">gi 90968485 emb AJ966479.1 </a>	<i>Clarkus</i>	99
178	<a href="#">gi 34105777 gb AY284581.1 </a>	<i>Coslenchus</i>	96
178	<a href="#">gi 239775238 gb FJ969129.1 </a>	<i>Miculenchus</i>	96
146	<a href="#">gi 34105777 gb AY284581.1 </a>	<i>Coslenchus</i>	98
146	<a href="#">gi 239775222 gb FJ969113.1 </a>	<i>Aglenchus</i>	98
146	<a href="#">gi 239775238 gb FJ969129.1 </a>	<i>Miculenchus</i>	98
20	<a href="#">gi 34105777 gb AY284581.1 </a>	<i>Coslenchus</i>	98
20	<a href="#">gi 239775238 gb FJ969129.1 </a>	<i>Miculenchus</i>	98
12	<a href="#">gi 34105852 gb AY284656.1 </a>	<i>Cruz nema</i>	100
45	<a href="#">gi 2921442 gb AF036643.1 </a>	<i>Diplogaster</i>	100
45	<a href="#">gi 2921439 gb AF036640.1 </a>	<i>Pristionchus</i>	100

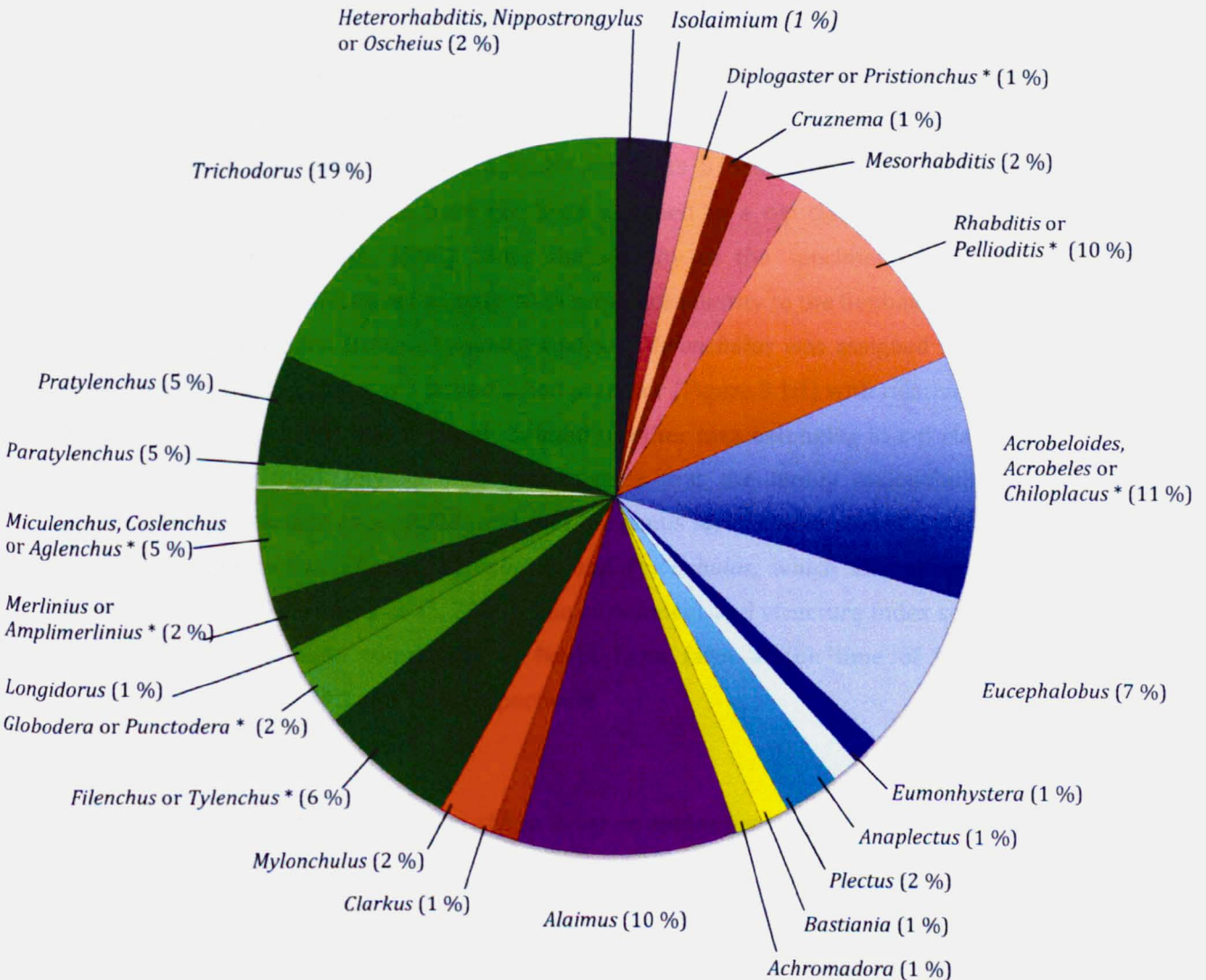
Table 5.3 continued:

Specimen	Closest matching GenBank sequence		% Identity
	Accession number	Taxon	
47	<a href="#">gi 34105861 gb AY284665.1 </a>	<i>Eucephalobus</i>	98
52	<a href="#">gi 34105861 gb AY284665.1 </a>	<i>Eucephalobus</i>	97
56	<a href="#">gi 34105861 gb AY284665.1 </a>	<i>Eucephalobus</i>	98
22	<a href="#">gi 34105861 gb AY284665.1 </a>	<i>Eucephalobus</i>	99
215	<a href="#">gi 34105861 gb AY284665.1 </a>	<i>Eucephalobus</i>	98
156	<a href="#">gi 34105861 gb AY284665.1 </a>	<i>Eucephalobus</i>	99
204	<a href="#">gi 51094022 gb AY593937.1 </a>	<i>Eumonhystera</i>	96
61	<a href="#">gi 34105787 gb AY284591.1 </a>	<i>Filenchus</i>	99
76	<a href="#">gi 34105787 gb AY284591.1 </a>	<i>Filenchus</i>	99
76	<a href="#">gi 164504672 gb EU306348.1 </a>	<i>Tylenchus</i>	99
29	<a href="#">gi 238734459 gb FJ949564.1 </a>	<i>Filenchus</i>	81
138	<a href="#">gi 34105787 gb AY284591.1 </a>	<i>Filenchus</i>	95
164	<a href="#">gi 34105787 gb AY284591.1 </a>	<i>Filenchus</i>	88
27	<a href="#">gi 34105816 gb AY284620.1 </a>	<i>Globodera</i>	99
27	<a href="#">gi 188039936 gb EU682391.1 </a>	<i>Punctodera</i>	99
133	<a href="#">gi 34105816 gb AY284620.1 </a>	<i>Globodera</i>	99
133	<a href="#">gi 188039936 gb EU682391.1 </a>	<i>Punctodera</i>	99
53	<a href="#">gi 2707749 gb AF036593.1 AF036593</a>	<i>Heterorhabditis</i>	83
53	<a href="#">gi 109390572 emb AJ920356.1 </a>	<i>Nippostrongylus</i>	83
173	<a href="#">gi 2707749 gb AF036593.1 AF036593</a>	<i>Heterorhabditis</i>	82
173	<a href="#">gi 109390572 emb AJ920356.1 </a>	<i>Nippostrongylus</i>	82
173	<a href="#">gi 8515058 gb AF083019.1 AF083019</a>	<i>Oscheius</i>	82
125	<a href="#">gi 45550257 gb AY552971.1 </a>	<i>Isolaimium</i>	90
182	<a href="#">gi 156068983 gb EF538760.1 </a>	<i>Longidorus</i>	99
100	<a href="#">gi 239775237 gb FJ969128.1 </a>	<i>Merlinius</i>	99
100	<a href="#">gi 164504675 gb EU306351.1 </a>	<i>Amplimerlinius</i>	99
100	<a href="#">gi 239775246 gb FJ969137.1 </a>	<i>Pratylenchoides</i>	99
50	<a href="#">gi 164504675 gb EU306351.1 </a>	<i>Amplimerlinius</i>	99
50	<a href="#">gi 239775237 gb FJ969128.1 </a>	<i>Merlinius</i>	99
31	<a href="#">gi 223950951 gb FJ716584.1 </a>	<i>Mesorhabditis</i>	96
89	<a href="#">gi 223950951 gb FJ716584.1 </a>	<i>Mesorhabditis</i>	95
82	<a href="#">gi 239775238 gb FJ969129.1 </a>	<i>Miculenchus</i>	99
37	<a href="#">gi 34105951 gb AY284755.1 </a>	<i>Mylonchulus</i>	99
38	<a href="#">gi 34105953 gb AY284757.1 </a>	<i>Mylonchulus</i>	99
49	<a href="#">gi 90968502 emb AJ966496.1 </a>	<i>Paratylenchus</i>	99
142	<a href="#">gi 34105892 gb AY284696.1 </a>	<i>Plectidae</i>	96
103	<a href="#">gi 2934879 gb AF036602.1 AF036602</a>	<i>Plectus</i>	98
83	<a href="#">gi 161209841 gb EU130801.1 </a>	<i>Pratylenchus</i>	99
169	<a href="#">gi 161209841 gb EU130801.1 </a>	<i>Pratylenchus</i>	99
69	<a href="#">gi 161209841 gb EU130801.1 </a>	<i>Pratylenchus</i>	98
186	<a href="#">gi 161209841 gb EU130801.1 </a>	<i>Pratylenchus</i>	98
64	<a href="#">gi 158827541 gb EU196007.1 </a>	<i>Rhabditis</i>	99
64	<a href="#">gi 16519208 gb AF430633.1 </a>	<i>Pellioiditis</i>	99
55	<a href="#">gi 158827541 gb EU196007.1 </a>	<i>Rhabditis</i>	98
55	<a href="#">gi 16519215 gb AF430640.1 </a>	<i>Pellioiditis</i>	98



Table 5.3 continued:

Specimen	Closest matching GenBank sequence		% Identity
	Accession number	Taxon	
23	<a href="#">gi 34105849 gb AY284653.1 </a>	<i>Rhabditis</i>	98
25	<a href="#">gi 158827541 gb EU196007.1 </a>	<i>Rhabditis</i>	99
25	<a href="#">gi 16519208 gb AF430633.1 </a>	<i>Pellioiditis</i>	99
143	<a href="#">gi 34105849 gb AY284653.1 </a>	<i>Rhabditis</i>	98
144	<a href="#">gi 34105849 gb AY284653.1 </a>	<i>Rhabditis</i>	98
118	<a href="#">gi 158827541 gb EU196007.1 </a>	<i>Rhabditis</i>	99
118	<a href="#">gi 16519208 gb AF430633.1 </a>	<i>Pellioiditis</i>	99
97	<a href="#">gi 158827541 gb EU196007.1 </a>	<i>Rhabditis</i>	98
97	<a href="#">gi 16519208 gb AF430633.1 </a>	<i>Pellioiditis</i>	98
58	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
71	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
81	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
42	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
131	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
198	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
213	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
201	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
214	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
153	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
137	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
174	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
101	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
110	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	99
154	<a href="#">gi 51699255 emb AJ439583.1 </a>	<i>Trichodorus</i>	97



**Figure 5.3:** Composition of the nematode community at North Lynn farm, Shropshire on 1<sup>st</sup> April 2009. Colours represent functional guilds: Ba<sub>1</sub> brown; Ba<sub>2</sub> blue; Ba<sub>3</sub> yellow; Ba<sub>4</sub> purple; Ca<sub>4</sub> red; plant-parasites green; animal parasites grey, where Ba = bacteriovore, Ca = carnivore and subscript numbers correspond to coloniser-persister scores. NB: The feeding guild and c-p value of *Isolaimium* have not been determined. Asterisks indicate cases in which identity is uncertain (see section 5.3.1).

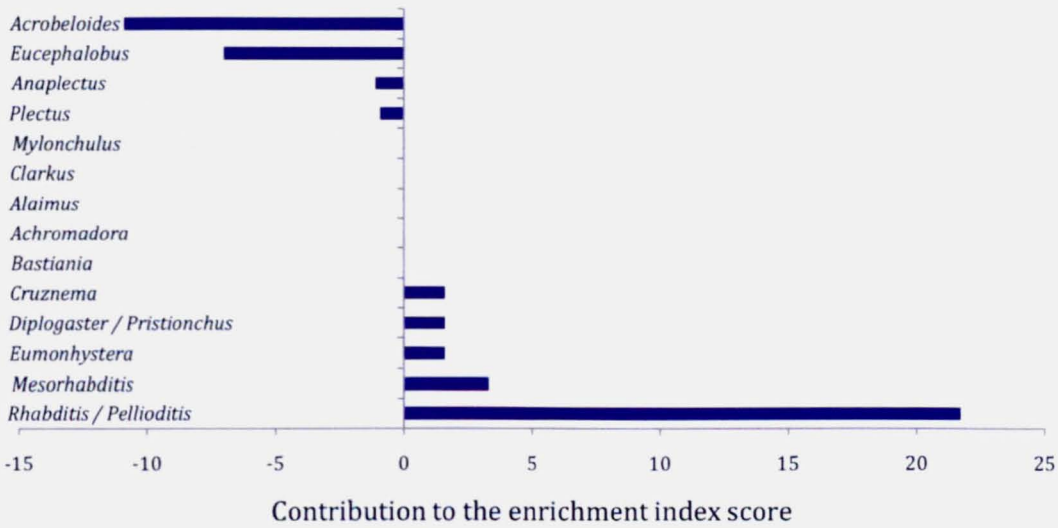
*Nippostrongylus* is a parasite of rodents (Taliaferro & Sarles, 1939) that exists in soil during the first three juvenile stages. *Heterorhabditis* is an insect parasite that exists in soil as infective juveniles (Poinar, 1976). *Oscheius* is a sub-genus of *Rhabditis*, at least some species of which have necromenic associations with insects (Stock *et al.*, 2005).

Only non-parasitic genera are considered for determination of SI and EI scores since endoparasites spend a considerable part of their life cycles outside the soil environment and since the abundance of parasites depends to a great extent on their hosts, potentially confounding measurement of the effects of disturbance on the community (Ferris, 2001). Of the free-living genera, 27 % belonged to c-p class 1, 41 % to c-p class 2, 5 % to c-p class 3 and 25 % to c-p class 4 (Figure 5.3). No c-p class 5 nematodes were found. The *Isolaimidae* and *Mylonchulidae* have not been assigned to a c-p class (Bongers, 1990, 1999; Bongers & Bongers, 1998). Since the identity of the specimen identified as *Isolaimium* was uncertain, based on only 90 % sequence identity to the Genbank sequence, this genus was excluded from community analysis. *Mylonchulus* was assigned a value of four, based on the fact that it is a large-bodied predator (Figure 5.1H) with relatively small gonads (Jairajpuri, 1970) that is closely related to other taxa belonging to c-p class four (Griffiths *et al.*, 2006). Phylogenetic data suggest that the family *Mylonchulidae* is polyphyletic (Holterman *et al.*, 2008) and that the genus *Mylonchulus* shares a clade with the genera *Anatonchus*, *Clarkus*, *Coomansus* and *Prionchulus*, which all belong to the *Mononchidae* (Ca<sub>4</sub>) (Griffiths *et al.*, 2006). The enrichment and structure index scores for the free-living nematode community at North Lynn farm at the time of the initial characterisation were 74.3 and 72.9, respectively.

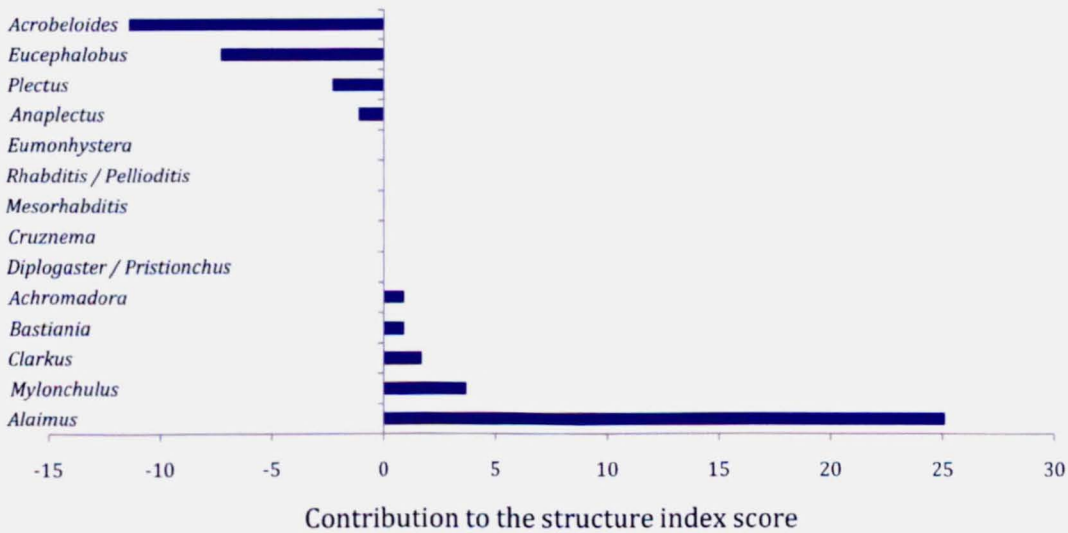
### 5.3.3 Primers for specific amplification of free-living nematode genera and families

Genera were prioritised for primer design based upon the magnitude of their contributions to the enrichment and structure indices (Figure 5.4). The importance of a genus to the index scores is determined by its abundance and weighting given to its functional guild (Tables 5.1 and 5.2). The index scores of the community present at North Lynn farm were determined largely by just four taxa. The abundant c-p 4 bacteriovore *Alaimus* made by far the greatest positive contribution to the structure index whilst the abundant c-p 1 bacteriovore *Rhabditis* made the greatest positive contribution to the enrichment index. For both indices, *Acrobeloides* and *Eucephalobus* made large negative contributions since these genera belong to the Ba<sub>2</sub> functional guild and are basal components of the community. It was considered that in order to accurately determine EI and SI scores, over 90 % of the free-living nematodes in the

A



B



**Figure 5.4:** Sensitivity of the enrichment (A) and structure (B) indices to the presence of particular nematode taxa. Enrichment and structure index scores were calculated for the soil nematode community at North Lynn farm. The scores were then recalculated several times, each time excluding a single taxon from the calculation in order to determine the contribution of that taxon to the index scores. Genera with negative contributions to the indices are those belonging to the Ba<sub>2</sub> and Fu<sub>2</sub> functional guilds, indicative of basal conditions.



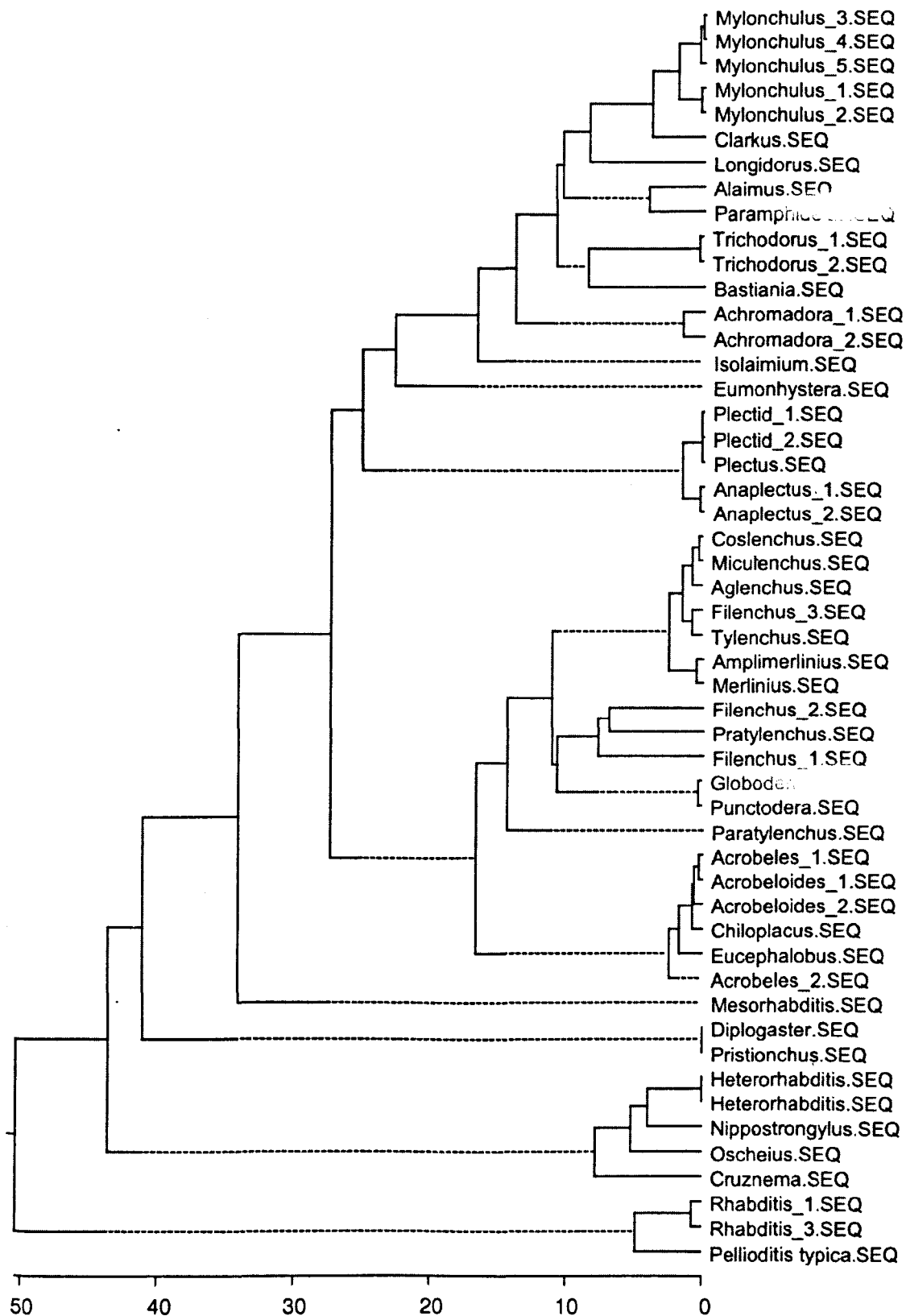
community would have to be identified. Thus primers were designed for *Acrobeloides*, *Acrobeles*, *Alaimus*, *Anaplectus*, *Chiloplacus*, *Clarkus*, *Cruzinema*, *Eucephalobus*, *Mesorhabditis*, *Mylonchulus*, *Pellioiditis*, *Plectus* and *Rhabditis*, which together comprised 40 out of the 44 free-living nematodes present. Primers were also designed and employed for *Isolaimium* and *Heterorhabditis*. However, due to the ambiguity of identification of these genera and the parasitic feeding habit of *Heterorhabditis*, data from qPCR with these primers sets was omitted from the final analysis.

The GenBank 18S rRNA gene sequences contained highly conserved and highly divergent regions. Divergence was greater between distantly related taxa and the genera generally clustered into monophyletic groups in phylogenetic analysis (Figure 5.5). Sequence alignment facilitated identification of regions of the 18S rRNA gene that were conserved within a genus but divergent between genera. Primers were located within these regions and tested for amplification of DNA from target and non-target genera. After several rounds of testing and redesign, primer sets were identified that specifically amplified target genera (Tables 5.4 and 5.5). In several cases, a primer set was designed for one genus but also amplified one or more genera within the same family. This was the case for *Rhabditis* and *Pellioiditis* (Rhabditidae), *Plectus* and *Anaplectus* (Plectidae) and for *Acrobeles*, *Acrobeloides*, *Chiloplacus* and *Eucephalobus* (Cephalobidae) (Table 5.5). Family level identification is sufficient for determination of structure and enrichment index scores and therefore family-specific primer sets are acceptable and indeed preferable to genus-specific sets. For each of these primer sets, amplification efficiency and amplicon size did not vary between the different target genera. Thus, the primers could be used to accurately quantify all target genera in mixed samples.

When a primer set amplified DNA from a non-target genus, it was usually rejected and another set was designed. In some cases, primer sets amplifying DNA from non-target genera were accepted because the efficiency of non-target amplification was low, resulting in Ct values at least four cycles later than with target DNA (Table 5.5).

#### 5.3.4 Effects of fumigation and biofumigation on the free-living nematode community

Fumigation with the methyl isothiocyanate-liberating soil fumigant Dazomet and biofumigation with *B. juncea* cv. ISCI99 both had a strong and lasting effect on the soil nematode community (Table 5.6; Figure 5.6). Ten days post treatment (dpt) with Dazomet or mustard, the total abundance of all nematodes was reduced to 20 % of the abundance in mock treated soil (Figure 5.6A). The strength of the effect was proportional to c-p value, the higher c-p taxa being reduced to a greater extent. At 10 dpt with Dazomet, the



**Figure 5.5:** Phylogenetic tree showing relatedness of nematode genera sampled from [redacted] farm. 18S ribosomal RNA gene sequences of sampled nematodes were amplified using primers SSU18A and SSU26R (Blaxter *et al.*, 1998) and sequenced using the forward primer SSU18A. The resulting ~400 bp sequences were searched for homologues on the National Centre for Biotechnology Information (NCBI) database. Database sequences (~1700 bp) with the greatest sequence identity to the query sequences were used to construct the phylogenetic tree. Horizontal distance along the solid lines represents percentage sequence divergence.

**Table 5.4:** Primers designed to specifically amplify 18S ribosomal RNA gene sequences of particular nematode taxa in real-time PCR.

Primer pair	Sequences	Annealing temp. (°C)	Target genera	Amplicon length (bp)
Alaim_F4c Alaim_R132	ACTTCGGTCCGCTCTTTGGT CATCTCGTTATTTTTTCGTCACTA	62 62	<i>Alaimus</i>	259
Cla_F9c Cla_R23	TCTTTATTGAGTGTCTTGAGTAG GAAGCTGCCCGCCGAGTC	62 62	<i>Clarkus</i>	366
Cruz_F6b Cruz_R42	GCTTCTGGCGTAGTTTGTG GTGAGGAACGGTCTTTTTATG	60 60	<i>Cruznema</i>	260
Ceph_F14 Ceph_R27c	AGCGTGCTGTATCCATTCTC CCCGGTTCAAGCCACTGC	60 60	<i>Acrobeloides</i> <i>Acrobeles</i> <i>Chiloplacus</i> and <i>Eucephalobus</i>	272
GUF2 * AplecR1 *	TTACGTCCCTGCCCTTTGTA CCCGAAAGCCCAACAGC	60 60	<i>Anaplectus</i> and <i>Plectus</i>	93
Het_F4d Het_R8f	TCGGACGAAGGAATGTTGAC CCTTTCGGAATGGTCTTTTTAC	60 62	<i>Heterorhabditis</i>	261
Isolai_F12 Isolai_R15	AAACGGCTACCACTTCTATG CGATAACGGGCCTCAAAGA	58 58	<i>Isolaimium</i>	110
Mesorhab_F1 Mesorhab_R42	ACTTCGGTGTCAATTTATTAGATT AGCAGTCTCGTTAGTTGTTGT	60 60	<i>Mesorhabditis</i>	287
Mylonch_F2 Mylonch_R9b	CTTCTTAGAGGGACGAGCAG ATCCCTATCCCAATCACGAA	62 62	<i>Mylonchulus</i>	175
Pell_F4b Pell_R10b	GACTTCCGATGGCCGTAAC TGGACCATGACTAACCTAAAAG	62 62	<i>Rhabditis</i> and <i>Pellioditis</i>	226

\* These primers were designed by Wang (2009).

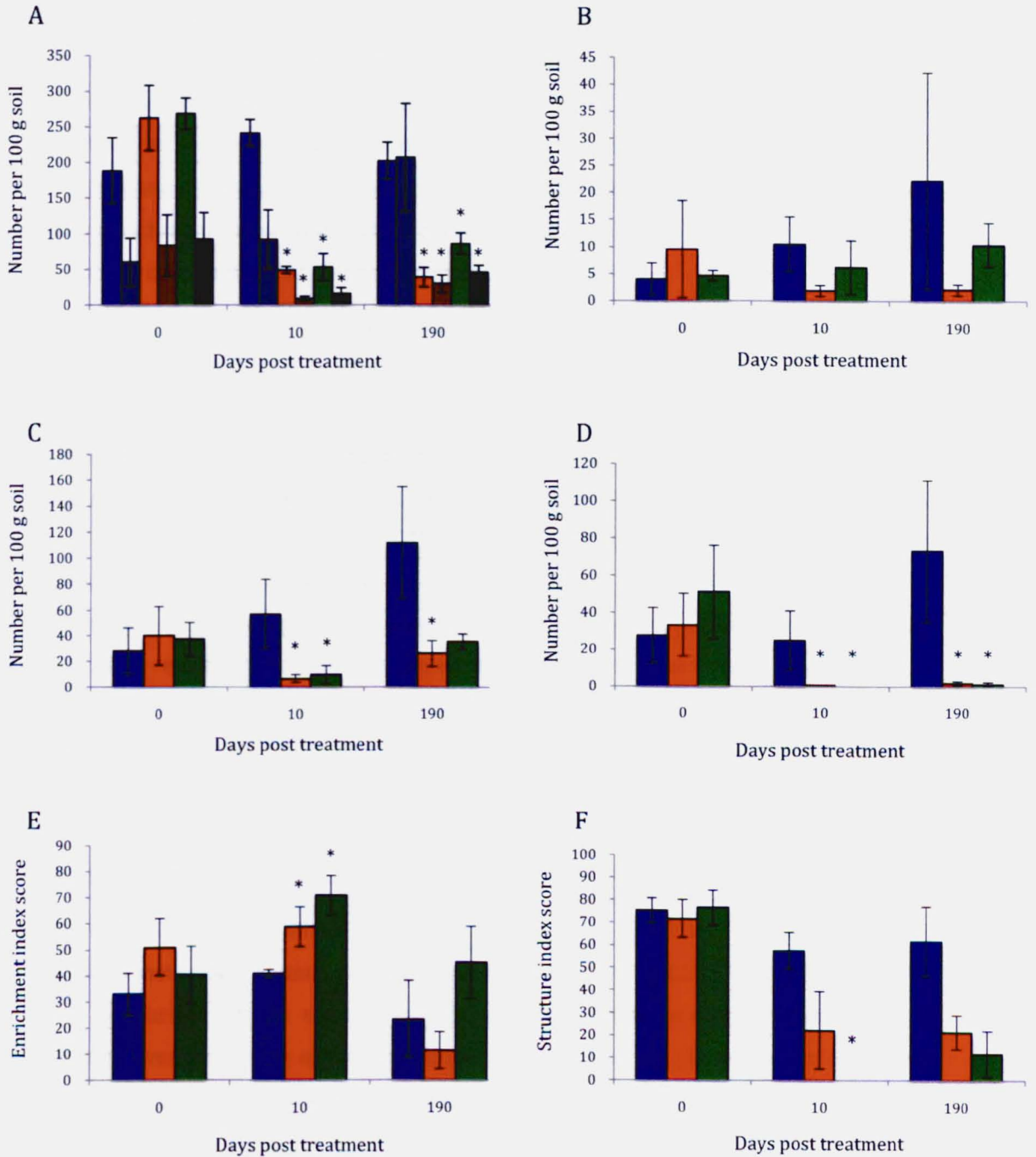






**Table 5.6:** Mean ( $\pm$ SEM) abundance of nine families of free-living nematodes in soil at 0 (immediately prior to treatment), 10 and 190 days post treatment with homogenised *Brassica juncea* cv. ISCI99 leaf material (5 % w/w), Dazomet (70 mg kg<sup>-1</sup> soil, a typical field rate) or nothing (mock treated soil). Coloniser-persister (c-p) values are shown in parentheses after family names. Significant differences from control at  $P \leq 0.05$  are highlighted in bold.

Family	Population density (number 100 g <sup>-1</sup> soil)								
	Pre-treatment			10 days post treatment			190 days post treatment		
	Dazomet	Mustard	Control	Dazomet	Mustard	Control	Dazomet	Mustard	Control
<i>Rhabditidae</i> (1)	9.1 $\pm$ 4.4	4.6 $\pm$ 0.9	3.9 $\pm$ 1.9	1.8 $\pm$ 0.4	6.2 $\pm$ 4.3	11 $\pm$ 5	2.0 $\pm$ 1.4	10 $\pm$ 3	22 $\pm$ 20
<i>Cephalobidae</i> (2)	36 $\pm$ 24	34 $\pm$ 13	22 $\pm$ 15	<b>6.6 <math>\pm</math> 2.8</b>	<b>9.3 <math>\pm</math> 6.8</b>	55 $\pm$ 27	<b>1.8 <math>\pm</math> 0.6</b>	<b>15 <math>\pm</math> 9</b>	96 $\pm$ 45
<i>Plectidae</i> (2)	4.3 $\pm$ 2.0	3.2 $\pm$ 1.5	6.3 $\pm$ 3.1	0.2 $\pm$ 0.2	0.4 $\pm$ 0.2	1.5 $\pm$ 0.6	24 $\pm$ 10	20 $\pm$ 6	16 $\pm$ 6
<i>Alaimidae</i> (4)	32 $\pm$ 17	42 $\pm$ 26	18 $\pm$ 13	<b>0.4 <math>\pm</math> 0.4</b>	<b>0 <math>\pm</math> 0</b>	23 $\pm$ 16	<b>0.4 <math>\pm</math> 0.4</b>	<b>0.5 <math>\pm</math> 0.3</b>	61 $\pm$ 39
<i>Mononchidae</i> (4)	0 $\pm$ 0	1.2 $\pm$ 0.7	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.6 $\pm$ 0.2	0.9 $\pm$ 0.9	7.9 $\pm$ 4.9
<i>Mylonchulidae</i> (4)	1.2 $\pm$ 0.5	8.1 $\pm$ 6.3	9.3 $\pm$ 7.0	0.2 $\pm$ 0.2	0 $\pm$ 0	1.7 $\pm$ 0.8	0.8 $\pm$ 0.8	0 $\pm$ 0	4.9 $\pm$ 4.9
<i>Isolaimiidae</i> (-)	0.8 $\pm$ 0.8	0.5 $\pm$ 0.5	0.4 $\pm$ 0.4	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Total free-living nematodes	83 $\pm$ 43	93 $\pm$ 36	60 $\pm$ 34	<b>9 <math>\pm</math> 3</b>	<b>16 <math>\pm</math> 8</b>	92 $\pm$ 41	<b>30 <math>\pm</math> 12</b>	<b>47 <math>\pm</math> 9</b>	207 $\pm$ 76



**Figure 5.6:** Changes in the nematode community following treatment of soil with homogenised *Brassica juncea* cv. ISCI99 leaf material (5 % w/w) (green columns), Dazomet (70 mg kg<sup>-1</sup> soil, a typical field rate) (red columns) or nothing (mock treated soil) (blue columns). A) Total nematode abundance, including plant parasites, determined directly by counting (light columns) and total free-living nematode abundance, determined by qPCR (dark columns); B) c-p 1 taxa; C) c-p 2 taxa; D) c-p 4 taxa; E) enrichment index; F) structure index. Soil was taken from North Lynn farm, Shropshire, and the experiment was carried out in pots plunged into soil outdoors at Leeds University experimental facility, Grove House Gardens, Leeds. Samples for the zero days post treatment timepoint were taken immediately prior to treatment. Significant differences from the relevant control at  $P \leq 0.05$  are highlighted with asterisks. Error bars show SEM. N = 5.



population densities of c-p 1, c-p 2 and c-p 4 taxa were reduced by 83, 88 and 98 % respectively, relative to mock-treated soil. At the same time after treatment with mustard, the reduction in population density was 41, 83 and 100 % for c-p 1, c-p 2 and c-p 4 taxa, respectively (Figures 5.6B – 5.6D). This increase in the relative abundance of c-p 1 taxa and decrease in the relative abundance of c-p 4 taxa is reflected in an increase in the enrichment index scores and a decrease in the structure index scores of Dazomet and mustard treated soil at 10 dpt (Figures 5.6E and 5.6F).

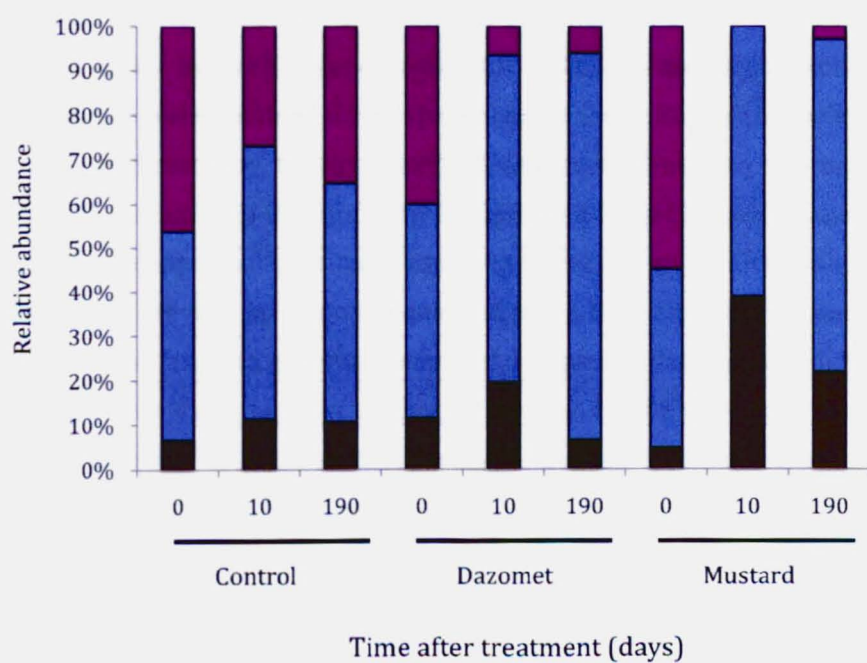
Between 10 and 190 days post treatment with Dazomet or mustard, populations of free-living nematodes had increased but remained low relative to control populations, which also increased in this period (Figure 5.6A). Population growth was more rapid after mustard than Dazomet, the population of free-living nematodes in mustard treated soil being 60 % greater than that in Dazomet treated soil at 190 dpt (Figure 5.6A, Table 5.6). The increase in abundance between 10 to 190 dpt in Dazomet and mustard treated soil was largely due to an increase in the abundance of c-p 2 taxa, mostly *Plectidae*. C-p 2 taxa increased in absolute and relative abundance during this period (Figures 5.6C and Figure 5.7), resulting in declines in the enrichment and structure index scores (Figures 5.6E and 5.6F). In contrast, increased total abundance in mock-treated soil during the same period was due to increases in the populations of all taxa (Table 5.6). The relative abundance of taxa belonging to each c-p class remained relatively constant in the mock-treated soil (Figure 5.7), as reflected in the relatively stable enrichment and structure index scores (Figures 5.6E and 5.6F).

The total abundance of nematode genera estimated by qPCR was consistently lower than total nematode abundance determined directly by counting (Figure 5.6A). The mean proportion of the total nematode abundance that was quantified by qPCR increased over the course of the experiment from 34 % at the pre-treatment timepoint to around 100 % by 190 days post treatment.

SI scores were recalculated without the data for *Mylonchulus* since this genus was assigned to a c-p class by this author. It was found that, due to its low relative abundance, misclassification of *Mylonchulus* would not have greatly affected SI values.

The total glucosinolate content of the *B. juncea* cv. ISCI99 leaf material at the time of incorporation was  $24.4 \pm 3.7$  (SEM)  $\mu\text{mol g}^{-1}$  d.w.





**Figure 5.7:** Changes in the relative abundance of c-p 1 (dark brown), c-p 2 (blue) and c-p 4 (purple) taxa after treatment of soil with homogenised *Brassica juncea* cv. ISCI99 leaf material (5 % w/w), Dazomet (70 mg kg<sup>-1</sup> soil, a typical field rate) or nothing (mock treated soil).

## 5.4 Discussion

### 5.4.1 Characterisation of the nematode community of a UK potato field by sequencing of 18S rRNA genes

Nematodes sampled from a potato field at North Lynn farm, Shropshire, were identified to genus level by sequencing an approximately 400 bp region of their 18S rRNA gene and comparing the resulting sequences to sequences of known origin on the National Center for Biotechnology (NCBI) GenBank database. As in previous studies (Waite *et al.*, 2003; Bhadury *et al.*, 2006; Griffiths *et al.*, 2006; Holterman *et al.*, 2006; Wang, 2009), sequencing of the 18S rRNA gene facilitated identification of the majority of the specimens. One hundred individuals were photographed, their DNA was extracted and a region of their 18S rRNA genes was PCR amplified and sequenced. Good quality sequence data were obtained for 81 specimens. Of these, 80 % could be identified with a high degree of certainty, by virtue of 98 % sequence identity to reference sequences (Table 5.3). Accuracy of identification is supported by qPCR amplification of DNA from identified specimens using primers designed to regions of the GenBank sequences that lie outside of the 400 bp region sequenced using DNA from those specimens. The 19 specimens that failed to generate meaningful sequence data appeared, by morphology, to belong to the same families as those identified by the molecular method (data not shown). There was no evidence that particular taxa were excluded during the process of DNA extraction, PCR amplification and sequencing. Morphological identification to family level agreed with the molecular identification for only 24 % of the 81 identified specimens, highlighting the difficulty of morphological identification.

The nematode community at North Lynn farm was dominated by bacteriovores (49 %) and plant parasites (45 %) (Figure 5.3). There was a small proportion of predators (3 %) and a small proportion (2 %) of nematodes tentatively identified as *Heterorhabditis*, *Oscheius* or *Nippostrongylus*, which are entomopathogenic, necromenic and animal parasitic genera, respectively (Taliaferro & Sarles, 1939; Poinar, 1975; Stock *et al.*, 2005). As parasites or necromenic organisms, these were not considered part of the free-living community. Low relative abundance of predators and omnivores is typical of cultivated soils but the total absence of obligate fungivores is unusual (Berkelmans *et al.*, 2003; Leroy *et al.*, 2009; Wang, 2009; Kapagianna *et al.*, 2010). Wang (2009) used the same 18S rDNA sequencing method as in the present study to characterise the nematode community at Leeds University farm, Headley Hall, West Yorkshire. The time of year when the soil was sampled was the same as in the present study (April) and the only noteworthy methodological difference is that Wang (2009) used the method of

Whitehead & Hemming (1965) for nematode extraction. The composition of the Headley Hall community differed from that at North Lynn in several respects. The Headley Hall community contained a smaller proportion of bacteriovores (29 %), a similar proportion of predators (2 %) and a greater proportion of plant parasites (57 %), omnivores (7 %) and fungivores (5 %) (Wang, 2009). The bacteriovore communities at the two sites were similar, the major difference being the much greater proportion of *Alaimus* at North Lynn. The genera *Acrobeloides*, *Anaplectus*, *Eucephalobus*, *Alaimus*, *Pellioiditis* and *Rhabditis* were common to both sites. The sole predatory genus at Headley Hall was *Anatonchus*, which belongs to the same family (Mononchidae) as *Clarkus* found at North Lynn. Despite the smaller relative abundance of high c-p class omnivores and predators at North Lynn, the structure index score there (72.9) was greater than at Headley Hall (61.8), due to the high relative abundance of *Alaimus* (Ba<sub>4</sub>). The enrichment index score was also greater at North Lynn (74.3) than at Headley Hall (44.4). The values of both indices for the North Lynn soil are towards the upper ends of the ranges generally observed for agricultural soils (Ferris & Matute, 2006; Dupont *et al.*, 2009; Leroy *et al.*, 2009; Liang *et al.*, 2009; Kapagianna *et al.*, 2010).

#### 5.4.2 Development of qPCR primers for specific quantification of free-living nematode taxa

The cost of carrying out sequencing reactions for hundreds of individual nematodes prohibits use of DNA sequencing for ecotoxicological studies. A qPCR method is much more economical. Firstly, individual qPCR are much less costly than sequencing reactions. More importantly, qPCR can be performed with DNA from pooled samples of hundreds of nematodes. Entire communities can be characterised in just a few reactions, one for each taxon quantified. The greatly increased volume of samples that can be processed more than compensates for the inevitable experimental error introduced by qPCR. Primer sets were designed that can quantify the abundance of 13 genera of free-living nematodes (Table 5.4). These 13 genera contained over 90 % of the free-living nematode abundance at North Lynn farm at the time of initial sampling in April.

As previously reported for sub-clades of the order Mononchida (Holterman *et al.*, 2008) and several distantly related genera of free-living and plant-parasitic nematodes (Wang, 2009), the degree of divergence between 18S rRNA gene sequences from different taxa enabled identification of primer sites that were conserved within a genus but divergent between genera. Sets of primers were identified that specifically amplified one or more genera from the community (Table 5.4). Since genera within a family share the same trophic guild and similar life history traits (Bongers, 1990, 1999), functional guilds are

assigned to families (Ferris *et al.*, 2001) and family level identification is sufficient. Where possible, primers were designed that would amplify several genera within the same family. One set of primers (Ceph\_F14 + Ceph\_27c) specifically amplified DNA of the Cephalobid genera *Acrobeloides*, *Acrobeles*, *Chiloplacus* and *Eucephalobus* (Table 5.5). Another set (GUF2 + AplecR1) was designed by Wang (2009) for specific amplification of DNA from the Plectid genera *Plectus* and *Ceratoplectus*. This primer set was found to also amplify *Anaplectus* DNA (Table 5.5). Finally, one set of primers (Pell\_F4b + Pell\_R10b) amplified DNA of the Rhabditid genera *Rhabditis* and *Pellioiditis* but not *Cruznema* or *Mesorhabditis*. Floyd *et al.* (2002) found Rhabditid genera to possess a high degree of heterogeneity in the 18S rDNA gene sequence. Divergence between 18S rDNA sequences of the Rhabditid genera from North Lynn farm (Figure 5.5) prevented design of a primer set that would comprehensively and specifically amplify DNA from all of these genera. Separate genus-specific primer sets were designed for *Cruznema* and *Mesorhabditis*.

Products of qPCR with SYBR Green should be between 100 and 400 bp in length. Product sizes were within this range for all primer pairs except GUF2 + AplecR1, which had a product size of 93 bp (Table 5.4). The predicted annealing temperatures of all primer pairs except Isolai\_F12 + Isolai\_R15 were between 60 and 62 °C, allowing reactions with different primer pairs to be carried out simultaneously in the same run.

Primer specificity was tested in qPCR using single nematode DNA digests as template. Many sets of primers were rejected due to non-target amplification. The primers that were retained did not amplify non-target genera in the vast majority of cases (Table 5.5). However, some primer sets were retained despite non-target amplification. In these cases, non-target amplification occurred with lower efficiency than target amplification, resulting in Ct values at least four cycles greater. This was deemed acceptable for the purposes of the study. Further study may be able to identify more specific primer sets if other parameters, such as annealing temperature, are compromised.

#### 5.4.3 Characterisation of the free-living nematode community by qPCR

The primers were used in real-time PCR to quantify the abundance of 13 genera of free-living nematodes in mixed samples of up to 100 individuals. Quantification of DNA from pooled samples of nematodes introduces additional sources of experimental error to the estimation of abundance. Technical error, such as variation in DNA extraction efficiency

and PCR conditions can be minimised by careful technique and replication. Good experimental design should ensure that such sources of error are randomised with respect to experimental treatments. A potentially confounding variable that is more difficult to control is the genome copy number per individual, which can vary greatly for r-strategists with abundant gametes. Genome copy number can vary by an order of magnitude between juvenile and mature hermaphrodite *C. elegans* (Golden *et al.*, 2007). It could be argued that abundance of gametes indicates future population size and that environmental disturbance that reduces fecundity ought to be taken into account. That the individual should be the unit quantified in nematode community analysis is not incontrovertible. Clearly all genomes do not possess equal fitness but nor do individuals of different developmental stage.

In the present study, the degree of variation in genome size between individuals was tested by determining Ct values in qPCR using DNA from different individual specimens and the Ct values in replicated qPCR using DNA from the same individual. Variation was no greater between individuals of the same genus than between repeated measurements of the same individual. There was no evidence that variation in genome size was greater for r- than for K-strategists. Thus, variation in genome copy number does not appear to have confounded the results of the study. However, translation of qPCR Ct values into abundance of nematodes could be improved by constructing calibration curves that relate Ct value to the abundance of nematodes in pooled samples containing varying known amounts of the target nematode. This would also control for the possible influence of non-target DNA on target amplification efficiency.

#### **5.4.4 Effects of biofumigation and fumigation on the soil nematode community**

Biofumigation with *Brassica juncea* cv. ISCI99 and fumigation with the methyl isothiocyanate-liberating fumigant Dazomet both had strong and lasting effects on the nematode community. The two treatments both reduced overall abundance by around 80 % and the abundance of sensitive taxa by almost 100 % (Table 5.3; Figure 5.6).

Both Dazomet and mustard caused SI scores to decrease due to a decline in the relative abundance of the c-p 4 genera *Alaimus*, *Clarkus* and *Mylonchulus* (Table 5.6; Figures 5.6 and 5.7). At 10 dpt, sensitive taxa had declined in relative abundance and SI scores in mock-, Dazomet- and mustard-treated soil were  $57 \pm 8$  (SEM),  $22 \pm 17$  and zero, respectively. Another methyl isothiocyanate-liberating soil fumigant, metham sodium has previously been shown to reduce the relative abundance of high c-p value

nematodes (Ettema & Bongers, 1993; Pen-Mouratov & Steinberger, 2005; Kapagianna *et al.*, 2010), as has methyl bromide (Wang *et al.*, 2006), which sometimes totally eliminates nematodes of all c-p classes (Yeates *et al.*, 1991). Normally, application of green manure to agricultural fields stimulates microbial production and causes a consequent increase in microbivorous nematodes but does not alter the community SI score (Ferris *et al.*, 1996; McSorley & Frederick, 1999; Colins *et al.*, 2006; Ferris & Matute, 2006; Wang *et al.*, 2006; Dupont *et al.*, 2009; Leroy *et al.*, 2009; Liang *et al.*, 2009; Gruver *et al.*, 2010). However, the green manures in previous studies lacked nematicidal activity. Even the brassicaceous materials used by Collins *et al.* (2006) and Gruver *et al.* (2010) showed no evidence of biocidal activity towards fungal pathogens or plant parasitic nematodes. In contrast, the *B. juncea* cv. ISCI99 leaf material used in the present study is high in 2-propenyl glucosinolate and is effective at reducing the population density of *G. pallida* (see Chapter 3). To this author's knowledge, this is the first report of the effects on the free-living community of a brassica green manure with a proven nematicidal effect towards a plant-parasitic species. In this case, incorporation of Dazomet or mustard green manure both reduced population densities of all nematode taxa observed. This is consistent with the fact that isothiocyanates are general biocides that are toxic to a wide range of organisms, including the free-living Ba<sub>1</sub> nematode *C. elegans* (Donkin *et al.*, 1995). The degree of population reduction was proportional to c-p class; sensitive c-p 4 taxa were most severely reduced and relatively tolerant c-p 1 taxa were least severely reduced, resulting in elevated EI scores and depressed SI scores at 10 days post treatment (Figures 5.6E and 5.7).

Once the toxins associated with fumigation or biofumigation had cleared from the soil, it was expected that there would be an enrichment response as microbial activity increased due to availability of organic matter in the form of dead meiofauna and, in the case of biofumigation, plant material. Between 10 and 190 days post treatment with mustard or Dazomet, nematode populations did increase but contrary to predictions, Ba<sub>2</sub> nematodes increased more dramatically than enrichment opportunist Ba<sub>1</sub> nematodes (Table 5.6). This resulted in a greater relative abundance of Ba<sub>2</sub> taxa and reduced EI values (Figures 5.6E and 5.7). It is possible that Ba<sub>1</sub> populations had already peaked by 190 days after addition of mustard green manure and had been succeeded by Ba<sub>2</sub> taxa since Ba<sub>1</sub> populations can fluctuate dramatically, peaking and returning to baseline levels within this timeframe (Ettema & Bongers, 1993; Ferris & Matute, 2006). For instance, by six months following incorporation of alfalfa green manure, Ba<sub>1</sub> Rhabditidae had undergone two cycles of population fluctuation while Ba<sub>2</sub> Cephalobidae steadily increased throughout the period (Ferris & Matute, 2006).

SI scores at 190 days post treatment with mustard or Dazomet were still substantially lower than in mock-treated soil. Ettema & Bongers (1993) found that c-p 3 to c-p 5 nematode populations had not recovered even one year after fumigation with metham sodium. High c-p class K-strategists have lower intrinsic rates of natural increase and hence require longer than r-strategists to reach carrying capacity following disturbance (Bongers 1990).

Over the six-month course of the experiment, the proportion of the nematode community that was quantified by qPCR increased (Figure 5.6A). The difference between total abundance and the abundance of genera quantified in qPCR was probably largely accounted for by plant parasites, which would have declined throughout the course of the experiment since there were no plants growing in the soil.

## 5.5 Summary

The main outcomes from this chapter are as follows:

1. The soil nematode community of a UK potato field was characterised by partial sequencing of the 18S rRNA genes of 81 randomly sampled specimens.
2. Comparison of the resulting sequences with those of identified specimens on the GenBank database enabled identification to genus.
3. Community enrichment and structure index values were determined according to Ferris *et al.* (2001).
4. Nine sets of primers were designed that enabled quantification of 13 genera of free-living nematodes. Together, these genera comprised over 90 % of the free-living nematode abundance and contained the key genera contributing to enrichment and structure index scores.
5. These primers were used in quantitative real-time PCR to determine the community composition in soil that had been mock-treated, treated with the fumigant Dazomet or treated with *B. juncea* cv. ISCI99 green manure (5 % w/w).
6. *B. juncea* cv. ISCI99 and Dazomet both had strong and lasting effects on the free-living nematode community. They reduced the total abundance of free-living nematodes by 80 % and the abundance of sensitive genera by almost 100 %.

## Chapter 6: General Discussion

*In vitro* and glasshouse experiments were performed to determine the effects of isothiocyanates and brassica leaf homogenates on the physiology and viability of the potato cyst nematode *Globodera pallida* (Chapters 2 and 3) and the fungal pathogen *Rhizoctonia solani* (Chapter 4). The results show that incorporation of brassica green mulches into soil can provide high levels of control of potato cyst nematodes through the release of toxic volatile isothiocyanates. In contrast, brassica green mulches had no significant effect on the inoculum density of *R. solani* or consequent disease on potato. The biosafety of the approach was examined by characterising the free-living nematode community before and after treatment of soil with the synthetic pesticide Dazomet or green manure of a *Brassica juncea* cultivar containing a high content of 2-propenyl glucosinolate (Chapter 5). Both Dazomet and *B. juncea* green manure had strong and lasting effects on the free-living nematode community, dramatically reducing the abundance of sensitive taxa. The implications of these findings will now be discussed in relation to the potential use of biofumigation in potato cultivation.

### 6.1 Synthesis of findings in relation to potential field application of biofumigation

#### 6.1.1 Attainment of effective doses of brassica biomass and isothiocyanates required to control *Globodera pallida* in the field

The results of toxicity assays with leaf extracts suggest that concentrations of brassica biomass effective at suppressing *G. pallida* second-stage juveniles (J2) can be attained in the field. J2 were highly sensitive to isothiocyanates, 50 % reduction in recovery through sand columns occurring after 24 h exposure to concentrations as low as 4  $\mu\text{M}$ . J2 were also highly sensitive to aqueous extracts of brassica leaves; over 90 % reductions in the rate of recovery through sand columns occurring after 24 h exposure to extracts from 1.8 mg dry leaf matter  $\text{ml}^{-1}$ . In the field, sufficient inhibition of motility to prevent root invasion would result in the death of the animal. Zasada *et al.* (2009) found that after 2 h exposure to 10  $\mu\text{M}$  benzyl isothiocyanate, *Meloidogyne incognita* J2 were still alive but their movement was inhibited. After this treatment, the number of J2 colonising roots of *Glycine max* was reduced by over 90 % and multiplication was reduced by over 75 %. Thus, whether or not concentrations found to inhibit motility in the present study were sufficient to cause direct mortality, attainment of such concentrations in the field at the time of nematode hatching would be expected to reduce root invasion and nematode multiplication. The concentration of dry leaf tissue



used in preparation of extracts was  $1.8 \text{ mg ml}^{-1}$  and several of these extracts caused over 90 % inhibition of motility. Previous observations of agricultural soils show moisture content at field capacity to be around 24 % by mass (Veihmeyer & Hendrickson, 1931) and bulk density to be around  $1.7 \text{ kg l}^{-1}$  (Leistra & Smelt, 1974; Sanchez-Moreno *et al.*, 2006; Laegdsmand *et al.*, 2007; Okada & Harada, 2007). Thus  $1.8 \text{ mg dry mass ml}^{-1}$  would be equivalent to  $2.2 \text{ tonnes dry matter ha}^{-1}$  to a depth of 0.3 m. Effective doses of brassica leaf homogenates towards nematodes have previously been found to be greater in soil than *in vitro* (Zasada & Ferris, 2004) since a smaller proportion of isothiocyanate is bio-available due to adsorption to soil constituents (Poulsen *et al.*, 2008). Predictions based on *in vitro* ED values underestimated by 70 % the amounts of biomass required to cause a given level of suppression (Zasada & Ferris, 2004). Thus, the amount of dry matter required to cause 90 % suppression in the upper 0.3 m layer in the field is estimated to be  $3.8 \text{ tonnes dry matter ha}^{-1}$ . Yields of epigeal parts of *B. juncea* grown for eight to nine weeks (until flowering) range from  $1.3$  to  $7.0 \text{ t d.w. ha}^{-1}$  (Larkin & Griffin, 2007; Friberg *et al.*, 2009; Motisi *et al.*, 2009b), the mean value from these studies being  $3.9 \text{ t d.w. ha}^{-1}$ .

Comparisons of the dose-response curves for J2 exposed to pure isothiocyanates with previous estimates of amounts of isothiocyanates achieved in soil also suggest that effective doses can be achieved in the field. One of the maximum published concentrations of isothiocyanate in soil after incorporation of a brassica green manure was  $91 \text{ nmol g}^{-1}$  soil (Gimsing & Kirkegaard, 2006). As stated above, the moisture content of agricultural soils at field capacity is typically around 24 % (Veihmeyer & Hendrickson, 1931). For a typical loam soil with 2 % organic matter and a moisture content of 20 % at  $12 \text{ }^\circ\text{C}$ , the proportion of methyl isothiocyanate in the water phase is calculated to be 80 % (Smelt & Leistra, 1974). Thus, the peak concentration of isothiocyanate in the water phase of soil during biofumigation could be as much as  $360 \text{ } \mu\text{M}$ , 30 times the  $\text{ED}_{80}$  values for benzyl, 2-phenylethyl and 2-propenyl isothiocyanate determined in the present study. The concentration of  $91 \text{ nmol g}^{-1}$  soil recorded by Gimsing & Kirkegaard (2006) was achieved using *B. juncea* cv. Nemfix, containing a high concentration of 2-propenyl glucosinolate, with a yield of  $5.6 \text{ t dry matter ha}^{-1}$  and several practices previously shown to increase the rate of glucosinolate hydrolysis, namely tissue pulverisation, immediate incorporation and irrigation. More typical concentrations attained range from 0.1 to  $22 \text{ nmol g}^{-1}$  soil (Gimsing *et al.*, 2006). The upper end of this range is still equivalent to seven times the  $\text{ED}_{80}$  value for 2-propenyl isothiocyanate determined here.

Based on these calculations, it should be possible to cause large reductions in the proportion of *G. pallida* J2 invading potato roots by incorporation of brassica green manures. However, this would require that peak isothiocyanate concentrations occur at a time when motile J2 are present in the soil, which would mean incorporating brassica tissues after potato planting. This is unfeasible and therefore encysted eggs rather than free-swimming juveniles would be the life-cycle stage targeted in practice.

Glasshouse experiments demonstrated that brassica leaf tissue incorporated into soil at 5 % (w/w) caused high levels of mortality to *G. pallida* encysted eggs, especially when the soil was covered with polyethylene sheeting. Over 95 % reductions in the population density of encysted eggs were observed in soil treated with several of the *B. juncea* cultivars and then covered. When treated soil was left uncovered, reductions of up to 85 % were observed. A rate of fresh brassica biomass equal to 5 % of the mass of soil was considered to be the upper limit of what could be achieved in the field and an appropriate amount for a “proof of concept”. The amounts of brassica biomass typically generated in the field would only be sufficient to treat a relatively thin layer of soil at this rate. Assuming once more a soil bulk density of 1.7 kg l<sup>-1</sup> and a brassica dry matter content of 10 %, a good yield of 7 t d.w. ha<sup>-1</sup> would generate sufficient biomass to treat only an 80 mm deep layer at 5 % (w/w). Given the sigmoidal shape of dose-response curves, it may be possible to generate substantial kill with considerably lower rates of biomass. For instance, the ratios of ED<sub>80</sub>/ED<sub>95</sub> for benzyl and 2-phenylethyl isothiocyanate towards *G. pallida* J2 were 0.33 and 0.45, respectively. By treating a thicker layer of soil at a lower rate, efficacy could be greatly increased. It would be useful to determine the dose-response relationships for effective cultivars such as *B. juncea* ISCI99 in soil in order to optimise incorporation depth for maximum efficacy. Let us assume that a 200 mm layer could be treated with sufficient biomass to cause 80 % mortality in that layer. Been & Schomaker (1999) found that the mean proportion of cysts in the surface 200 mm layers of six fields was 73 %. Thus, 80 % mortality in this layer would be equivalent to 58 % mortality of the whole population.

### 6.1.2 Attainment of effective doses of brassica biomass and isothiocyanates required to control *Rhizoctonia solani* in the field

*In vitro* toxicity assays showed *R. solani* to be relatively tolerant of isothiocyanates compared with *G. pallida*. ED<sub>50</sub> values for *R. solani* growth inhibition were on average 60-fold greater than ED<sub>50</sub> values for inhibition of *G. pallida* J2 motility. After exposure to doses of isothiocyanate sufficient to cause 100 % growth inhibition, *R. solani* mycelium

transferred to toxin-free medium recovered and continued to grow normally. Given that isothiocyanates only persist in soil for a few days at the very most (Warton *et al.*, 2003), sub-lethal doses would not provide practical control; concentration-time products would have to be sufficient to kill the inoculum. The most toxic isothiocyanate to *R. solani* was 3-(methylthio)propyl isothiocyanate. The minimum concentrations of this compound required to permanently inhibit the growth of isolates UN (AG3) and Rs08JL (AG3), representative of clones infecting potato in the UK, were 0.3 and 1.0 mM, respectively. For the more commonly produced, 2-propenyl isothiocyanate, these values were 0.5 and 2.0 mM. As stated above, one of the highest isothiocyanate concentrations in soil recorded was approximately equivalent to 360  $\mu$ M. Concentrations required to cause a given effect on fungal mycelium are likely to be greater in soil than *in vitro*, as they are for nematodes (Zasada & Ferris, 2004). Also, mycelium growing from sclerotia or colonised plant matter is more tolerant of isothiocyanates than mycelium growing from agar plugs (Yulianti *et al.*, 2006). Therefore, it seems unlikely that isothiocyanates released from brassica green manures could reliably control tuber- or soil-borne inoculum of *R. solani* in potato fields.

A glasshouse trial confirmed that brassica green manures incorporated at 5 % (w/w) failed to reduce the density of *R. solani* soil-borne inoculum or to suppress disease on subsequently planted potatoes. It seems that *Rhizoctonia solani* is not amenable to control by biofumigation, in the strict sense of suppression due to isothiocyanates. However, as discussed in section 4.4.2, recent findings have shown significant suppression of disease caused by *R. solani* on sugar beet and apple through mechanisms unrelated to isothiocyanates (Cohen & Mazzola, 2006; Motisi *et al.*, 2009a). Green manuring has been hypothesised to bring about changes in the soil microbiota that result in reduced saprophytic growth of *R. solani* (Yulianti *et al.*, 2007) and induction of host resistance to *R. solani* in apple (Cohen & Mazzola, 2006). Such biological mechanisms of disease suppression were not investigated in the present study.

### 6.1.3 Mechanisms involved in nematode suppression by brassica green manures

Results of *in vitro* and glasshouse experiments suggested that both isothiocyanates and other compounds were involved in suppression of *G. pallida*. Measurements of total leaf glucosinolate were used to determine the quantities of isothiocyanate potentially available in toxicity assays with leaf extracts (section 2.4.3). These quantities were compared with the dose-response curves with pure isothiocyanates. For some of the

brassicas, such as *Brassica juncea* cv. Nemfix and *Nasturtium officinale*, the amounts of isothiocyanate potentially available could have caused the observed suppression. In other cases, such as *Raphanus sativus* cv. Weedcheck, greater levels of suppression were observed than predicted from glucosinolate content alone, suggesting the additional involvement of other compounds.

In a glasshouse trial, a strong correlation ( $r^2 = 0.89$ ) was observed between the concentration of isothiocyanate-producing glucosinolate in incorporated leaf tissues and the percentage reduction in PCN egg viability in open soil, indicating that isothiocyanates were responsible for the effect. In covered soil, the correlation was weaker ( $r^2 = 0.50$ ) and there appeared to be a baseline of 60 % suppression regardless of glucosinolate content. Even the non-brassica wheat caused significant suppression in covered soil, showing that other mechanisms besides isothiocyanate production were involved. As discussed in sections 1.9.4.3 and 3.4.3, volatile sulphur containing toxins unrelated to glucosinolates can be generated by microbial degradation of sulphur-containing organic compounds that are present in high abundance in brassicas, particularly under anaerobic conditions (Lewis & Papavizas, 1970; Bending & Lincoln, 1999; Wang *et al.*, 2009). The amounts of these compounds produced during decomposition vary greatly between brassicas (Wang *et al.*, 2009) and this variation could have affected the levels of suppression observed. Anaerobic conditions may have developed in the covered soil, due to reduction of gas exchange and evaporation by the polyethylene covering and resulting waterlogged conditions.

#### 6.1.4 Toxicity of brassica tissues in relation to glucosinolate profiles

Total glucosinolate content of leaves was a poor predictor of suppressive activity towards *G. pallida* J2, suggesting that quality of glucosinolate and other factors are important determinants of bioactivity (see section 2.4.3 *Toxicity of brassica leaf extracts to G. pallida and relationship with glucosinolate content*). Brassicas containing glucosinolates that do not degrade to isothiocyanates were found to be ineffective at controlling *G. pallida* encysted eggs in soil.

In soil, the *B. juncea* and *B. rapa* cultivars were significantly more effective than other brassicas. These plants contain high concentrations of 2-propenyl or 3-butenyl glucosinolate, which are hydrolysed to the corresponding isothiocyanates. Consistent with findings for other nematodes (Lazzeri *et al.*, 1993, 2004; Buskov *et al.*, 2002; Zasada & Ferris, 2003), 2-propenyl isothiocyanate was amongst the most toxic

compounds to *G. pallida* *in vitro* but was not quite as toxic as 2-phenylethyl or benzyl isothiocyanate. Despite the slightly lower toxicity of aliphatic isothiocyanates in direct contact toxicity tests, they are more toxic than the aromatic isothiocyanates to the insect *Naupactus leucoloma* in soil due to their greater volatility and hence bioavailability (Matthiessen & Shackleton, 2005). The results of the present work suggest that the same may be true in toxicity to nematodes, despite the fact that nematodes inhabit the aqueous phase, rather than the air phase of soil. Soil fumigation studies with methyl isothiocyanate suggest that it disperses through soil in air-filled pores (Leistra & Smelt, 1974) (see section 1.9.4.1). As stated above, concentration-time products of methyl isothiocyanate and 1,3-dichloropropene can be well in excess of lethal amounts to PCN at the depth at which they are incorporated (Leistra & Smelt, 1974; Smelt *et al.*, 1974). Hence dispersal rate of the fumigants can be more important than their toxicity in determining population reduction of PCN. Poor performance of methyl isothiocyanate relative to the less toxic but more volatile 1,3-D can sometimes result from heterogeneous distribution in the horizontal and vertical planes (Leistra & Smelt, 1974; Smelt *et al.*, 1974). During biofumigation, macerated brassica tissue is dispersed throughout a soil layer rather than injected at a constant depth. Nevertheless, dispersal rate of isothiocyanates may be an important factor in the bioavailability of the toxins.

#### 6.1.5 Comparison of the effects of brassica green manures on plant parasitic and free-living nematodes and the biosafety of the approach

When field soil was amended with green manure of *B. juncea* cv. ISCI99 at the same rate that had been used in the glasshouse experiment with PCN, a strong effect on the free-living nematode community was observed. This is consistent with the theory that isothiocyanates are general biocides with non-specific effects (Brown & Morra, 1997). The family to which *Globodera* belongs, Heteroderidae, is classified as intermediate on the coloniser-persister scale, belonging to c-p class 3 (Bongers, 1990). Families in lower c-p classes are predicted to be less sensitive and families in higher c-p classes more sensitive to disturbance. This appeared to be the case since the abundance of Cephalobidae (c-p 2) was reduced by 83 % and the abundance of Alaimidae (c-p 4) was reduced by virtually 100 %, compared with a 95 % reduction in *G. pallida* abundance after an almost identical biofumigation treatment. The effects of biofumigation on the free-living nematode community were very similar to the effects of Dazomet. Metham sodium, another methyl isothiocyanate-liberating pesticide has been found to be more harmful to the free-living nematode community than the organophosphate fenamiphos

(Pen-Mouratov & Steinberger, 2005). Thus, it appears that biofumigation is at least as detrimental to the soil nematode community as granular nematicides. Ecosystem services performed by free-living nematodes, such as biological control may be inhibited by biofumigation. For instance, Ramirez *et al.* (2009) found that mustard green manures reduced the infection of bioassay insects by endogenous entomopathogenic nematodes in potato fields in Washington State, USA. These nematodes contribute to biological control of Colorado Potato Beetle, which is a major pest of potato in the USA (Berry *et al.*, 1997) but not in the UK.

The potential of biofumigation to harm human health or aquatic ecosystems is almost certainly much less than for granular nematicides due to the low environmental persistence of isothiocyanates. Current carbamate and organophosphate nematicides are much more persistent in the environment than isothiocyanates, having half-lives of up to 70 d (Pantelelis *et al.*, 2006; Osborne *et al.*, 2010). Oxamyl has been detected in groundwater (Leistra & Boesten, 1989) and fosthiazate has the potential to leach into groundwater from acidic soils with low organic matter content (Karpouzas *et al.*, 2007). In contrast, isothiocyanates are rapidly degraded in soils, half-lives from one to 40 hours being reported (Borek *et al.*, 1995; Warton, 2003; Gimsing & Kirkegaard, 2006; Gimsing *et al.*, 2007a;) and are considered to pose little threat of leaching (Gimsing & Kirkegaard, 2009). Isothiocyanates are already consumed in the diet in brassica vegetables. The greatest risk from exposure to isothiocyanates from brassica green manures would be during and immediately after incorporation. Saeed *et al.* (2000) estimated the potential intake of methyl isothiocyanate by farm workers in fields during the first 35 h following subsurface injection of metham sodium to be 0.4 mg person<sup>-1</sup> day<sup>-1</sup>. Permissible exposure limits have not been established for this compound and a 48 h re-entry period is recommended as a precaution. The isothiocyanates produced by brassicas are less volatile than methyl isothiocyanate (Sarwar *et al.*, 1998) and the maximum concentrations of isothiocyanate in soil during biofumigation are less than 20 % of the concentration of methyl isothiocyanate produced during treatment with metham sodium (Brown & Morra, 1997). Nevertheless, farmer workers operating vehicles during incorporation of mustard green manures have complained of eye irritation (Andy Barker, pers. comm.). The problem could probably be solved with the use of personal protective equipment but is worthy of further investigation.

## 6.2 Integration of biofumigation into current potato farming practice

### 6.2.1 Biofumigation requires little change to current practice

Adoption of a novel control measure by farmers clearly relies upon the technique being reliably effective. In addition, farmers are more likely to adopt a new practice if it involves minimal change to current practice in terms of agronomy, capital investment and knowledge. Biofumigation would easily integrate into current potato farming. Firstly, brassica crops such as *Brassica* spp., *Raphanus sativus*, *Sinapis alba* and *Eruca sativa* grow rapidly, reaching maturity within eight to nine weeks during the growing season. They are also reasonably cold-hardy. These qualities allow them to be grown during periods that would otherwise be fallow. In Washington State, USA, mustard cover crops are sown immediately after wheat harvest in August and ploughed under in late October when the soil is still reasonably warm (Gies, 2004; McGuire, 2004). In the UK, winter cereals are harvested in July, providing an even longer window of opportunity. Alternatively, the brassica crop can be sown in the autumn, allowed to over-winter and ploughed under in April after a period of spring growth. Such an approach was examined as part of this study in collaboration with Harper Adams University College. On commercial potato farms in Shropshire, a mustard mixture (*B. juncea* and *S. alba*) was sown in October after wheat. The plants grew to a small stature (5-10 cm) in autumn, survived the winter well despite temperatures below -10 °C and put on new growth in the spring. Unfortunately, dry spring conditions lead to poor growth and resulted in growers deciding to plant their potato crops early. Thus, the mustard was incorporated before it had attained substantial biomass. Research into optimal agronomic practice is ongoing.

Growing a mustard biofumigant crop costs £100 ha<sup>-1</sup> for seed plus an additional £200-300 ha<sup>-1</sup> for husbandry. This compares favourably with the cost of fumigation with 1,3-dichloropropene before it was withdrawn, £ 600 ha<sup>-1</sup>, making biofumigation economically viable so long as satisfactory PCN control is provided. Since rape is one of the main crops grown in rotation with potatoes, growers already have the necessary equipment and knowledge for brassica cultivation. No new equipment would be required for incorporation either. The equipment recommended for incorporation of a biofumigant crop is a rotary flail, followed by a disk harrow and a roller, all of which would be in the possession of most British potato growers. As discussed in the next section, results may be improved by creating a better surface seal using a powered roller. Such equipment is not usually owned by potato growers but by specialist contractors who carry out soil fumigation.

### 6.2.2 Soil sealing methods to improve the efficacy of biofumigation

In order to maximise soil concentrations of isothiocyanate during biofumigation, it is recommended that the brassica crop is thoroughly pulverised, rapidly incorporated and irrigated, facilitating glucosinolate hydrolysis and reducing volatile losses (Gimsing & Kirkegaard, 2006). A glasshouse trial with brassica green manures showed that PCN are controlled more effectively when the soil is covered with polyethylene. Polyethylene sheeting, which costs around £500 ha<sup>-1</sup>, is not economically viable in broad-acre farming and, as discussed above, polyethylene would have only slowed the rate of loss of isothiocyanates, not prevented it completely. With pesticides producing methyl isothiocyanate, such as Dazomet, a tractor pulling a powered roller, spinning at a faster rate than the tyres of the vehicle, provides a sufficiently good surface seal to reduce volatile losses and increase concentration-time products in the upper soil layers (Leistra & Smelt, 1974; Smelt *et al.*, 1974). As discussed in Chapter 3, it remains to be determined to what extent the increased kill seen in covered soil was due to trapping of volatile isothiocyanates or the generation and trapping of additional volatile toxins under anaerobic conditions. Further research to answer this question would help to determine whether efforts to improve biofumigation should be directed at increasing the concentrations of isothiocyanate or other brassica-derived compounds.

### 6.2.3 Biofumigation in integrated pest management

Any combination of *G. pallida* control measures must reduce the population density by 50 times between successive potato crops in order to negate multiplication of the nematode (Haydock *et al.*, 2006). Currently, this is achieved with a combination of crop rotation and granular nematicides. Until recently, the fumigant 1,3-dichloropropene (1,3-D) was used to reduce the population density of particularly heavy infestations. 1,3-D and the granular nematicide aldicarb have now been withdrawn from use within the European Union and the future availability of the remaining granular nematicides is uncertain (see section 1.3.4). *G. rostochiensis* would be relatively easy to control without nematicides; fully resistant cultivars, such as Maris Piper, could provide complete control. *G. pallida* is an increasing and much more difficult problem to control due to a lack of resistant cultivars (Trudgill *et al.*, 2003). Alternative methods for controlling *G. pallida*, such as biofumigation, are currently required to fill the deficit caused by the withdrawal of 1,3-D and would be required to provide even greater levels of control in the absence of granular nematicides. Increasing rotation lengths would control the pest but would also reduce farm profitability (Jatoo *et al.*, 2008).



The effects of different levels of *G. pallida* mortality imposed by biofumigation on sustainable rotation lengths were explored with a simple model of the exponential decline of the nematode in the absence of a host (Figure 6.1, Table 6.1). A sustainable rotation length is defined as the length of rotation at which the population density does not increase between successive crops and remains below the damage threshold. Two scenarios were considered. Scenario A represents the present situation with granular nematicides available while scenario B represents a situation in which granular nematicides are unavailable. The rate of natural decline is a key parameter in determining sustainable rotation lengths. Rates typically vary between 15 to 25 %  $y^{-1}$  between different populations of *G. pallida* (Whitehead, 1995). Thus, under each scenario, two different populations were considered, population 1 with a decline rate of 15 %  $y^{-1}$  and population 2 with a decline rate of 25 %  $y^{-1}$ . Granular nematicides were assumed to reduce the proportion of hatched juveniles that successfully invade host roots by up to 90 %, according to Philips & Trudgill (1998).

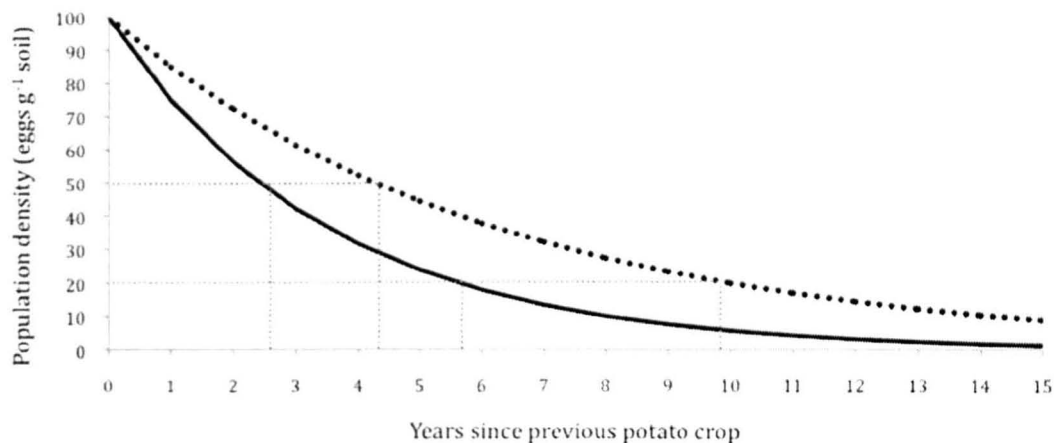
With a granular nematicide available, biofumigation would be required to cause a mortality rate of 50 % in order to reduce rotation length to an economically desirable four to six years (Table 6.1). The results of the present study suggest that this could be achieved with biofumigation (see section 6.1.1) although field validation is required. Without granular nematicides, other strategies, including biofumigation, would be required to cause 90 to 95 % mortality in order to maintain rotation lengths below eight or nine years (Table 6.1). This level of mortality is unlikely to be achieved using biofumigation alone. A second biofumigant crop within the same rotation would not reduce total *G. pallida* population density by the same proportion as the first crop because it could only feasibly treat a layer 200 – 300 mm deep and approximately 15 % of the PCN population generally resides below this depth. In addition, enhanced biodegradation of methyl isothiocyanate has been observed (Warton *et al.*, 2003) and the efficacy of biofumigation may decrease if it is used frequently (Matthiessen & Kirkegaard, 2006). Biofumigation could be combined with other control measures, such as partially resistant cultivars and trap cropping. The latter would affect PCN in lower soil layers, due to the mobility of hatching factors (Ryan & Devine, 2005). Halford *et al.* (1999) found that a potato trap crop provided the greatest rate of *G. pallida* population reduction and produced the greatest tuber yield when planted in early August and grown for six weeks. A biofumigant crop sown in the spring and grown for three to four months would produce very substantial biomass and would be incorporated at an ideal soil temperature. The combined effects of a trap crop and a brassica green manure with a high glucosinolate yield may be great enough to justify forfeiting a cereal cash crop if

granular nematicides were unavailable. Partially resistant cultivars provide a good complement to measures such as biofumigation or trap cropping, which reduce the initial population density before a potato crop ( $P_i$ ) (Trudgill *et al.*, 2003). This is because measures that reduce  $P_i$  reduce damage to the crop but only have a small impact on the population density after the crop ( $P_f$ ) due to the density dependence of multiplication (Trudgill *et al.*, 2003). Partially resistant cultivars reduce multiplication and hence  $P_f$ .

#### 6.2.4 Additional effects of cover crops

Besides disease suppression and reduced pesticide use, green manures have other benefits, including sequestration of nutrients (Eriksen & Thorup-Kristensen, 2002; Kristensen & Thorup-Kristensen, 2004; Dean & Weil, 2009; Ryans & Rosenfeld, 2010), improvement of soil structure (Chan & Heenan, 1996; McGuire, 2004) and reduction of erosion (Gies, 2004; McGuire, 2004). They also have the potential to cause problems. Besides the costs of growing and incorporating the crop, green manure crops can act as hosts to pests and diseases (Lu *et al.*, 2010) and they have the potential to become weeds themselves (Rayns & Rosenfeld, 2010).

Winter cover crops can dramatically reduce leaching of nitrate, which is important to prevent eutrophication of aquatic ecosystems. Ryans & Rosenfeld (2010) report a 95 % reduction of nitrate leaching by a *Secale cereale* (rye) cover crop. Early establishment of the root system before heavy winter rains was found to be crucial (Ryans & Rosenfeld, 2010). Brassicas such as *Raphanus sativus* (forage radish) have fast-growing roots that can reach depths of over 2 m (Kristensen & Thorup-Kristensen, 2004) and *R. sativus* and *B. napus* (rape) are at least as effective as rye at reducing nitrate leaching (Kristensen & Thorup-Kristensen, 2004; Dean & Weil, 2009). Sequestered nitrogen is stored in crop biomass and released when the crop is incorporated as green manure. Based on petiole nutrient sampling, a potato crop grown after a mustard green manure, required only 180 kg N ha<sup>-1</sup>, compared with the usual 280 kg N ha<sup>-1</sup> (McGuire, 2004; Hartz *et al.*, 2005).



**Figure 6.1:** Natural decline of *Globodera pallida* populations in the absence of a host crop modelled as  $P_y = P_0(1-h^y)$ , where  $P_y$  is the population density at year  $y$ ,  $P_0$  is the population density at year zero,  $h$  is the annual rate of natural decline and  $y$  is the number of years since year zero. Two hypothetical populations are shown, one with an annual decline rate of 15 % (dotted line) and one with an annual decline rate of 25 % (solid line). The number of years required to cause 50 % and 80 % reductions in population density are indicated.

**Table 6.1:** Predicted effects of different levels of mortality imposed by biofumigation on sustainable rotation lengths for control of two hypothetical *Globodera pallida* populations with annual decline rates of 15 %  $y^{-1}$  (population 1) or 25 %  $y^{-1}$  (population 2) under two different scenarios. Scenario A represents a situation in which granular nematicides reduce the proportion of hatched juveniles that successfully invade host roots by 90 %. Scenario B represents a situation in which granular nematicides are unavailable. Predictions are based on the model of population decline shown in Figure 6.1.

Mortality imposed by biofumigation (%)	Sustainable rotation length (y)			
	Scenario A		Scenario B	
	Population 1	Population 2	Population 1	Population 2
0	10	6	>20	14
50	6	4	20	12
60	5	3	19	11
70	2	1	17	10
80	2	1	15	9
90	1	1	11	6
95	1	1	6	4

Agronomic advice from the USDA (1998) suggests that 50 % of the nitrogen incorporated in a green manure crop will become available for the following crop, which is especially important for organic farmers. Other nutrients are also scavenged by cover crops and brassicas are especially effective at preventing leaching of sulphate and making it available to subsequent crops (Eriksen & Thorup-Kristensen, 2002).

Addition of organic matter to soil results in increased microbial activity (Ferris *et al.*, 1996). Soil microorganisms produce organic compounds that bind particles to produce aggregates, increasing soil stability, porosity, aeration and permeability (Hendrix *et al.*, 1990). Use of brassica winter cover crops in the USA and Australia has reduced erosion and increased the water permeability of the soil (Gies, 2004; McGuire, 2004; Chan & Heenan, 1996). McGuire (2004) found that water infiltration rates in a wheat/mustard-potato rotation were higher than in a sugar beet-corn-wheat-potato rotation and attributed at least part of the benefit to the incorporation of the mustard as a green manure. Improved water infiltration reduced run-off and associated problems, including erosion and disease problems due to pooling of water in hollows (McGuire, 2004).

Several studies have reported the agronomic practices carried out in cultivation of brassicas for biofumigation purposes without mentioning any need for protection from pests or diseases (Blok *et al.*, 2000; Wiggins & Kinkel, 2005; Larkin & Griffin, 2007; Snapp *et al.*, 2007; Friberg *et al.*, 2009). Lazzeri *et al.* (2003) specifically mention that pest and weed treatments were unnecessary during cultivation of *B. juncea* and *E. sativa*. Nevertheless, brassicas are susceptible to various fungal disease, including blackleg, caused by *Leptosphaeria maculans* (West *et al.*, 2001), wilt diseases caused by *Fusarium oxysporum* f. sp. *conglutinans* and *F. oxysporum raphani* (Garibaldi *et al.*, 2006) and to clubroot caused by *Plasmodiophora brassicae* (Johnston, 1968). The cultivars *B. juncea* ISCI99 and *E. sativa* cv. Nemat, which were effective at controlling *G. pallida* in the present study, are both susceptible to *F. oxysporum* f. sp. *conglutinans* and the latter is also susceptible to *F. oxysporum raphani* (Lu *et al.*, 2010). Growth and incorporation of susceptible plants in infected soil resulted in increases in soil inoculum density of these pathogens whilst growth and incorporation of resistant cultivars did not (Lu *et al.*, 2010). Naseri *et al.* (2008) found that the frequency of recovery of *L. maculans* from buried stubble of *B. rapa* decreased by 60 % over a 12 month period. Thus, if biofumigation is to be practiced in rotations involving brassicas, such as oilseed rape, the resistance status of the biofumigant and cash crops toward these fungal pathogens and the placement of the crops in the rotation must be considered.

### 6.3 Future directions

This work has demonstrated that biofumigation has the potential to cause a high degree of mortality to encysted eggs of PCN in soil and could therefore be a valuable tool in the integrated management of this important pest. However, as discussed in section 1.9.4.2, promising results in glasshouse experiments have not always lead to reliable and effective control under field conditions. Field trials with biofumigant crops are the logical next step in this work. The most promising plants from the present study are the *B. juncea* cultivars Nemfix, Fumus and ISCI99 and a *B. rapa* variety. The fodder radish *R. sativus* and the cress *N. officinale* also caused high levels of mortality to PCN and possess other qualities that may be of use. *R. sativus* has a trap-cropping effect on the beet-cyst nematode *Heterodera schachtii* (Schlathoelter, 2004). *N. officinale* has a low, trailing growth habit, very different to the other biofumigant crops studied, and could perform well in mixtures with other brassicas. It would be interesting to investigate the performance of mixtures due to the potential for synergy in terms of biomass production and phytochemical activity. Agronomic experiments are also required to determine the effects of biofumigation in different soils, to optimise the cultivation and incorporation of the crop and to optimise the position in the rotation, with particular attention to possible allelopathic effects or multiplication of pathogens of brassicas. The RNA-based method of quantifying viable eggs developed as part of the current work will be a useful tool in these studies.

The present study has shown that mortality of PCN eggs in soil treated with brassica green manures correlated with the content of isothiocyanate-yielding glucosinolates in those tissues. There was also evidence that other compounds unrelated to glucosinolates were involved in suppression in covered soil, possibly due to the generation of anaerobic conditions. Brassicas are known to produce volatile nematicidal sulphur compounds unrelated to glucosinolates but the role of these compounds in biofumigation has been studied very little compared with the glucosinolate catabolites (see section 1.9.4.3). Wang *et al.* (2009) recently demonstrated the involvement of methyl sulphide and dimethyl disulphide in suppression of the fungi *V. dahliae* and *F. oxysporum* and the nematode *T. semipenetrans* during biofumigation. Further glasshouse and field experiments should investigate the relationship between mortality of PCN and the concentrations of each of the known volatile nematicidal compounds produced by brassicas.

Plant breeders have been successful in producing Brassica cultivars yielding very high levels of glucosinolate. For instance, *B. juncea* cv. ISCI99 bred at the Italian Research

Centre for Industrial Crops (ISCI) contains over 2 % glucosinolate by mass and produces substantial biomass. Other crops that were almost as effective as *B. juncea* cv. ISCI99, such as the wild *N. officinale* accession or *R. sativus* contain much lower concentrations of glucosinolate and may be improved through breeding. In addition to conventional breeding, genetic engineering opens up some exciting possibilities for engineering glucosinolate biosynthesis, even producing novel compounds not found in nature. The first step in biosynthesis of glucosinolates, the oxidation of an amino acid to form an aldoxime, catalysed by a cytochrome P450 79 (Grub & Abel, 2006), is in common with the first step in biosynthesis of the cyanogenic compound dhurrin from sorghum (Kristensen *et al.*, 2005). However, dhurrin is synthesised from tyrosine, while there are no glucosinolates with tyrosine as the parent amino acid. By inserting sorghum *CYP79A1*, which catalyses conversion of tyrosine to p-hydroxyphenylacetaldoxime, into *Arabidopsis*, Kristensen *et al.* (2005) were able to generate the novel, tyrosine-derived p-hydroxybenzyl glucosinolate since the post-aldoxime enzymes have low specificity for the R-group and are evidently able to use tyrosine derivatives as substrates. Furthermore, this appeared to have very little effect on the transcriptome and metabolome besides the targeted effect (Kristensen *et al.*, 2005).

This initial step in glucosinolate biosynthesis, the oxidation of an amino acid, appears to be the rate-limiting step (Kristensen *et al.*, 2005) and therefore increased expression of the appropriate *CYP79* should result in increased levels of glucosinolates from that particular parent amino acid. Refinements to expression of downstream enzymes could then be used to alter the fate of the aldoxime substrate to generate different R-group modifications. Total glucosinolate production is the product of biomass and glucosinolate concentration so breeding biofumigant crops for high biomass will also be important.

## 6.4 Concluding remarks

Biofumigation has the potential to provide substantial levels of control of PCN in potato cultivation in the UK. Matthiessen & Kirkegaard (2006) have warned against overstating the potential of biofumigation based upon laboratory and glasshouse trials but the results presented here indicate that further study of biofumigation to control PCN in field trials is certainly warranted. If biofumigation can cause 50 % reductions in PCN population density in the field as predicted based upon currently available evidence then it would provide a useful addition to the repertoire of measures available for the sustainable management of PCN.

The practice of green manuring has probably been utilised by farmers for millennia. Unbeknownst to its practitioners, incorporation of green manures in some situations would have inadvertently reduced populations of plant parasitic nematodes and pathogens. With the advent of chemical fertilisers, green manuring fell into decline in the industrialised world and since then chemical pesticides have been developed and adopted on a large scale. Now, as the environmental and human health effects of some of these compounds are becoming better understood, demand for chemical free agriculture is growing and green manuring has seen a resurgence in the organic sector. The mainstream paradigm has also shifted away from heavy reliance on chemicals to integrated pest management, which aims to use appropriate quantities of synthetic chemicals. Research into the use of green manures has shown that they are not only useful as fertility-building crops but can also have pest suppressive effects. The application of modern analytical chemistry enables the exact compounds responsible for toxicity of plant materials to be identified and greater understanding of soil ecology is helping to elucidate the biological mechanisms of pathogen suppression by green manures. They are by no means a panacea but have the potential to play a significant part in sustainable agricultural production in the future.

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