Management of the stem and bulb nematode (*Ditylenchus* spp.) in winter beans (*Vicia faba* L) using biofumigant Brassica spp. and other allelopathic cover crops

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Declaration

The work presented in this thesis is an original compilation of the author and is in line with the registered title of the research programme. All the relevant sources of information referred to in this thesis are citied within the text and the sources appropriately referenced. None of the findings reported herein have been previously presented for award of a degree or other qualification in another institution.

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Abstract

Stem nematodes, *Ditylenchus gigas* and *D. dipsaci*, are major pests in field bean (*Vicia faba* L.) production in the UK. Currently, there are no recommended management measures for their control in *V. faba* production. A potential sustainable solution that has been used successfully for other plant parasitic nematodes is biofumigation using brassica cover crops. The active ingredient in biofumigant brassicas are isothiocyanates (ITC), which are produced from glucosinolates after disruption of brassica tissues. ITC have an affinity for proteins and can therefore cause mass disruption of the functional systems within target pests including nematodes.

It is believed that concentrations of 50 µg/ml ITC are achievable in biofumigated soils. *In vitro* studies with allyl ITC identified that 50-100 µg/ml of 2-phenethyl ITC and benzyl ITC caused up to 100% mortality against the two stem nematodes after 24 h exposure whilst sulforaphane had no effect. Experiments to understand the effect of sub-lethal ITC concentrations were developed by exposing stem nematodes to doses of 3-100 µg/ml. In a 23% pluronic gel, stem nematodes were examined for their ability to move to the roots of their host, *V. faba*. The ITC affected the stem nematodes form by stimulating them to coil while limiting their foraging activities at sub-lethal (12.5-25 µg/ml) concentrations. At 25 µg/ml, 2-phenethyl ITC and benzyl ITC reduced migration towards the host by over 90% while sulforaphane had no effect on the stem nematodes' ability to find their host even at 100 µg/ml.

Brassica species were investigated under controlled conditions to determine whether they are non-hosts or hosts of stem nematodes. Three separate experiments were conducted for five brassica plants. The results showed that all brassica plants tested were poor hosts for *D. gigas* while certain brassica plant such as *Sinapis alba* and *Eruca sativa* can support the reproduction of *D. dipsaci*.

Brassica plants that are associated with nematotoxic isothiocyanates were investigated for their suppressive effect in two field experiments. Brassica-legume mixtures and brassica grown alone were considered in field experiment 1, while field experiment 2 investigated the effect of three brassica species. The biomass of these brassicas was low in field experiment 1 because of a delayed sowing date and absence of N and S fertilizers although brassica plants grown in a mixture with legumes produced significantly greater biomass than brassicas sown alone. Field experiment 2 showed that brassica plants such as *Brassica juncea*, *B. carinata* and *Sinapis* alba could suppress *Ditylenchus gigas* compared to a fallow control.

In conclusion, this study has demonstrated the potential of using brassica cover crops against *Ditylenchus* spp. in *V. faba* production.

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Finally, I thank God for seeing me through this PhD.

Common Abbreviations

AFLP	Amplified fragment length polymorphism
bp	Base pair
CTAB	Cetyl trimethylammonium bromide
FE	Field Experiment
gDNA	Genomic DNA
GSL	Glucosinolate
ITC	Isothiocyanates
ITS-rDNA	Internal transcribed spacer-Ribosomal DNA
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
N	Nitrogen
NTC	No template control
PCR-ITS-RLFP	Polymerase Chain Reaction- Internal transcribed spacer- Restriction Fragment Length Polymorphism
qPCR	Quantitative polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
UK	United Kingdom

Research Output

Oral presentations

a) The use of biofumigation for managing *Ditylenchus* spp. in *V. faba.* Grower's meeting, January 2020 at Harper Adams University, UK
b) Management of *Ditylenchus* spp. in field bean production using Brassicaceae plants. PCGIN stakeholder meeting, November 2020 held online
c) Isothiocyanates associated with Brassicas impedes the survival and foraging activities of *Ditylenchus* spp. Biofumigation 7 Symposium. March 2021 held online
d) The application of a biofumigation system to manage stem nematodes, *Ditylenchus dipsaci* and *Ditylenchus gigas*, of field beans *Vicia faba*. European Society of Nematology PhD and Post-doc conference, May 2021 held online

Poster Presentations

a) The susceptibility of the stem nematode *Ditylenchus gigas* to isothiocyanates associated with Brassicas. European Society of Nematologist, Conference. September 2018 at Ghent University, Belgium
b) Sub-lethal concentrations of isothiocyanates associated with brassica species impedes the host finding activity of the stem nematodes *Ditylenchus gigas* and *D. dipsaci*. Advances in Nematology Conference, December 2020 held online

Press release

a) "The use of biofumigant cover crops to manage stem and bulb nematodes in field beans (page14)" PGRO Summer Pulse (June 2021)
b) "How a new test helps bean growers tackle stem nematode" Farmers Weekly (December 2020)
c) "Ground breaking research on nematodes" The Vegetable Farmer (June 2020)

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1.1 Field bean, *Vicia faba* L.

1.1.1 Origin and distribution to the UK

Field bean, *Vicia faba* is one of the earliest domesticated legumes in the world. Its origin, however, is ambiguous and has been debated for a long time. Several authors relied on species with rudimentary features or archaeological remains to suggest its possible origin. Ladizinsky (1975) proposed an origin in Afghanistan relying on the distribution of a population (*paucijuga*) that bears a set of primitive characteristics. This suggestion, however, was disputed by Cubero (1984) because a different population present in the Mediterranean countries shared similar primitive features to *paucijuga* (Cubero, 1984). Therefore, the author concludes that a true centre of origin should not only have a primitive cultivar but ancestral archaeological remains to support it.
Archaeological remains of field bean from the Neolithic period (dated 6250 BC) were exhumed from a field in Jericho (Hopf, 1983) and recently a wild progenitor was recovered from Mount Carmel (Caracuta *et al.*, 2016). This finding may have established the origin of field beans as Near East Asia (Ladizinsky, 1975).
Field bean seed and its cultivation were dispersed to other world regions through different routes. From the Near East, where its farming began, the crop spread to the UK and Europe through Anatolia (Turkey). Field bean archaeological remains, dating back to the Iron Age, were recovered in Glastonbury Somerset, UK (Smartt, 1990). Its cultivation has increased and was established in the UK before 1000 BC (Link *et al*., 2009).

1.1.2 Field bean morphology and its varieties

Field bean is a leafy annual plant, having robust and erect stems that grow up to 2 m in height. Tillers originate from the base of the stem. It has compound leaves with ovate leaflets. The majority of the flowers produced are allogamous. Their pods, indehiscent, can reach 20 cm in length, while seeds per pod vary from two to eight (Bond *et al.*, 1985; Duc, Gérard *et al.*, 2015)
Seed characteristics such as size and shape form the basis for intraspecific classification. There are four populations *major, equine*, *minor* and *paucijuga* (Cubero, 1973). The large-seeded types, *Vicia faba major*, or broad bean has a 1000-seed weight of ≥ 1 kg; while small-seeded types, *Vicia faba minor*, *found* in Ethiopia, has a 1000-seed weight ≤ 500 g and the medium seeded types, *Vicia faba equine*, are commonly grown in Egypt (Duc, G., 1997). This legume belongs to the family Fabaceae, subfamily Faboideae, and

tribe Fabeae. Genetically, it is diploid (2n = 12 chromosomes) with a chromosome size of 13,000 Mbp (Duc, 1997; Kwon *et al.*, 2010).

Because field beans have adapted to different growing seasons they are classified as Mediterranean, winter, and spring varieties. In the UK, two crop types are grown: spring and winter. Winter varieties have some advantages over the spring beans and hence are commonly grown. They can produce 47% more yield over spring beans with a lower seed rate, have efficient use of available soil moisture and can tolerate drought (Link *et al.*, 2010). In addition, winter varieties can quickly recover from any form of mechanical injury (Herzog and Geisler, 1991; Ghaouti, 2007). Winter varieties are autumn sown and have a long growing period while spring varieties are spring sown. Hence, field beans are exposed to pest and pathogens in all seasons (Stoddard *et al.*, 2006).

1.1.3 Global production of field beans

Field beans are predominantly grown for their seed with China being the leading producer globally, contributing almost half of worldwide production. Figure 1.1 illustrates that the UK has the highest global production in yield (kg/ha). It exports up to 60% of its produce to Egypt, North Africa and Middle East (Metayer, 2004; Duc *et al.*, 2010).

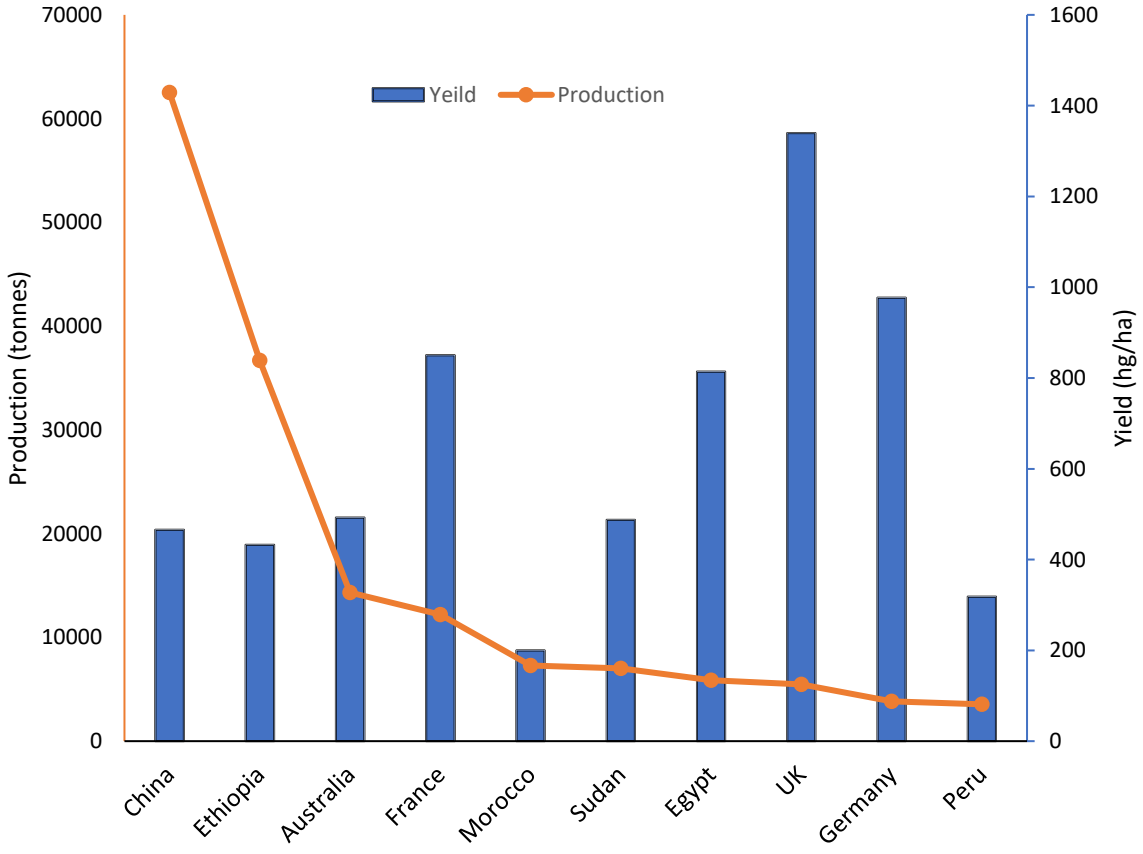

Figure 1.1. The production (tonnes) and yield (hg/ha) of *Vicia faba* (field beans) in ten of the highest producing countries

Field bean seed is processed and used in several ways, with beans either boiled, eaten fresh or canned. The bean is a staple food in the Middle East, countries in the Mediterranean region, China and Ethiopia. In North Africa and West Asia, it is well valued as the 'poor man's meat' (Van de Wouw *et al.*, 2001). Field beans also have a strong significance in Serbia, where special meals made from field beans are served during Christmas meals as a substitute to meat, eggs or dairy, which are restricted by the Christian Orthodox Church (Mikić *et al.*, 2011). The seed is also included in the diet of poultry. The seed contains 30% protein and rich in lysine and low in methionine, cysteine and tryptophan (Duc *et al*., 1999).
Immature pods and plant tops are used as vegetables while the whole bean plant is used before maturity as either livestock feed or green manure. The crop residues can be used as fodder or ploughed into the soil (Van de Wouw *et al*., 2001).

As a leguminous crop, beans contribute nitrogen to the rhizosphere and thereby play a key role in cropping systems in a symbiotic relationship with the soil bacterium *Rhizobium leguminosarum* (Duc *et al*., 2010; Jensen *et al*., 2010; Kharrat and Ouchari, 2011). This greatly reduces the dependence of growers on extensive use of synthetic fertilizers thereby protecting soil and water quality. Field beans were found to contribute over 13-fold more Nitrogen (N)/ha to a field than adzuki bean (Rochester *et al.*, 1998). Intercropping nitrogen fixing species such as field beans in with cereals lessen the reliance on fossil energy.

1.2 Field beans production constraints

In most field bean growing countries, the interest of farmers has moved from legumes to cereals because of better economic profits. This factor has largely contributed to the decline in areas under field bean cultivation. The farmers still engaged in this crop production struggle with variable yield due to biotic and abiotic factors (Preissel *et al.*, 2015).

1.2.1 Abiotic Stress

Spring and autumn sown field beans are susceptible to prevailing weather conditions associated with each growing season. Frost, for instance, is a major abiotic stress affecting field beans in winter. It induces loss of leaf turgidity and chlorophyll content leading to a reduction in survival, yield production and seed quality (Rowland, 1978; Arbaoui *et al*., 2008; Sallam and Martsch, 2015). Winter varieties are particularly affected, as their reproductive and vegetative stages may be exposed to such harsh conditions

(Ghaouti, 2007; Maqbool *et al.*, 2010), typically not a concern in the UK. A few genotypes, however, can withstand freezing temperatures -9.6ºC (Inci and Toker, 2011) and -22ºC if previously hardened (Duc, 1997).

Field beans are very sensitive to soil moisture stress. It requires more water than other crops. It performs poorly compared to peas and chickpeas under soil moisture stress (Loss and Siddique, 1997; McDonald and Paulsen, 1997; Loss *et al*., 1997; Amede *et al*., 2003). Drought conditions induce changes in the leaf stomatal conductance (Alghamdi *et al.*, 2015) with leaf wilting seen after three days of drought (McDonald and Paulsen, 1997). Drought conditions occurring between the first pod appearance and full pod set can result to 45% yield loss (Xia, 1994).

1.2.2 Biotic Stress

Field beans are host to a wide variety of insect and nematode pests, fungal and viral pathogens as well as parasitic weeds. These contribute to the suppression of yield and quality of field beans.

Broomrapes (*Orobanche crenata* and *O. foetida*) have an association with field beans called holoparasitism (Pérez-de-Luque *et al.*, 2010; Stoddard *et al.*, 2010) and are estimated to cause losses ranging from 50% to 80% in fields with medium and high levels of infestations, respectively (Gressel *et al.*, 2004). This parasite is important in field beans production in the Mediterranean basin, Northern Africa and Sub-Saharan African countries (Maalouf *et al.*, 2011).

Fungal diseases such as chocolate spot (*Botrytis fabae* and *B. cinerea*), rust (*Uromyces viciae-fabae*) and ascochyta blight (*Asochyta fabae*) are damaging to field beans. Other fungal diseases including downy mildew (*Peronospora visicae*) (oomycete) and foot rot (*Fusarium* spp). There are specific environmental conditions required for each disease. Chocolate spot predominantly caused by *B. fabae* infection is severe in warm (15-25ºC), wet and humid conditions (Villegas-Fernández *et al.*, 2009; Stoddard *et al.*, 2010), while cool-moist (5-15ºC) conditions encourage ascochyta blight (*A. fabae*) (Hanounik, S., 1980). Chocolate spot can reduce field bean yield by 67-100% (Bouhassan *et al.*, 2004; Sahile *et al.*, 2010; Teshome and Tagegn, 2013), while yield loss associated with ascochyta can be 30-90% (Ondrej and Hunady, 2007).

Aphids (*Aphis fabae* and *A. craccivora*) are also important in field beans production. They attack young leaves and stem tissue, which results in stunted and deformed terminal growth. Yield reductions can be as high as 86%-100% (Way, 1967; Nuessly *et al.*, 2004;

Banks and Macaulay, 1967). Additionally, they transmit plant viruses (Heathcote and Gibbs, 1962) such as bean yellow mosaic virus (BYMV), bean leaf roll virus (BLRV), faba broad bean, mottle virus (BBMV) and broad bean stain virus (BBSV) which inflict yield loss of up to 80% (Makkouk *et al.*, 2002; Pande, 2009).

1.2.3 Nematode pests of field beans

Field beans suffer under plant nematode parasitism; different nematode species infect and cause damage to every plant part. Plant parasitic nematode of field beans include the stem and bulb nematode (*Ditylenchus* spp.), the pea cyst nematode (*Heterodera goettingiana*), root-knot nematode, *Meloidogyne* spp. (Sikora and Greco, 1990). Others include *Pratylenchus* spp., *Tylenchorhynchus dubius*, *T. latus, Radopholus similis, Hoplolaimus* spp*.,* and *Belonolaimus longicaudatus* (Riggs and Niblack, 1993; Hooper, 1983a).

Different nematode genera give rise to different symptoms on beans. The presence of brown lesions and galls on the roots of field beans, particularly on root bifurcations, indicate symptoms of *Meloidogyne* spp. The main species infecting beans include *M. artiellia, M. javanica* and *M. incognita* (Lombardo *et al.*, 2011). Generally, they are considered to be less important in field bean production (Sikora, R. A. and Greco, 1990). The root lesion nematodes (*Pratylenchus neglectus*, *P. pinguicaudatus* and *P. thornei*) cause damage to field beans. However, there are some resistance varieties available. For example, Di Vito *et al*. (2002) found three varieties of field bean to be resistant against *P. thornei* and two varieties against *P. neglectus* and against *P. pinguicaudatus*. In a different study, Ismail *et al*. (2013) screened twenty varieties and found ten with some form of resistance to *Pratylenchus* spp.

The roots of field bean are subject to infection by the pea cyst nematode, *Heterodera goettingiana* (Beane and Perry, 1984). This nematode is important in many temperate regions, especially during cool-growing seasons. Field beans can tolerate this nematode at density low as 0.8 eggs/g of soil. However, at 5, 15 and > 64 eggs/g of soil, yield can be reduced by 20, 50 and 100% respectively (Greco *et al*., 1991).

Further nematode pests of field beans include the stunt nematode, *Tylenchorhynchus latus* and the reniform nematode, *Rotylenchulus reniformis*. Tolerant field bean varieties have been reported (Ismail *et al.*, 2013).

1.3 Stem and bulb nematodes, *Ditylenchus* spp.

Stem and bulb nematodes *Ditylenchus* spp. are the most important nematode pest infecting field beans. These nematodes are obligate migratory endo-phytoparasites that rely on their host's parenchymatous tissues for food and reproduction (Duncan and Moens, 2013). This plant-parasitic nematode causes economic losses to many countries because it is polyphagous and parasitic on plants.

1.3.1 Global Occurrence

Stem and bulb nematodes are widely recognised as important pests for temperate agriculture. In 1857, Kühn initially described this genus infecting the heads of teasel (*Dipsacus fullonum*) in Germany. Since then, *Ditylenchus* spp. have been reported in other countries including the United Kingdom, France, Canada, and the United States of America (Goodey, 1951; Hajihassani *et al*., 2017; Chitambar, 2018). They have also been reported in the Mediterranean region in countries such as Algeria, Morocco, Portugal, Italy, and Turkey (Yavuzaslanoğlu *et al.*, 2015) and in some Asian countries such as Japan, China among others (Goodey, 1951; Chitambar, 2018; Ikuyo *et al*., 2018). The occurrence of the species *D. gigas* correlates with the areas where field beans are grown.

1.3.2 Life cycle and survival

Most literature describing the life cycle of *Ditylenchus* spp. (Figure 1.2) focusses on *Ditylenchus dipsaci* on onions. This nematode completes its life cycle within 19 to 23 days at 15˚C (Yuksel, 1960). Reproduction is by amphimixis with each male having the potential to fertilize more than one female (Sturhan, Dieter and Brzeski, 1991) . Females lay 200 to 500 eggs within host plant tissue. First moult occurs in the egg and thereafter the second stage juvenile's hatch. Three further moults occur in quick succession, with the juveniles developing into adults in four to five days. Adults live for 45 to 73 days. The nematode reproduces at a soil moisture of 61-94% field capacity and 90-100% relative humidity with a temperature below 20 ˚C (Griffin, 1987; Griffith, Gwyn S. *et al.*, 1999). Juveniles are characterised by a shorter body length, while the presence of vulva and spicule in adults separates female from male.

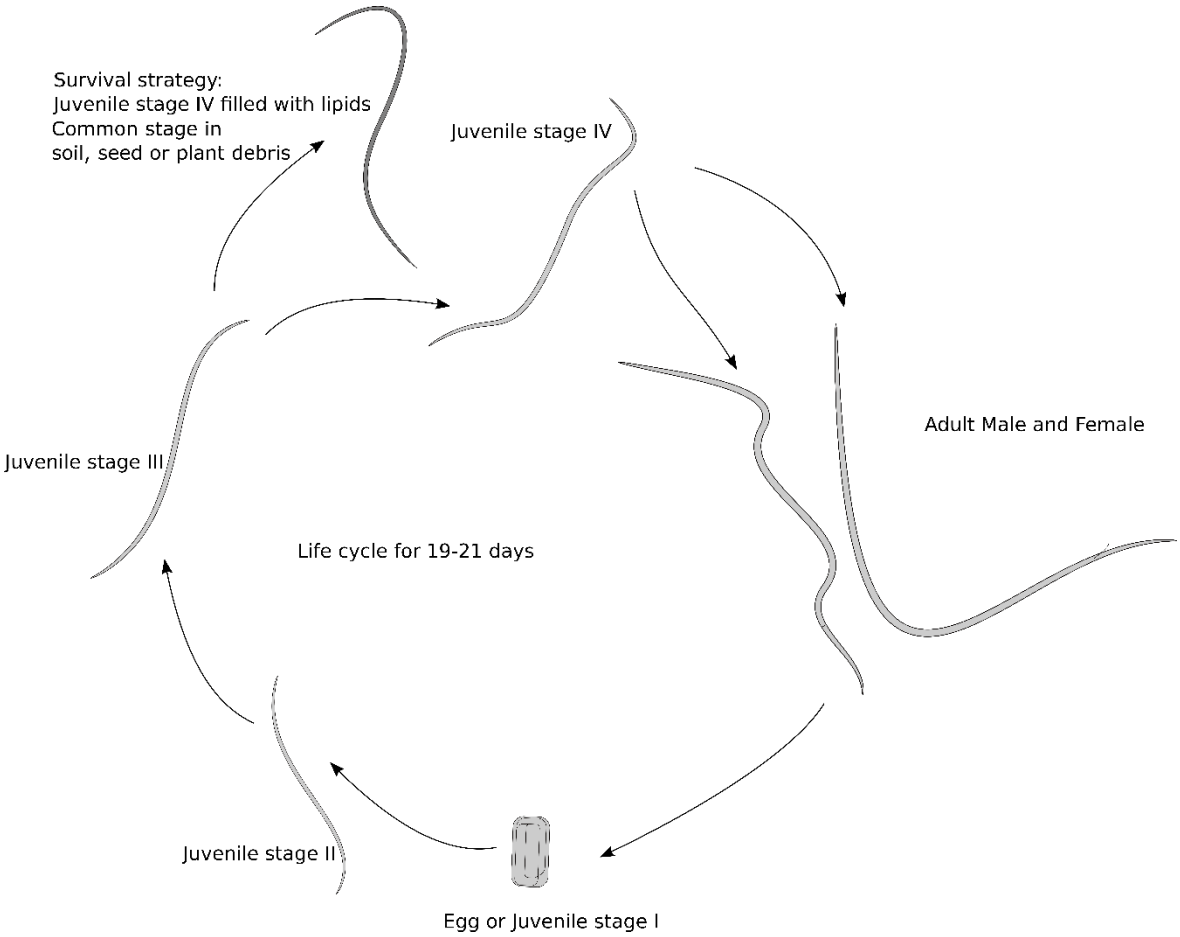

Figure 1.2. The generalised life cycle of *Ditylenchus* spp.. All stages including adult male, and female develop in their host plant while the Juvenile stage four is common in soils

Stem nematodes (mostly J4) aggregate and coil as plant senescence sets in or in adverse conditions (Figure 1.3). This strategy enables them to survive adverse conditions with no food supply. The fourth stage juvenile (J4) is both the infective and survival stage. They survive drought conditions for up to five years by entering an anhydrobiotic state (Perry, R., 1977). Wilson and French (1975) , found them persisting in clay soil after seven years, whilst Hooper (1983) recorded a survival period of ten years. In field beans, they survive underneath the seed coat, and recover to infect germinating seedling (Powel 1974).

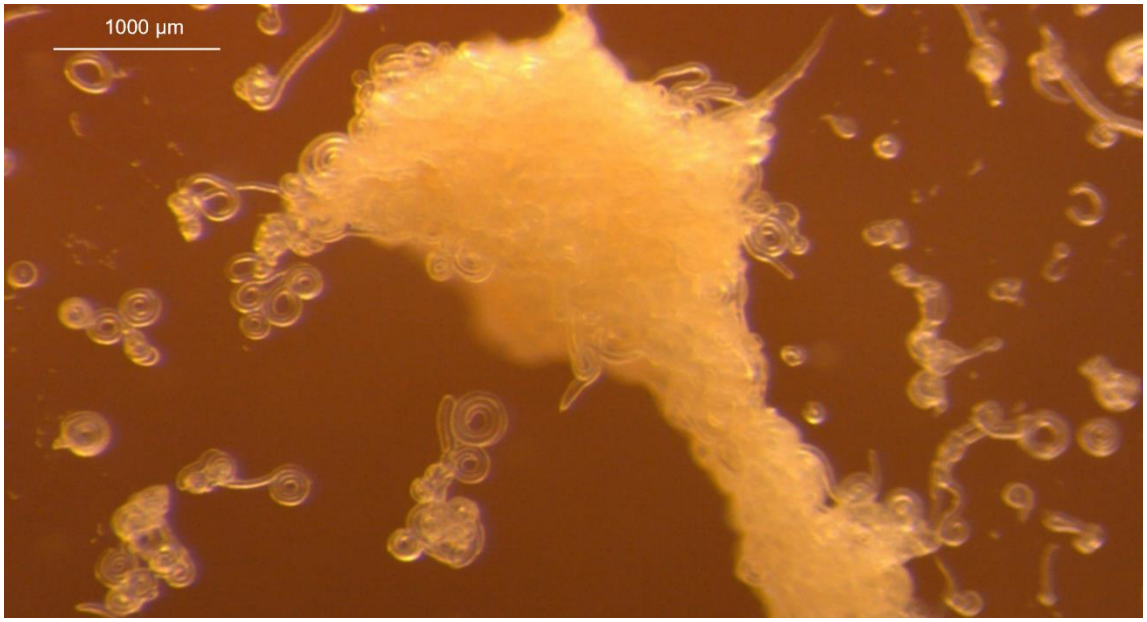

Figure 1.3. A photograph of stem nematodes *Ditylenchus dipsaci* coiling and aggregating to form a clump of 'eelworm wool'

1.3.3 Hosts of *Ditylenchus* spp.

Ditylenchus spp. affect a wide range of host plants, including cultivated plants and weeds that encompass the majority of plant families. *Ditylenchus dipsaci* is the main species causing economic damage to plants in the Poaceae and Fabaceae amongst others (Sikora *et al*., 2005; Sousa et al., 2003; Castillo *et al*., 2007; Tomasi, 2012; Goodey, 1951). *Ditylenchus gigas* attacks a smaller range of crop plants and wild plants/weeds (Vovlas *et al*., 2011; Augustin and Sikora, 1989). However, both species are important pests of *Vicia faba* (Table 1.1).

Table 1.1. List of host plants for *Ditylenchus gigas* and *D. dipsaci*

Ditylenchus spp.	Host
D. dipsaci	*Allium cepa* (Onion), *Allium sativum* (Garlic), *Anthriscus sylvestris* (Parsley), *Atriplex patula* (Patula), *Avena sativa* (Oat), *Beta vulgaris* (Beet), *Chenopodium foliosum* (Spinach Strawberry Sticks), *Cirsium acaule* (Thistle), *Cucumis melo* (Melon), *Dipsacus spp.*(Teasel), *Fragaria x ananassa* (Strawberry), *Galanthus nivalis* (Snowdrop), *Hyacinthus orientalis* (Hyacinth), *Lamium amplexicule* (Deadnettle), *Lolium perenne* (Ryegrass), *Medicago sativa* (Alfafa), *Narcissus* spp. (Daffodil), *Nicotiana tabacum* (Tobacco), *Pisum sativum* (Pea), *Polygonum persicaria* (Redshank), *Solanum tuberosum* (Potato), *Spinacia oleracea* (Spinach), *Stellaria media* (Chickweed), *Solanum lycopersicum* (Tomato), *Trifolium* spp. (Clover), *Tulipa* spp. (Tulip), *Vicia faba* (Field beans), *V. sativa* (Common vetch), *V. villosa* (Hairy vetch), *Veronica chamaedrys* (Speedwell)
D. gigas	*Avena sterilis* (Sterile oat), *Convolvulus arvensis* (Bindweed), *Lamium spp.* (Deadnettle), *Ranunculus arvensis* (Buttercup), *Vicia faba* (Field beans)

1.3.4 Epidemiology and symptoms on infested plants

Stem nematodes rely on water films to infest plant parts. They penetrate and feed (Figure 1.4) on plant buds, petioles, lenticels, or stomata and afterwards move intercellularly through the middle lamella of parenchyma causing stem swelling and deformation, leaf and petiole necrosis and the production of smaller, distorted seed (Sikora and Greco, 1990; Palomares-Rius *et al*., 2017).

On field beans in particular, the swellings, distortions on the stem turn from green, to red to and then black with age (Figure 1.5). Shortened internodes resulting in stunted plants (Figure 1.6). As the plant matures, the nematodes migrate to the seeds; infected seeds exhibit dark patches and with wool-like deposits present in heavily infestations, (Powell, 1974) . The seeds are shrivelled, shrunken, and show patches of discoloration on the seed coats (EPPO, 2008; Whitehead and Tite, 1987) see Figure 1.7. Such symptoms can be confused with some fungal diseases such as *Fusarium* basal foot rot (Greco, Nicola, 1993; Chitambar, 2018).

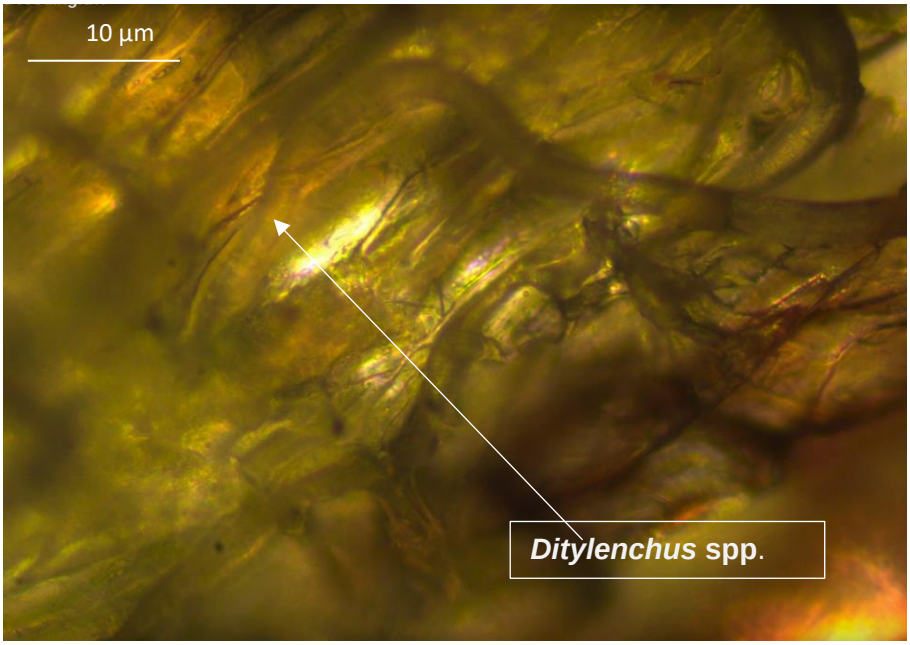

Figure 1.4. *Ditylenchus* spp. feeding on a *Vicia faba* cell

Figure 1.5. Early(left) and late (right) symptoms of *Ditylenchus* spp. infection on field beans

Figure 1.6. Stunted field beans plant with a swollen crown (yellow arrow) caused by *Ditylenchus* spp.

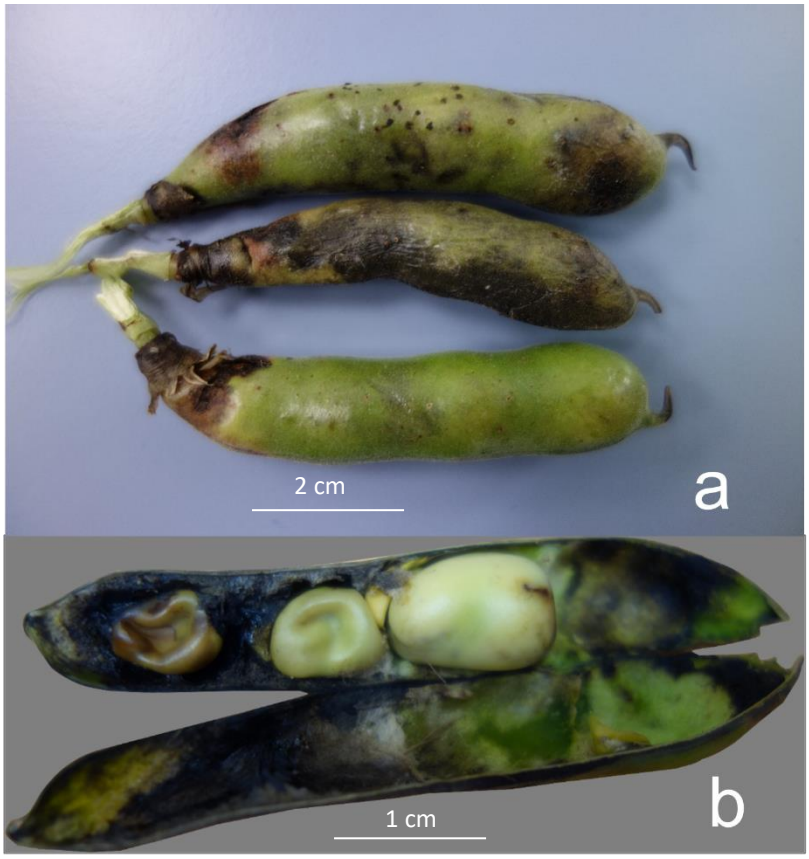

Figure 1.7. A damaged pod with brown patches (a) and shrunken seeds (b) caused by *Ditylenchus* spp.

1.3.5 Economic Damage

In general, crop yield loss correlates with the density of stem nematodes in a field and the type of crop grown. Initial population densities below two individuals per gram of soil, causes damage to onions (Seinhorst, 1955; Bridge and Starr, 2007). Moreover, an area of 120 cm^2 containing ten individuals of stem nematodes has been reported to seriously affect the performance of alfalfa plants (Palo, 1962). Stem nematode infestation can lead to a 37% yield reduction in oats (Whitehead *et al.*, 1983) and a 68% reduction in field bean yield in the UK (Hooper, 1983b ;Biddle and Cattlin, 2007). Not only is the performance of an infested plant reduced, stem nematodes also cause the death of bean seedlings particularly in high population densities under UK conditions (Hooper, 1983) resulting in patches (Figure 1.8). Most damage and yield loss associated with stem nematodes in field beans is attributed to *D. gigas* (Hooper, 1983; Sikora and Greco, 1990) due to its greater prevalence in the UK (Stawniak, 2011).

Feeding damage caused by stem nematodes favours secondary infections by other soil pathogens including fungi (*Fusarium* spp., *Verticillium* spp. and *Rhizoctonia* solani) and bacteria. These pathogens are opportunistic and utilise wound openings on the plant

created by *Ditylenchus* spp.. For example, disease complexes involving *Ditylenchus* spp.have been reported with *F. oxysporum* f. sp. *medicaginis* on alfalfa (Griffin, 1990) and *R. solani* on sugar beet (Hillnhütter *et al.*, 2011). In addition, an association between the pathogenic bacterium *Corynebacterium insidiosum* and *D. dipsaci* has been documented on alfalfa. The bacterium attaches itself to the nematode cuticle and infects plant tissue as the nematode invades (Hawn, 1963; Hawn, 1971).

Figure 1.8. A *Ditylenchus* spp. infested field showing patches of stunted growth in field beans. Source: Hutchison Crop Specialists

1.3.6 Distribution and dispersal

The stem nematodes are motile at most stages of their lifecycle, moving in a sinusoidal motion by contractions of their muscles. Their movements allow them to move through soil pores and swim along moisture films, even though they can only cover a few centimetres per year (Prot, 1980). These limited dispersal methods do not account for their widespread spread from an infected plant to different fields. Through passive means and the anhydrobiotic survival mechanisms of stem nematodes, a wider distribution can be facilitated. As a result of rain or irrigation, stem nematode-infested soils and plant debris are spread across the field (Cook and Yeates, 1993). Furthermore, infected soils and debris are transported by farm machinery such as tractors, harvesting equipment, and their wheels and tyres. Spread could also occur during seed storage from a few infected seeds to the entire stock.

1.3.7 Taxonomy and phylogeny of *Ditylenchus* spp.

The genus *Ditylenchus* is positioned within the family Anguinidae. Members of this family share common features such as a median bulb with or without valve, a post-uterine sac that is present or absent, mature females that are not swollen and a fungivores or plant parasitic feeding habit (Fortuner and Maggenti, 1987) . Members of the Anguinidae include *Anguina*, *Afrina*, *Subanguina*, *Nothotylenchus* and *Ditylenchus* among others. However, it is more difficult to distinguish between *Nothotylenchus* and *Ditylenchus*. A useful feature that aids in separating the two genera is the presence or absence of median bulb valves. Ditylenchus-like species with and without median bulb valves are classified as *Ditylenchus* and *Nothotylenchus* respectively. However, that feature is not solely reliable as other *Ditylenchus* spp. such as *D. myceliophagus* have weak valve too (Brzeski, 1981; Fortuner and Maggenti, 1987). Fortunately, *D. dipsaci* and *D. gigas* have pronounced median values, which assists with their identification. The scientific classification of *Ditylenchus* is given below:

Phylum Nematoda
Class Chromadorea
Subclass Chromadoria
Order Rhabditida
Suborder Tylenchina
InfraorderTylenchomorpha Vovlas
Superfamily Sphaerularioidea
Family Anguinidae
Subfamily Anguininae
Genus *Ditylenchus* Filipjev, 1936
(Manzanilla López and Marbán Mendoza, 2012)

1.3.8 Morphology and morphometrics of *Ditylenchus* spp.

The genus *Ditylenchus* has over 82 species identified and described. Of the 82, a few are parasitic on plants and cause serious economic damage to plants. Plant parasites include the stem and bulb nematodes (*Ditylenchus dipsaci*, *D. gigas and D. weischeri*), the potato rot nematode (*D. destructor*), the peanut pod nematodes (*D. africanus* and *D. angustus*) (Sturhan and Brzeski, 1991; Chizhov *et al.*, 2010; Vovlas *et al.*, 2011). The majority are mycophagous, for example the mushroom spawn nematode *D. myceliophagus* (Goodey, 1958) and *D. halictus* (Ye *et al.*, 2010) which is also entomophagous.
In a broad sense, the species within this genus can be distinguished from each other based on host preferences. This characteristic is not reliable for *D. dipsaci* or *D. destuctor*

that both infect potatoes. That said, their pathogenicity on potato differs; *D*. *dipsaci* attacks the stems, leaves and tubers while *D. destructor* attacks only stolon and tubers (Brodie *et al.*, 1993). The stem nematode *D. dipasci* also shares similar host (field beans) and symptoms with *D. gigas*. A number of morphological features are used to separate these species.

The genus *Ditylenchus* is characterised with low lip region with no striation, flattened and not offset. They also possess cylindrical isthmus and slightly swollen posterior bulb and few annulations on the body. The adult male and female possess characteristics that are used to distinguish between species. *Ditylenchus dipsaci* and *D. gigas* share similar features with their tail terminus slightly pointed and four lateral lines down the body length. These species are differentiated based on length; *D. gigas* is 1.5 -2 times longer than *D. dipsaci* (Figure 1.9). Although morphometrics and morphology allows species recognition (Table 1.2), the features used are variable, with only a few reliable for taxonomic purposes (Fortuner, 1982). Therefore, the use of molecular diagnostics can further confirm a species identity.

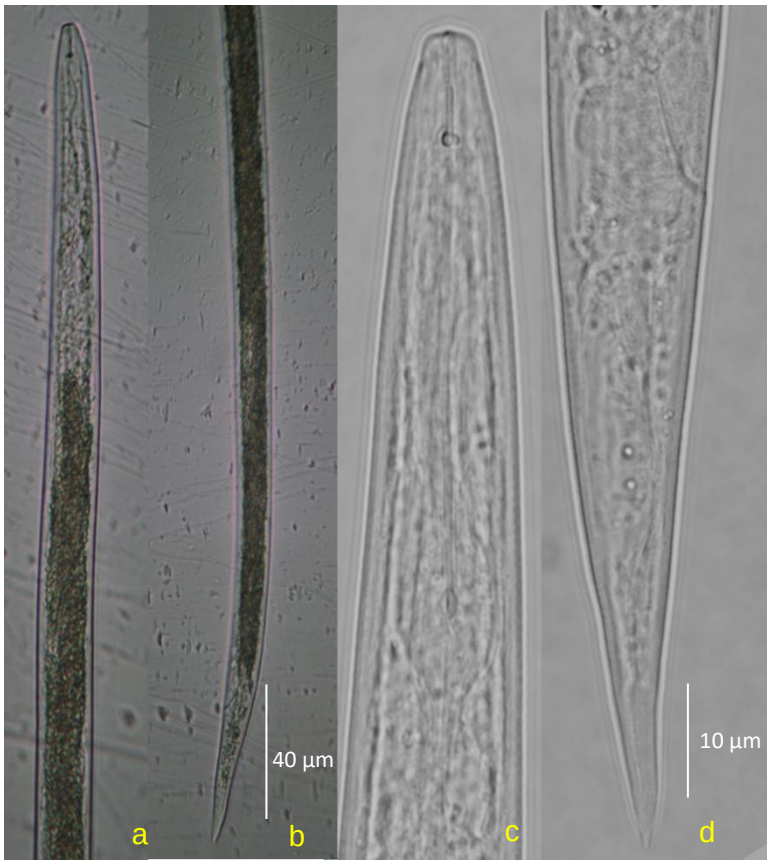

Figure 1.9. *Ditylenchus gigas* anterior (a) and posterior (b) at x100 magnification and *D. dipsaci* anterior (c) and posterior (d) at x400 magnification

Table 1.2. Main morphometric features used to distinguish females of *Ditylenchus gigas* and *D. dipsaci* (µm)

Character	*D. gigas*		*D. dipsaci*	
	Female	**Male**	**Female**	**Male**
Body length	1561-1932	1373-1716	1.2-1.4	1.0-1.3
a	43.0-56.4	34.3-63.0	31.3-45.5	39.7-53.5
b	7.3-9.3	6.7-10.7	6.4-8.2	6.3-7.8
Tail length	69-103	74-96	88-91	83-89
c	16.8-27.6	5.7–20.0	13.5-19.5	13.9-16.3
Stylet length (µm)	11.5-13.0	11.0–12.5	10-12	10-12
PUS	81-150		40-70	
V%	80–83		75-79	
Spicule length		23.5–28		22-28

a= overall body length/maximum body width; b= total body length/pharyngeal length; c= total body length/tail length, PUS= post uterine sac, V%= percentage distance of vulva from anterior

1.3.9 Molecular characterization of *Ditylenchus* spp.

Studies on the species in the genus *Ditylenchus* shows a paraphyletic relationship between them. Researchers have identified a *D. dipsaci* complex as *Ditylenchus dipsaci sensu lato* with several host races. Amongst them, the adult nematodes isolated from infested field beans were perceived larger than “normal race” and named “giant race” Debray and Maupas (1896) . Sturhan and Brzeski (1991) reported that the giant race did not cross with races from tobacco (*Nicotiana tabacum*), spinach (*Spinacia oleracea*) and sea plantain (*Plantago maritima*) and so suggested that it be recognised as a different species. Phylogenetic analysis and restriction enzyme-based profiling have also indicated the two races of importance in field bean production (Subbotin *et al*., 2005; Kerkoud *et al*., 2007). Furthermore, the chromosome number of the “normal race” and “giant race” were also diploid 2n=24 and tetraploid 2n=48-60 respectively (Sturhan, 1969; Sturhan, 1970; Vovlas *et al*., 2011). Further studies using PCR-ITS-RLFP, protein isoelectric focussing, RAPD, AFLP and ITS-rDNA gene sequence strengthen the claim that the giant race was a separate species (Wendt *et al*., 1993; Tenente and Evans, 1997; Esquibet *et al*., 2003; Marek *et al*., 2005). Finally, Volvas *et al,* (2011) analysed the molecular sequences of the ITS1-5.8S-ITS2, the D2-D3 region of the 28S gene, the small 18S subunit, the partial mitochondrial gene for cytochrome c oxidase I (mtCOI), and *hsp90* gene sequences and

supported the description of the new species as *Ditylenchus gigas.* Hence, the two species are namely *D. dipsaci sensu stricto* and *D. gigas* (Volvas *et al.,* 2011).
Across the globe, the different populations of a single species can differ genetically. For instance, the *D. gigas* populations from Poland differs to populations recovered from the countries surrounding the Mediterranean Sea. Therefore, finding conserved regions across their populations is essential. Several authors have used molecular diagnostics to distinguish *Ditylenchus gigas* from *D. dipsaci* (Table 1.3). Southern-blot technique was developed to specifically detect *D. dipsaci* using radioactively labelled DNA probes (Wendt *et al*., 1993). Similarly, using Sequence Characterized Amplified Region primers, it is possible to distinguish *D. gigas* from *D. dipsaci* (Esquibet *et al.*, 2003). Furthermore, PCR based methods have been developed for *D. dipsaci* (Madani *et al.*, 2015; Marek *et al.*, 2005; Subbotin *et al.*, 2005). Moreover, a qPCR and PCR assay for distinguishing the two species is available (Jeszke *et al.*, 2015).

Table 1.3. PCR and qPCR specie-specific primers used in molecular diagnostics of *Ditylenchus gigas* and *D. dipsaci*

Species	Names	Region	Sequence (5' to 3')	bp	Author
D. dipsaci	PF1	ITS	AAAGGCTCTGTTGGGTTCTAT	327	Marek *et al*.,2005
D. dipsaci	PR1		ATTTACGACCCTGAGCCAGAT		
D. dipsaci	PF2	ITS	TCG CGA GAATCA ATG AGT ACC	396	Marek *et al*.,2005
D. dipsaci	PR2		AAT AGC CAG TCG ATT CCG TCT		
Ditylenchus spp.	U831	hsp90	AAYAARACMAAGCCNTYTGGAC	182	Madani *et al*., 2015
D. dipsaci	Dip_hsp90 R		GWGTTAWATAACTTGGTC		
D. dipsaci	DitReal-timeR2		CAGAGTGAAATAGCCAGTCGATTC	263	Subbotin *et al*., 2005
D. dipsaci	DitNF1		RGCTTATGACAAATTCATGGCGG		
Ditylenchus spp.	DITuniF	ITS	CTGTAGGTGAACCTGC	148	Jeszke *et al*., 2015
D. gigas	DITgigR		GACCACCTGTCGATTC		
D. dipsaci	DITdipR		GACATCACCAGTGAGCATCG	270	

1.4 Stem nematode management

The challenges associated with managing *Ditylenchus* spp. and suppressing their damage include their short life cycle, rapid development, survival strategy (anhydrobiosis), and wide host range that allows them to maintain a large population density.

1.4.1 Preventive strategies

Stem nematodes survive on seed and plant debris and can easily be spread by when these infected materials are introduced to uninfected sites (Hooper and Southey, 1978). Precaution to minimise this risk is necessary. Such approach includes inspecting all plant hosts for any surviving stem nematodes. Seed inspection should be carried out before planting (Southey, 1978; EPPO, 2008; Stoddard *et al.*, 2010). Planting nematode-free seeds on non-infested sites is a vital part of good management against stem nematodes. In the UK, stem nematode seed inspection is voluntary, so few seed companies participate in this program (Stawniak, 2011).
Farm hygiene that minimises stem nematode build-up is essential. At the end of growing season, the infested plant stalks should be disposed by incinerating where practicable (Stoddard *et al*., 2010). Feeding infected stalks to farm animals is problematic since the nematodes can survive after passing through the gut (Palmisano *et al.*, 1971). In summary, inspecting field bean seeds for *Ditylenchus* spp. is necessary for avoiding introducing the nematode to uncontaminated land.

1.4.2 Physical crop protection strategies

Physical measures considered against stem nematodes include hot water treatment and soil solarization. Hot water is applied as a pre-plant treatment. This method involves immersing planting materials such as seeds, bulbs, cloves, seedlings, tubers, rootstocks in hot water for a given time. In principle, the heat acquired will be lethal to nematodes. Infection of narcissus bulbs with stem nematodes was suppressed after exposing the bulbs to water at 44.4ºC for 3 h (Hanks and Linfield, 1999). Sometimes, hot water treatment is combined with chemicals such as formaldehyde, abamectin and sodium hypochlorite to improve control (Green, 1963; Qiu *et al*., 1993; Roberts and Matthews, 1995). However, this measure fails because stem nematodes can evade treatments by surviving in the core of plant tissues (Gratwick and Southey, 1986). Moreover, Winfield (1970), when applying hot water treatment, noted an increase in onion bulb damage with improved mortality of stem nematodes. Likewise, there is the risk of seed damage when applying this method to disinfect *Ditylenchus* spp. infested field bean seed (Qiu *et al*., 1993).

Soil solarisation is the process of generating heat from the radiant energy from the sun under transparent plastic sheets to suppress soil borne pathogens. This measure is detrimental to stem nematodes (Greco, 1993). However, the method is only applicable during summer (Greco, 1993). Such set-up reduced the density of stem nematodes after 6 weeks in Italy (Greco *et al*., 1985) and 8 weeks in Israel (Siti *et al.*, 1982). Maximum soil temperature up to 47°C will effectively suppress *D. dipsaci* in garlic field. This method affects stem nematodes to up to 10-20 cm depth of soil (Siti *et al*., 1982; Greco, 1993). Summers in sub-tropical and tropical countries are often dry and hot but the UK have short dry and hot periods. Moreover, there is no information available on whether soil solarization is feasible to minimise *Ditylenchus* spp. population densities in the UK for farmers.

1.4.3 Cultural strategies

Crop rotation is employed to reduce nematode population density below economic level; before reintroducing the desired crop. There are two setbacks in using this measure: disease development at low initial population (undetectable) and the wide host range of stem nematodes. Physical factors such as latitude and soil type affect the rate at which pest population decline in crop rotation (Brown, *et al*., 1978). *Ditylenchus dipsaci* are more persistent in heavy clay soils than in light sandy soils (Seinhorst, 1956). Wilson and French (1975) found that *D. dipsaci* surviving on weeds can cause damage on oats 7 years after growing a susceptible crop. On the other hand, non-host crop species for *D. dipsaci*, if found, may not have a high market value (Whitehead, 1997) and may build up the abundance of other damaging nematode species. However, rotating non-hosts for up to 10 years intervals between field beans reduces *D. gigas* (Caubel *et al*., 1999). Crop rotation for 3–4 years has been estimated to supress *Ditylenchus dipsaci* population in a field below an economic threshold. This effect can last 5–6 years before the nematode population build up and reach a density that causes economic crop loss.
Other cultural control measures including weeding, removing volunteer crops, wide row spacing and, time of planting, are less effective (Greco *et al.*, 1991).

1.4.4 Natural and induced plant resistance

Nematode resistance refers to any gene of a plant that hinders or prevents nematode multiplication (Trudgill, 1991). Using stem nematode resistant crops should be cost efficient as they ensure stable production of a crop without compromising its yield. In resistant crops, there may be modifications in the expression of defence compounds, such as isoflavenoids, in response to *D. dipsaci* infection.

The cultivation of field beans with resistance to *Ditylenchus* spp. disrupts reproduction. This was true for a cultivar INRA 29H used in Morocco that reduced their reproduction by 70% (Andaloussi, 2001). Field beans cultivars screened at Syria and France were also resistant to a *D. gigas* population (Hanounik *et al*., 1986; Caubel and Leclercq, 1989a; Caubel and Leclercq, 1989b). However, a virulent stem nematode population was shown to overcome the resistance in many of these cultivars (Andaloussi 2001). Moreover, no cultivar screened by Stawniak (2011) had complete resistance to stem nematodes. Though the knowledge and technology of introducing artificial resistance to field crops is available, there is little information on field bean cultivars with resistance against stem nematodes. Hence, no recommended field beans cultivars are available to UK famers with stem nematode problems.

1.4.5 Biocontrol agents and biopesticides

Biopesticides are derived from natural resources such as fungi and bacteria. They cause lethal infections to the pest species or produce bioactive compounds that disrupt pest activity. They also suppress nematode densities through antagonistic interactions, playing a key role in suppressive soils. Two important genera are the rhizobacteria, *Bacillus* and fluorescent *Pseudomonas*.

The species in two genera (*Bacillus* and *Pseudomonas*) alter the quality of plant root diffusates by producing enzymes, antibiotics and siderophores making the host plant less attractive to nematode pests (Chitwood, 2002; Tian *et al*., 2007; Turatto *et al*., 2018). The activity of these biocontrol agents, *Pseudomonas* spp. (CBSAL05) and *Bacillus* spp. (CBSAL02), can also cause nematode mortality. Isolates of these genera caused 55.19% and 53.53% mortality of *Ditylenchus* spp. respectively (Turatto *et al.,* 2018). Even though these biocontrol agents are effective, their performance may depend on species. It was reported that *D. dipsaci* can tolerate *B. firmus* more than root-knot nematodes *Meloidogyne incognita* and burrowing nematodes *Radopholus similis* (Mendoza *et al.*, 2008). Furthermore, fungal agents such as *Drechmeria coniospora* destroys *D. dipsaci* too. It attaches its conidia to the cuticle of the nematode and parasitizes it (Jansson *et al.*, 1987).

Despite the potential of biopesticides against stem nematodes, there are no products available on the market, mainly because they are difficult to mass produce.

1.4.6 Fumigants and non-fumigants nematicides

Nematicides are synthetic chemicals with toxic activity against plant parasitic nematodes and so protect plants from being attacked or reduce the level of damage caused. There are two classes of nematicides:

1) Fumigants (halogenated aliphatic hydrocarbons and methyl isothiocyanate liberators)
2) Non-fumigants (organophosphate and organocarbamate).

1.4.6.1 Fumigant

Methyl bromide is a broad-spectrum pesticide with severe toxicity against plant parasitic nematodes. In previous research it was used as fumigant to reduce stem nematodes surviving in the seed of alfafa (Page *et al*., 1959; Mouttet *et al*., 2014) and shallots (Hague, 1968). When applied to field bean seed at 2500 mg/l stem nematodes, the infection was reduced but there was a negative effect on seed germination. Reducing the concentration of methyl bromide to concentrations 1000-2000 mg/l did not effectively suppress the survival of the nematodes especially their J4 stage (Powell, 1974; Stoddard *et al.*, 2010).

As shown in another study, the control of stem nematodes by fumigant application has been demonstrated by using 20-80g/m^3 methyl iodide for 24 hrs at 15^{o}C on alfalfa seeds (Ciesla *et al.*, 2010). Improved yield performance was observed on narcissus bulbs that received a combination a fungicide, thiabendazole with either formaldehyde or peroxyacetic acid (Hanks and Linfield, 1999). And more recently, up to 99% of stem nematodes on garlic were suppressed after treatment with 20 g/m^3 Hydrogen cyanide (HCN) for up to 24 h (Zouhar *et al.*, 2016).

1.4.6.2 Non-fumigants

This group of nematicides can be soil or foliar applied. Foliar application of nematicides is thought to diminish the probabilities for ground water contamination over soil-applied nematicides (Westerdahl *et al.*, 1991). Foliar application of oxamyl to the leaves and bulbs of daffodil (*Narcissus* spp.) effectively reduced stem nematode levels (Westerdahl *et al.*, 1991). Application as seed treatment with alfalfa seeds immersed in 0.5% oxamyl for 10 h resulted in free of stem nematode (Gray *et al.*, 1994). However, when applied to soil in field beans production, oxamyl showed less bioactivity against the nematodes (Whitehead and Tite, 1987).

Ethoprophos is another non-fumigant nematicide that has been shown to have suppressive activity against stem nematodes. For example, Greenwood *et al*. (1984) recorded a reduction in *Ditylenchus* spp. populations by 98% after applying ethoprophos in the production of alfafa. A similar effect against stem nematodes was observed with carbofuran on alfafa (Greenwood *et al.*, 1984; Gray *et al.*, 1994), diazinon on *Phlox subulata* (LaMondia, 1999), and aldicarb (carbamate) on field beans and strawberry productions (Lewis, 1979; Whitehead and Tite, 1987).

Abamectin is a non-fumigant nematicide with a different mode of action. This nematicide is a product in the fermentation of the soil bacterium *Streptomyces avermitilis*. Its effectiveness against *D. dipsaci* has been recorded in Lamium and Phlox production (Becker, 1999; LaMondia, 1999). However, its effectiveness against stem nematodes on field beans is unknown.

Though nematicides are undoubtedly effective against stem nematodes. Most crop managers generally regard the application of nematicides as not being cost-effective for field beans (Greco 1993; Andaloussi, 2001). Furthermore, the high fecundity of stem nematodes combined with the variation in efficacy of products, due to soil and weather conditions makes nematicides less attractive (Chitwood, 2002). Finally, their use is restricted by European pesticide usage policies (Regulation (EC) No. 1107/2009) since they pose a threat to human health (as carcinogens) and the environment. As such many synthetic nematicides mentioned above have been withdrawn from use completely in the UK for controlling phytonematodes including *Ditylenchus* spp. (Hague, 2009; Stoddard *et al.*, 2010; Mouttet *et al.*, 2014). Hence, these restrictions on the use of chemical nematicides necessitate the development of alternative management strategies (Ploeg, 2008).

1.4.7 Botanical nematicides

In a bid to find alternatives to the chemical nematicides, various studies have investigated naturally available bioactive compounds in plants. There are several groups of plant secondary metabolites with promising nematicidal activity including aldehydes, ketones, alkaloids, glycosides, isothiocyanates, limonoids, quassinoids and saponins. Others are organic acids, phenolics, flavonoids, quinones, piperamides, polyacetylenes, polythienyls, and terpenes. (Chitwood, 2002).

1.4.7.1 Botanicals used in Stem nematode management

Under *in vitro* conditions, phytochemical compounds tested against stem nematodes have shown nematotoxic properties. A phytochemical extracted from marigold (*Tagetes nana*), α-terthiophene (thiophene), for example, was shown to cause *Ditylenchus* spp. mortality with LD_{50} of 0.05% (w/v) (Uhlenbroek and Bijloo, 1959; Uhlenbroek and Bijloo, 1958). Similarly, an LD50 of 0.01% (w/v) was recorded when a phytoalexin (rishitin), obtained from potato discs, was tested against stem nematodes (Zinovieva and Chalova, 1987). In experiments using 0.1% (w/v) tricyclic carbamate from calabar bean *Physostigma venenosum*, the infectivity of stem nematode, *D. dipsaci*, on pea seedlings was suppressed (Bijloo, 1965).

Furthermore, chemical compounds found in essential oils such as carvacrol and thymol processed by distillation from *Eugenia caryophyllata, Origanum compactum, O. vulgare, Thymus vulgaris,* and *T. mastichina* have been shown to have nematicidal properties. When tested against *Ditylenchus dipsaci* at concentrations of 0.5 and 0.75 % (w/v) mortality was 80-100% (Zouhar *et al.*, 2009). Recently, methanolic extracts from *Morina persica* (Caprifoliaceae) 0.025% and 0.05% (w/v) caused 100% mortality of *Ditylenchus dipsaci* after 24 h exposure (Onaran and Sağlam, 2017).

In soil, such plants derived products can retain their nematotoxicity. For instance, soil incorporating plant biomass containing flavonoids reduced stem nematodes by 93% and by 75.5%, respectively, when Artemisia dracunculus (Asteraceae) and Chelidonium majus (Papaveraceae) were used (Timchenko and Maiko, 1989).

However, stem nematode toxicity is not applicable to all plants. It is important to note that not all plants are toxic to stem nematodes. This was the case when the nematotoxic acids costic and isocostic (acids) derived from *Inula viscosa* leaf powder were effective against other plant parasitic nematodes in sand but not against stem nematodes (Oka *et al*., 2001).

Lectins are carbohydrate-binding proteins that can bind to the surface of nematodes and damage them. They are present in plants, fungi and defend them against pathogens and phytophagous insects (Trigueros *et al.*, 2003). In addition, they are involved in mediating the adhesion between nematode-trapping fungi and nematode cuticles (de Ulzurrun and Hsueh, 2018). In a study by Zhao *et al*., (2009) lectins isolated from the fungi, *Xylaria hypoxylon* and *Agrocybe cylindracea* showed nematicidal activity against *D. dipsaci* with LD_{50} of 0.47 and 0.9% (w/v) respectively. In the same study, lectins obtained from the fungi *Tricholoma mongolicum*, *Ganoderma lucidum* and *Boletus edulis* and plants *Pseudostellaria heterophylla, Solanum lycopersicum* had more nematotoxic activity against soybean cyst *Heterodera glycines* than *D. dipsaci* (Zhao *et al.*, 2009).

In conclusion, many of these studies have identified botanicals with potential activity against stem nematodes with up to 100% mortality reported *in vitro*. However, no studies have shown their application on field populations of *Ditylenchus* spp. For several of the methods described, the protocol required to achieve contact between the pest and the bioactive compound is laborious and may also require large amounts of biomass that are not practicable in the field. Hence, a different approach, biofumigation, is considered for nematode pest management.

1.5 Biofumigation

Biofumigation is a pest management method in crop production that uses plant-based compounds to manage pathogens, nematode pests, and weeds (Figure 1.10). The process works by exploiting biocidal compounds, most importantly isothiocyanates (ITCs), released through the hydrolysis of glucosinolates (GSLs) contained in certain plant families, principally the Brassicaceae. The term was first coined by Kirkegaard *et al*. (1993) who investigated the technique for managing soil-borne pests and pathogens in horticulture and agriculture using brassica plants. The term was revised and now includes pest suppression derived from the incorporation of plant tissues even of non-brassica plants that containing bioactive metabolites (Kirkegaard and Matthiessen, 2004). The following sections will largely focus on suppression linked to the application of glucosinolate-containing-plants and briefly highlight other obtainable benefits in the biofumigation system.

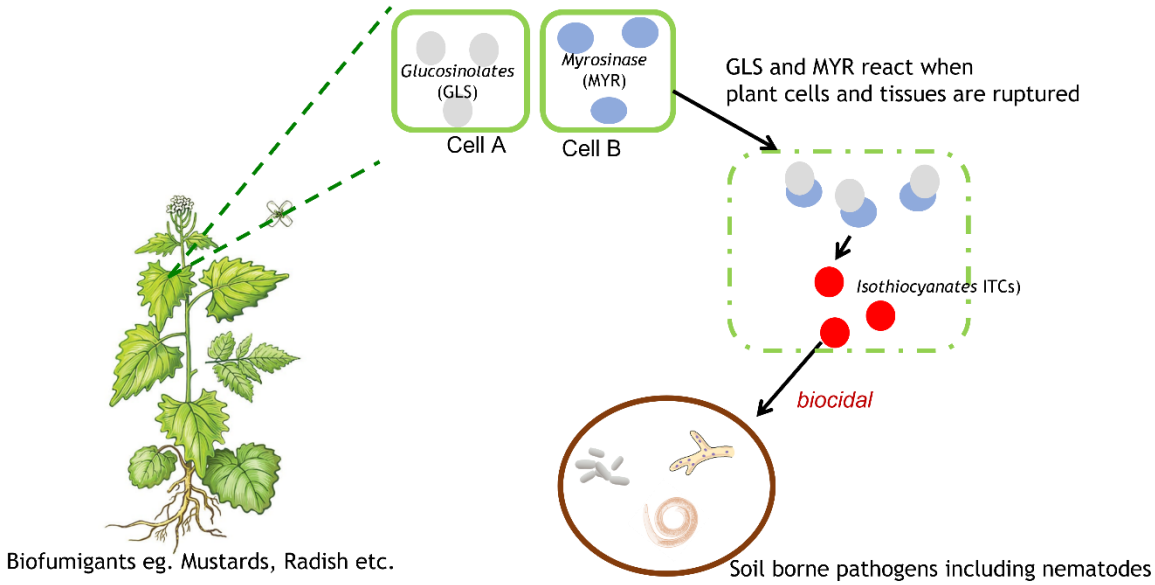

Figure 1.10. The concept of biofumigation showing how the use brassica can suppress soil borne plant pathogens and nematode pests

1.5.1 Glucosinolate types and classification

Glucosinolates are phytoanticipins (secondary metabolites) produced in plants belonging to the family Brassicaceae (Halkier and Gershenzon, 2006; USDA,2018) with ca. 200 types (Clarke, 2010). The scientific classification of Brassicaceae is as follows:

Kingdom Plantae – Plants

Subkingdom Tracheobionta – Vascular plants

Superdivision Spermatophyta – Seed plants

Division Magnoliophyta – Flowering plants

Class Magnoliopsida – Dicotyledons

Subclass Dilleniidae

Order Capparales

Family Brassicaceae - Mustard family

(USDA, 2018)

The members of Brassicaceae are beneficial in several processes. This includes, but is not limited to:

1) Reducing the incidence of cancers in humans after consumption (e.g. broccoli, kale and brussels sprouts) (Becker and Juvik, 2016) and

2) Suppression plant pests and pathogens, including nematodes under the biofumigation process (Björkman *et al.*, 2011; del Carmen Martínez-Ballesta *et al.*, 2013).

1.5.2 Biosynthesis of glucosinolates

Glucosinolates (GSLs) are derived from amino acids and are classified into three groups (Table 1.4) namely aliphatic, aromatic and indolic (Avato and Argentieri, 2015).

Table 1.4. Different glucosinolate categories based on their chemical structure

Glucosinolates **category**	**Amino acid derivative**
Aliphatic	alanine, leucine, isoleucine, methionine or valine
Aromatic	phenylalanine or tyrosine
Indolic	tryptophan

The pathway of their biosynthesis is in three phases

1) side-chain elongation of amino acid precursor,

2) development of the core glucosinolate structure, and

3) secondary modifications of the sidechain (Grubb and Abel, 2006).

This process involves several genes which have been identified by Sønderby *et al*. (2010). The basic structure of glucosinolates consists of a β-D-thioglucose moiety, a sulphonated oxime group and an aglycone side chain (Figure 1.11).

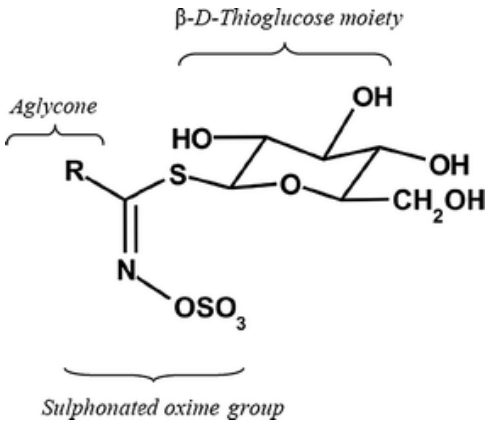

Figure 1.11. Glucosinolate basic chemical structure (Avato and Argentieri, 2015) .

1.5.3 Brassica glucosinolate profile

Kirkegaard and Sarwar (1998) screened 80 brassica plant species for potential application in biofumigation systems, they found that the total glucosinolate content ranged from 0.8 to 45.3 mmol m^{-2} which correlated with their biomass. The shoot of indian mustard, *Brassica juncea*, for instance, contains 50% more glucosinolate than the shoot of rocket *Eruca sativa* (Antonious *et al.*, 2017). This variation is also seen among cultivars of same species (Rosa *et al.*, 1996; Park *et al.*, 2017) as observed in two cultivars of *B. juncea*, 'Pacific Gold' and 'Arid' that contain 300 µmol g^{-1} and 10 µmol g^{-1} GSL respectively (Brown *et al*., 2004; Malhi *et al*., 2007). There are rare instances of this variation within a given cultivar (Kirkegaard and Sarwar, 1998).

As brassica plants grow, the type and concentration of glucosinolate vary with growth stage and plant organs. In most cases, the total GSL concentration increases with growth and reaches its highest concentration during reproductive development (Antonious *et al*., 2017) or at 50% flowering stage (Bellostas *et al.*, 2007; Doheny-Adams *et al.*, 2018). When brassica plants approach maturity, glucosinolate concentrations decrease in the foliage and roots but increase in reproductive tissues, leading to seed possessing the highest concentration of glucosinolate. The seed of *B. juncea* is known to have more than 100% more GSL than the leaves at this stage (Rangkadilok *et al.*, 2002; Oliveira *et al.*, 2011). Hence, selecting of brassica plants for biofumigation based on their seed GSL content is not recommended (Kirkegaard and Sarwar, 1998). In addition, it highlights the importance of selecting the right growth stage when using biofumigants.

Glucosinolates are more abundant and diverse in brassica roots than their shoots, which suggests that plants have developed this strategy to avoid constant exposure to soil pathogens (Van Dam *et al*., 2009). Although brassica roots have higher glucosinolate concentration over shoots, their use in biofumigation systems is limited since their

biomass is low i.e. an average of 23.6% of the total plant GSLs (Kirkegaard and Sarwar, 1998). Furthermore, glucosinolate compositional differences occur in the root region. The root epidermis that is in contact with the soil contains more glucosinolate than the inner tissues as observed in *R. sativus* (Kim *et al*., 2013).

The quality of glucosinolate also changes with the growth of Brassica plants. The seeds of brassica generally contain aliphatic GSL and rarely contain aromatic GSL (gluconasturtin) (Baenas *et al.*, 2012; Bellostas *et al.*, 2007) but modifications in glucosinolate structures can occur. In *B. napus*, the aliphatic GSL concentration decreased, as indolyl and aromatic GSL formed a week after planting (Bellostas *et al*., 2007). As brassica plants grow, there are qualitative changes in GSL composition in the different plant organs (Table 1.5), with aliphatic GSLs and aromatic GSLs predominantly found in the shoot and root respectively, while indolyl GSLs are found in all plant tissues but at low concentrations (<1 mol g^{-1}) (Kirkegaard and Sarwar, 1998).

Table 1.5. Glucosinolates associated with brassica plants.

Glucosinolate	Common names	Brassica species	Plant organ	Author(s)
			Aliphatic	
Gluconapin	Wild mustard	*Brassica rapa*	Shoot	Park *et al.* (2017)
Sinigrin	Indian mustard	*Brassica juncea*	Shoot	Zasada and Ferris (2004); Ngala *et al.* (2015)
	Ethiopian mustard	*Brassica carinata*	Shoot	Potter *et al*. (1998)
Sinalbin	White mustard	*Sinapis alba*	Shoot	Hopkins *et al*. (1998)
	White mustard	*Brassica hirta*	Shoot	Zasada and Ferris (2004)
Glucoerucin	Rocket	*Eruca sativa*	Shoot	Di Gioia *et al.* (2018)
Glucobrassicanapin	Wild mustard	*Brassica rapa*	Shoot	Kim *et al*. (2010)
Progoitrin	Canola	*Brassica napus*	Seed	Cartea *et al*. (2008)
Glucoraphanin	Radish	*Raphanus sativus*	Shoot	Ngala *et al*. (2015)
	Rocket	*Eruca sativa*	Shoot	Di Gioia *et al.* (2018)
			Aromatic	
Gluconasturtiin	Indian mustard	*Brassica juncea*	Root	Park *et al*. (2017)
	Radish	*Raphanus sativus*	Root	Ngala *et al*. (2015)
Glucotropaeolin	White mustard	*Sinapis alba*	Root	Sawar *et al*. (1998)
	White mustard	*Brassica hirta*	Root	Zasada and Ferris (2004)
Glucosinalbin	White mustard	*Sinapis alba*	Seed	Borek and Morra (2005)
			Indolyl	
Neoglucobrassicin	Canola	*Brassica napus*	Shoot	Potter *et al*. (1998)
4-methoxyglucobrassicin	Canola	*Brassica napus*	Shoot, Seed	Potter *et al*. (1998)

1.5.4 Glucosinolate decomposition

At the cellular level, plant cells referred to as S-cells are rich in glucosinolates with their vacuole containing the metabolite (Koroleva *et al.*, 2010). Adjacent to them are a different set of plant cells called myrosin cells (Husebye *et al.*, 2002). These cells contain glycosylated thioglucosidases otherwise referred to as myrosinases (Chen and Yan, 2007; Kissen *et al.*, 2009)

Upon plant tissue disruption, glucosinolates are hydrolysed by the myrosinase enzymes with cofactors (epithio- and nitrile specific proteins) to produce an array of chemicals (Bjorkman *et al*., 2011). Two levels of reactions are catalysed by the myrosinase; 1. the glycosylation process, in which two intermediary products are formed in a sequence, glycosyl-enzyme and then aglycone; 2. the deglycolyation process, in which the glycosyl-enzyme is hydrolysed by a water molecule (Burmeister *et al*., 1997). Indole glucosinolate breakdown products differ from others due to the instability of the initially formed isothiocyanates at neutral or slightly acidic pH (3-7) resulting in the production of indole-methanols, ascorbic acid conjugates, and oligomeric mixtures (Agerbirk *et al.*, 1998). Another form of glucosinolate degradation can involve soil-borne microbes or temperature. There are other enzymes recovered from the fungus, *Aspergillus niger* that have similar activity to myrosinase and can degrade glucosinolates (Ohtsuru *et al.*, 1973; Hanschen *et al.*, 2015). At temperatures over 60ºC, myrosinase becomes inactivated (Tian *et al*., 2018) but the glucosinolate degradation in such instance is temperature driven (Hanschen *et al.*, 2012). More discussion on the degradation of GLS is contained in the review by Bones and Rossiter (2006).

1.5.5 Isothiocyanate release

The hydrolytic products (Figure 1.12) of glucosinolate include isothiocyanates, oxazolidine-2-thiones, nitriles, epithionitriles, and thiocyanates nitriles, which may function as defence bioactive metabolites against plant pathogens, nematodes, and insects. Their release is largely based on soil properties such as pH. Isothiocyanates, however, have received most attention among the hydrolytic products since they are the most bioactive (Gimsing and Kirkegaard, 2009). In many reports, the amount of isothiocyanates released is in the range of 1% to 50% of the potential amount based on glucosinolate concentration (Gardiner *et al*., 1999; Morra and Kirkegaard, 2002; Gimsing and Kirkegaard, 2006).

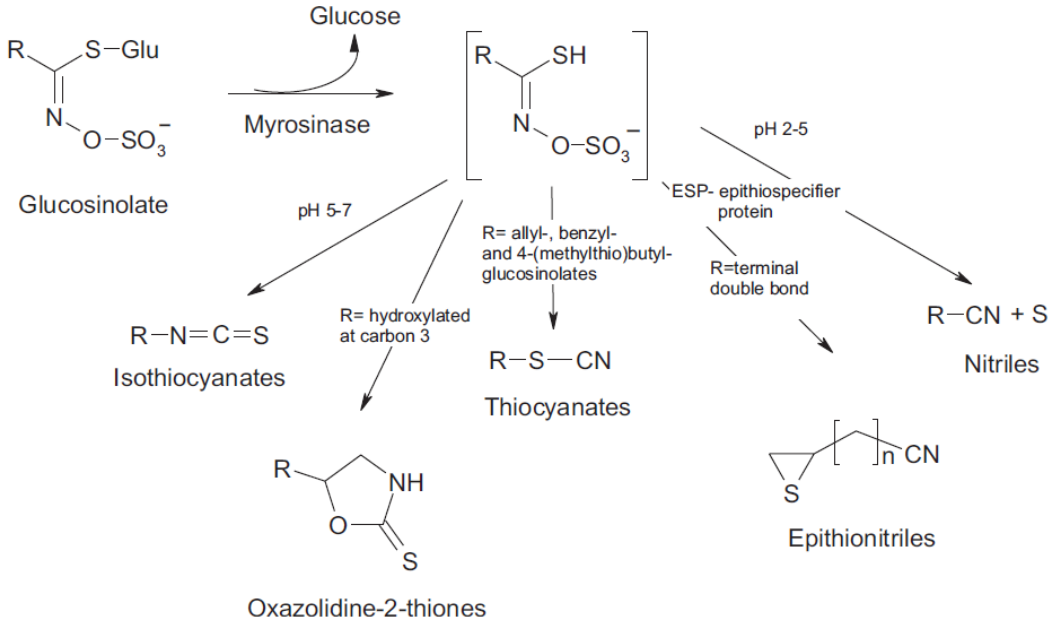

Figure 1.12. Structure of possible glucosinolate degradation products (Radojčić Redovniković *et al.*, 2008).

1.5.6 Effect of biofumigation on nematodes

Significant negative behavioural defects have been observed after exposing *Meloidogyne incognita* and *M. hapla* to isothiocyantes resulting in reduced egg production whilst also inhibiting hatch as well as the infectivity of their juveniles on soybean (Yu *et al*., 2005; Zasada *et al*., 2009). Isothiocyanates can interfere with protein molecules by irreversibly reacting with nucleophiles such as the thiol and amine group of enzymes (Lazzeri *et al*., 1993; Brown and Hampton, 2011; Avato et al., 2013) and cause DNA damage (Fouché *et al.*, 2016) . Excessive energy loss resulting from the expression of nematode heat shock proteins which are stimulated by 2-propenyl isothiocyanate could also be the cause of nematode inactivity and mortality (Brolsma *et al.*, 2014).

1.5.7 Managing plant parasitic nematodes using Biofumigation

The application of biofumigants to suppress nematode pests of crops is receiving increasing attention. Such studies have focussed on the management of the potato cyst nematode *Globodera* spp.. (Ngala *et al.*,2015) and root-knot nematodes *Meloidogyne* spp. (Aydınlı and Mennan, 2018) among others have been investigated. This section will cover the success of biofumigation under three conditions–*in vitro*, glasshouse and field.

1.5.7.1 *In-vitro* studies

The set-up of *in-vitro* assays are simple for identifying the relative toxicities of dominant ITC which is important for assessing the effectiveness of potential biofumigants against plant parasitic nematodes. Isothiocyanates have been reported to have nematotoxic effects. For example, 2-propenyl isothiocyante at 0.1 % (w/v) elicited 100% mortality of potato cyst nematodes *Globodera rostochiensis* juveniles (J2) after 24 h exposure (Pinto *et al.*, 1997) and *G. pallida* after 72 h and 24 h exposure to 0.0025 and 0.005% (w/v) respectively (Wood *et al*., 2017). Beside using pure isothiocyanates, freeze dried (roots and leaf) can be applied, such was the case for *Brassica juncea* which gave lethal doses (LD_{50}) against *G. pallida* as 0.002% (w/v) (Brolsma *et al*., 2014) and 0.0027% (w/v) (Ngala, Woods *et al.*, 2015) after 2 h and 96 h exposure respectively.

Glucosinolates are not bioactive even under *in vitro* conditions. However, when myrosinase is added their hydrolytic product is generated before bioactivity is observed. For instance, benzyl glucosinolates at 1 mg/ml with added myrosinase was toxic to *G. rostochiensis* leading to 100% mortality after 24 h exposure (Buskov *et al.*, 2002). Similar effect was observed when *G. pallida* J2 were exposed to 0.05 mg/mL with a record of 96% mortality (Wood *et al*., 2017). This compound is also nematoxic to the juveniles of *H. glycines* with lethal dose effect (LD_{50}) of 0.015 mg/ml (Schroeder and MacGuidwin, 2010). Furthermore, several plant-parasitic nematodes have been shown to be susceptible to pure isothiocyantes including *Pratylenchus penetrans*, *P. neglectus* (Yu *et al*., 2005) and *Tylenchus semipenetrans* (Zasada and Ferris, 2004). In summary, pure and derived isothiocyanates under *in-vitro* conditions are undoubtedly nematicidal or nematostatic to a wide range of economic important plant-feeding nematodes.

1.5.7.2 Field and glasshouse studies

There are many of reports of biofumigation success in literature under controlled environmental and field conditions. Generally, Brassica plants tissue are chopped to 2-5cm in length or seed milled and homogenised with soil having the target nematode pests present. This procedure facilitates the production of isothiocyanates.

Studies have shown that a strong nematode suppression is possible with dried, fresh foliage and seed meals of brassicas. In glasshouse experiments, three *Brassica juncea* lines (Nemfix, Fumus, and ISCI99) at 5% (w/w) led to over 95% mortality of encysted eggs of *G. pallida* in polyethylene-covered soil (Lord *et al*., 2011). Similarly, soil incorporated with air dried leaf meal of *B. juncea* at 1.6% (w/w) suppressed the number of galls, egg masses and eggs of *Meloidogyne incognita* on tomato plants by over 90% (Oliveira *et al*., 2011). While the seed meal of *B. juncea* at 0.06% resulted in > 90% suppression of *Pratylenchus penetrans* and *Meloidogyne incognita* (Zasada *et al*., 2009). Under field conditions, Ngala *et al*., (2015) recorded a reduced *G. pallida* multiplication (*P*f/*P*i =0.92)

on plots where *B. juncea* were grown and incorporated. The seed meal of *B. juncea* suppressed the hatching of the juvenile of *G. pallida* by 90% (Dandurand *et al.*, 2017) Positive effects of biofumigation are not recorded in every case. Although *Eruca sativa* reduced *Meloidogyne hapla* population levels by over 95% (Riga, 2011), it increased the development of *M*. *incognita* in another study (Engelbrecht, 2012).
In conclusion, studies have supported the use of biofumigation against different species plant-parasitic nematode. But its efficacy will depend on the target species and the type of brassica applied. The translation of one successful biofumigation effect to another will not be direct as several factors can impede its performance.

1.5.8 Factors affecting the success of biofumigation

The soil where the biofumigation process occurs is complex, consisting of solid grains with pores as well as chemical compounds and micro- and macro-organisms that interact to always and in different ways affect the soil composition. Physical and chemical soil properties play key roles in determining the release, retention, and activity of isothiocyanates, during brassica growth and following biomass incorporation. Therefore, the efficacy of biofumigation will largely depend on edaphic factors as they influence the concentration of bioactive isothiocyanates in soil.

1.5.8.1 Soil fertility

Brassica performance depends on soil nutrition during the growing season. Brassica plant agronomic characteristics such as biomass and glucosinolate concentrations are improved by increasing nitrogen and sulfur fertilization rates respectively (Taylor *et al*., 1991 ; Öztürk, 2010; Björkman *et al*., 2011). For example, when sulphur was applied at 30 kg/ ha to *B. juncea canola* cv. Arid, *B. juncea* canola cv. Amulet, *B. juncea* mustard cv. Cutlass, and *B. napus* cv. InVigor 2663 hybrid canola, they significantly produced quality biomass (Malhi *et al.*, 2007) Similarly, sewage sludge rich in these nutrients supported more production of GSLs than no-mulch bare soil (Antonious *et al.*, 2017). To achieve maximum potential of Brassicas, it is essential that soil is adequately fertilized.

1.5.8.2 Biomass maceration and incorporation

Biofumigation involves the use of machinery to chop and incorporate biofumigants. They should sufficiently damage (cut and bruise) plant tissues, thereby allowing the glucosinolates to interact with water and myrosinase leading to isothiocyanate production. When biofumigants are pulverised and incorporated, up to 56% of the glucosinolates were converted to isothiocyanates as compared to <1% when whole plants were incorporated into the soil (Morra and Kirkgaard, 2002; Gimsing and Kirkgaard 2006). A recent study

reported that by using a flail-spader implement combination, biofumigation efficacy against *Globodera pallida* was increased by 7% compared to a than a roll conditioner-rotavator combination (Watts, 2018). Therefore, achieving maximum cell disruption is essential to enhance the release efficiency of glucosinolate into isothiocyanate. Further details on the important maceration and incorporation implements can be found in Watts (2018).

1.5.8.3 Soil organic matter

Soil organic matter consist of plant and animal residues at various stages of decomposition. They contain nucleophilic chemicals such as phenols, amines, alcohols, carboxylic acids and thiols (Brown and Hampton, 2011). Isothiocyanates react with these compounds. In a biofumigation system, the major forms of isothiocyanates are aromatic and aliphatic and aromatic isothiocyanates react with soil organic matter more than aliphatic isothiocyanates (Matthiessen, John N. and Shackleton, 2005). The efficacy of biofumigation is, however, not affected when soil organic matter content is within the range of 2.2% to 6.1% (De Cauwer *et al.*, 2019). Moreover, as different soil types contain varying percentages of soil organic matter, results from one field are not easily compared to another.

1.5.8.4 Air and soil temperature

Temperature can facilitate or impede the reactive processes in biofumigation. For instance, the production and activity of methyl isothiocyanate was uniform across a range of temperatures (10-20^{o}C) but declined to 50% at low temperature of 5°C. Such low soil temperatures particularly below 12°C reduce their volatility while increasing solubility in water, therefore influencing their bioactivity against the target pest (Lembright, 1990; Lane and Trudgill, 1999). A higher temperature is hypothesized to increase the concentration of ITC in the soil through stimulation of enzymatic conversion of glucosinolates to isothiocyanates (Gardiner *et al.*, 1999). Low temperatures, however, slows the process.

1.5.8.5 Soil Moisture

Soil moisture content refers to the percentage of water present in the soil. It has some influence in biofumigation. Adequate soil moisture condition is believed to stimulate the breakdown of the Brassica tissue, hydrolysis of glucosinolates and release of isothiocyanates (De Cauwer *et al*., 2019) and help retain ITC by turning them to soil solution hence limiting gaseous loss (Simpson *et al.*, 2010).

Morra and Kirkegaard (2002) and Matthiessen *et al.* (2004) respectively recorded a doubling and ten-fold increase of the isothiocyanate concentration as the soil moisture content rose from field capacity to saturation. Another study by Gimsing and Kirkegaard

(2006) suggested otherwise and found no effect of soil moisture content on *B. juncea* biofumigation when the moisture content was raised from wilting point to field capacity. However, this study reported no application of a soil roller (an important activity after biofumigation) at the time of incorporation.
In a recent study by Watts (2018), a soil moisture of 50% of field capacity was found to be more effective for biofumigation than 0, 25, 75 and 100% of field capacity, and water saturated soil. This could be due to optimal water to air ratio within soil pores for VOC diffusion, and retention within the soil (Watts, 2018).

1.5.8.6 The chemical nature and properties of the volatiles

The bioactivity of biofumigation-volatiles generally declines rapidly in soils. This rapid loss is also linked to their chemical nature and properties. Glucosinolates are water soluble organic anions (Avato and Argentieri, 2015) and are hence quickly degraded and leached before conversion to isothiocyanates. According to Gimsing and Kirkgaard (2006), their retention time is 30 minutes after incorporation. Papadopoulous and Alderson (2007) recorded a rapid decrease of ITC within three days of soil incorporation of Brassica and with half-life of <10 mins (Aissani *et al.*, 2013) . Moreover, ITC could be microbially degraded (Gimsing and Kirkgaard 2008) hence lost.

1.5.9 Conclusion on factors on biofumigation

Improving the conversion process of glucosinalates to isothiocyanates will lead to higher isothiocyanate in the soil. Therefore, soil-borne pests will be exposed to a more toxic environment. This can be achieved by extensive tissue disruption, ensuring suitable soil conditions that favour the isothiocyanate production such as a neutral pH, temperatures within (10-20◦C) and moisture content in the range of (25-75%) field capacity.

1.5.10 Supplementary benefits of biofumigation

Brassicas used as biofumigants also provide additional benefits when applied to soil. Though their application as biofumigants is generally aimed for isothiocyanate release for their more toxic properties, other bio-toxic compounds are produced such as phenolics, tocopherols, dimethyl disulphide and dimethyl sulphide that also play a role in pest suppression (Avato and Argentieri, 2015). In addition, soil changes resulting from such biofumigation include an increase in the soil biotic community which favour the populations of plant-pest-antagonists and enhancing nutrient cycling. Finally, brassica plants can absorb heavy metals and are important for phytoremediation (Szczygłowska *et al.*, 2011).

1.5.11 Negative impacts of biofumigation

Biofumigation is not always considered beneficial. Firstly, biofumigant species could be host to some important pests. For example, growing *Brassica juncea* and *B. napus* has been shown to increase soil population of *Meloidogyne javanica* by 23% (Stirling and Stirling, 2003). However, the key to avoiding such pest build up is selecting brassicas that are known to be poor hosts to the target (Matthiessen and Kirkegaard, 2006). Another detriment in applying biofumigation is shown when the activity of beneficial organisms such as entomopathogenic nematodes (Henderson *et al.*, 2009) and arthropods (Jensen *et al*., 2010) were suppressed. There are, however, some beneficial organisms, particularly nematodes, that have a shorter life cycle (R strategist) compared to plant parasitic nematodes and which can have a quicker recovery rate in a biofumigation system when compared to the target plant parasitic nematodes.

1.6 Research objectives

This research aims at determining whether biofumigation is a feasible management measure against the two stem nematodes, *Ditylenchus dipsaci* and *D. gigas*. The following objectives are considered:

1. To determine the bioactivity of different isothiocyanates against *Ditylenchus* spp. *in-vitro*
2. To distinguish the two stem nematodes, *Ditylenchus dipsaci* and *D. gigas* using molecular methods
3. To investigate the Brassica host suitability for *Ditylenchus dipsaci* and *D. gigas*
4. To access the biofumigation effect of selected Brassica plants against *Ditylenchus dipsaci* and *D. gigas* under field conditions

2.0 General materials and methods

2.1 Introduction

Detailed information is presented in this chapter about the general materials and methods used in the culturing, extraction, and identification of stem nematodes. The individual chapters describe the specific technique(s) that were added to achieve their stated aims. All procedures were undertaken at the Crop and Environment Research Centre, Harper Adams University, Newport, UK.

2.2 Stem nematode culture

Suspensions of the nematodes *Ditylenchus dipsaci* and *D. gigas* were used for inoculum in glasshouse experiments (Chapter 5), for molecular identification (Chapter 7) and in the host finding assays (Chapter 4), using two different types of methods to culture them.

2.2.1 Culturing of *Ditylenchus dipsaci* on carrot discs

A population of *Ditylenchus dipsaci*, originally isolated from infected narcissus bulbs, was reared on carrot discs following the procedure of Kaplan and Davis (1990). Carrots were obtained from a local supplier (Waitrose Supermarket, UK), washed and surface sterilised by passing carrots, sprayed with ethanol, through a flame. Thereafter, the carrots were peeled using a sterile surgical blade and cut into <1 cm thick discs (Figure 2.1). Discs were then aseptically transferred to sterile 35mm mini-Petri dishes (Sarstedt, Germany) and incubated in the dark at 23 ±1◦C for two weeks. A concentration of 0.2 % streptomycin sulphate was used to sterilise *D. dipsaci* a day prior to disc inoculation. Then, carrot discs with no visible contamination from fungi or bacteria were inoculated with *D. dipsaci* after incubation. The set-up was further incubated for 2-3 months before use. At this time, stem nematodes, *D. dipsaci*, were found to egress from the carrot disc and aggregate onto the lid of the Petri dishes. The carrot discs were cut into smaller sizes of <0.3 mm to allow efficient nematode extraction (Section 2.3.1)

2.2.2 Culturing of *Ditylenchus gigas* on field beans

Field beans plants *Vicia faba* (cv Fuego), were grown in John Innes No. 2 sterilised loam-based compost and maintained in the glasshouse at 21±2 °C in a 12:12 (Figure 2.2). Two weeks after planting, emerged plants were inoculated with ca. 1000-2000 *D. gigas* in 1-2ml of tap water suspension, introduced to the stipule(s) and crown of the plants. Plants were watered every two days until required. Plants were occasionally fed with water-soluble NPK fertilizer (Chempak No. 2; 25:15:15 + TE) and maintained for 3-4 months.

The stems of *D. gigas* infected *Vicia faba* plants were cut into to 2-3 cm pieces before nematode extraction (Section 1.3.1).

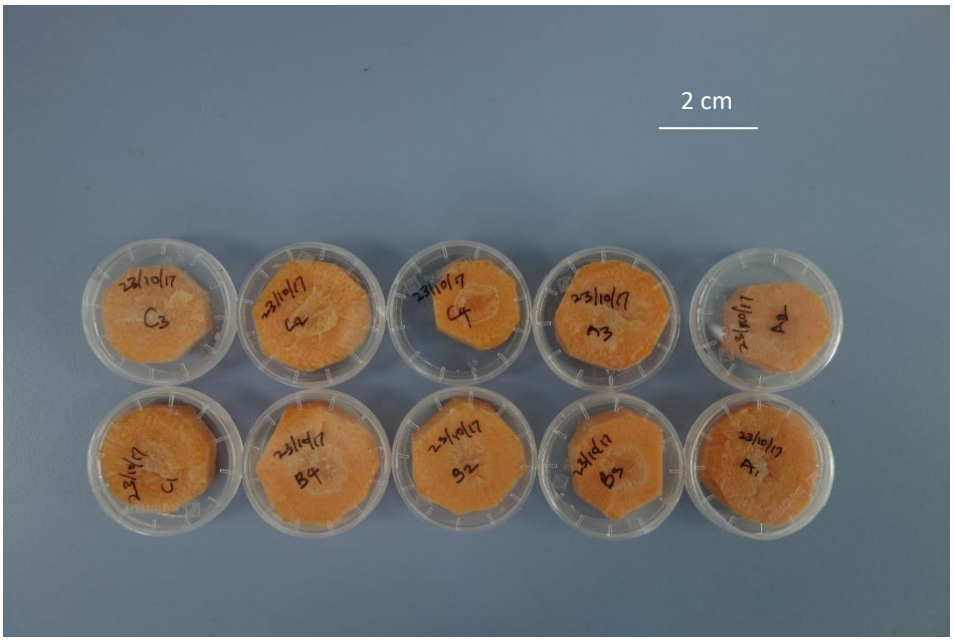

Figure 2.1. Carrot discs prepared for inoculation with *Ditylenchus dipsaci*

Figure 2.2. Young field bean plants inoculated with *Ditylenchus gigas*

2.3 Nematode extraction

2.3.1 Nematode extraction from plant and carrot discs

For all the plant samples and carrot discs, a modified Whitehead tray method (Whitehead and Hemming, 1965) was used to obtain a suspension of active stem nematodes (*D.*

gigas or *D. dipsaci*) in water. The technique relies on nematodes' motility and their tendency to move toward moisture.

Subsamples of plant tissues (<100 g) were spread on a standard facial tissue (20 x 20 cm.) over a plastic wire mesh (1 mm) on a plastic-coated wire letter tray (40 x 30 x 8 cm), resting in a pool of shallow tap water in a rectangular plastic tray (56 X 40 X 12 cm). Extraction was performed at 20° C. Nematodes were collected every 2–6h intermittently for 3 days. The nematodes were then concentrated by passing the nematode suspension through a 38 µm mesh sieve, and then rinsed to collect the nematodes into 50 ml falcon tubes using 35 - 40 ml of distilled tap water.

2.3.2 Nematode extraction from soil

Stem nematodes were extracted from soil samples collected from controlled environment or field experiments using the Seinhorst two-flask technique (Seinhorst, 1955) (Figure 2.3). This method relies on the principle of density. Nematodes are generally less dense in comparison to soil particles.

Each of the bagged samples was homogenised by gently mixing them. Soil cores, formed by the soil auger during sampling, were also loosened in the process and large pebbles taken out. A subsample of 200 g was used for extraction. Procedures were performed as described by Van Bezooijen (2006).

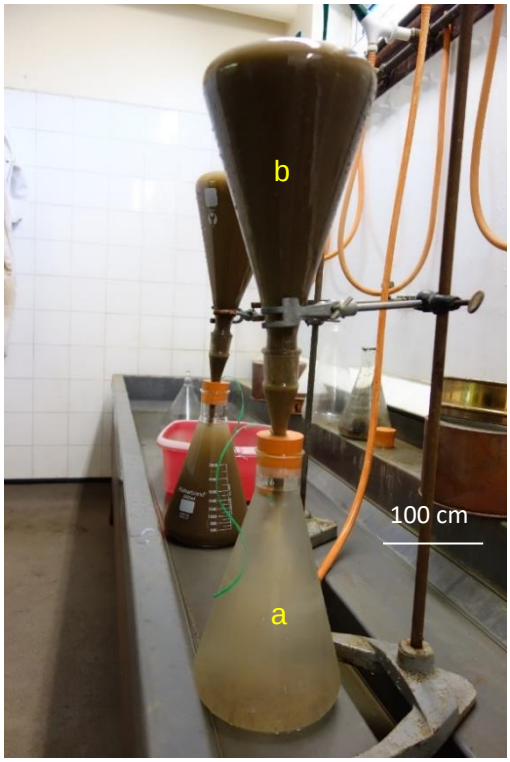

Figure 2.3. Seinhorst two-flask method showing the sedimentation process. Soil particles with more density than nematodes are collected in flask (a) while nematodes remain in flask (b)

2.4 Identification of specimens

Following extraction, the nematode suspensions were concentrated to 10 ml for each sample. To do this, the suspension was first allowed to settle for 3–6 hours, followed by pipetting out the supernatant until the volume was reduced to 10 ml. For each sample, the suspension was poured into a gridded counting dish. After that, the suspension was examined for the presence of any *Ditylenchus* spp. and counted using a Leica DMi1 stereomicroscope (Leica microsystems Milton Keynes, UK) at 40X magnification
For suspensions from the field, the identity of a specimen that matched the description of *Ditylenchus* spp. was picked and placed in a drop of water on a glass slide. Afterwards, the specimens were covered with a glass slip and sealed by coating the edges of the glass slip with nail varnish. Using a compound microscope (Meiji MX5300L, Meiji Techno Co. Ltd) the slides were examined and the species were identified using taxonomic keys and monographs (EPPO, 2008; Vovlas *et al*., 2011).

2.5 Data analysis

Statistical analysis of collected data were carried out using the R programming language (R Studio 4.0.2). The specific details on the choice of analysis are described in the materials and methods of the relevant chapters.

3.0 The effect of isothiocyanates associated with Brassica species on the survival of stem nematodes

3.1 Introduction

Appreciable crop production losses observed in many countries, including the United Kingdom, are a direct result of nematode feeding damage. Plant parasitic nematodes have been estimated to cause a global yield loss of 12.3 percent, which equates to a value of $157 billion dollars (Hassan *et al.*, 2013). In the UK, the key nematodes affecting field bean production are the stem and bulb nematodes, *Ditylenchus gigas* and *D. dipsaci* (Hooper, 1983; Stawniak, 2011). Measures to manage these pests on crops in the field have been practiced including hot water treatment (Hesling, 1972), breeding for resistance (Andaloussi, 2001; Plowright *et al.*, 2002) and the use of nematicides (Whitehead *et al.*, 1979; Whitehead and Tite, 1987). However, no applicable management option is available for beans production in the UK (Stawniak, 2011). The widely used fumigant nematicide, methyl bromide, was banned in developed countries from 2006 (according to the Montreal protocol) due to its negative impact on the ozone layer (Mouttet *et al.*, 2014) and thereafter environmentally suitable substitutes have become the focus (Bello *et al.*, 2001). Alternatives strategies to chemical nematicides include biofumigation which is viewed as a safer and efficient measure against soil borne pests and pathogens including nematodes (Bello *et al*., 2001; Matthiessen and Kirkegaard, 2006). Biofumigation involves exploiting isothiocyanate generation from macerated brassica plants containing glucosinolates and myrosinase. Isothiocyanates are toxic volatile compounds that are biocidal to nematodes and other soil borne pests and pathogens (Ntalli and Caboni, 2017). Various laboratory, glasshouse and field studies have shown that isothiocyanates are toxic to plant-parasitic nematodes (Wu *et al*., 2011; Wood *et al*., 2017). However, not all isothiocyanates associated with brassica plants are suppressive to nematodes (Wood *et al.*, 2017). Previous work has established that different isothiocyanates vary in effectiveness against different pathogens, with no single isothiocyanate (ITC) achieving a broad spectrum of control (Smith and Kirkegaard, 2002; Taylor *et al*., 2014).

The type and concentration of isothiocyanates released depends very much on the brassica plant species and variety (Daxenbichler *et al.*, 1991; Fahey *et al.*, 2001). Three chemical categories of parent glucosinolates associated with isothiocyanates are namely aliphatic, aromatic and indole. Important isothiocyanates with nematotoxic effects include allyl isothiocyanates (Wood *et al.*, 2017), 2-phenylethyl isothiocyanate, benzyl isothiocyanate (Zasada *et al.*, 2009) and sulforaphane (Wood *et al.*, 2017) and were reported to have toxic effect against *Meloidogyne* spp., and *Globodera pallida* (Wu *et al.*, 2011; Wood *et al.*, 2017). Moreover, most brassicas plants used in biofumigation systems

can release more than 50 µg/ml effective isothiocyanates concentrations under field conditions (Wood *et al.*, 2017).

3.2 Objectives

a) Determine the degree of stem nematode mortality at a range of ITC concentrations obtainable in the field
b) Find the concentration of pure isothiocyanates required to cause 50% mortality (LD_{50}) of the stem nematodes *D. gigas* and *D. dipsaci*
c) Determine which isothiocyanates have the most nematicidal effect against stem nematodes
d) Compare the effects of isothiocyanates between the two species of stem nematodes

3.3 Null hypothesis

Isothiocyanates have no effect on the mortality of the stem nematodes *Ditylenchus gigas* and *D. dipsaci.*

3.4 Materials and methods

3.4.1 Isothiocyanates (ITCs)

The choice of isothiocyanates for this study was dependent on previous reports of their toxicity to nematodes (Zasada *et al*., 2009; Wood *et al*., 2017) and their association to brassica plants used in biofumigation systems (Lord *et al*., 2011; Ngala *et al*., 2015). Pure ITCs were obtained from Sigma-Aldrich, UK. Allyl ITCs and sulforaphane represent aliphatic ITCs with increasing side chain length, while benzyl and 2-phenylethyl ITCs have aromatic side chains (Table 3.1). Stock solutions of each ITC were prepared in 1% dimethyl sulfoxide (DMSO).

3.4.2 Stem nematode mortality assays

Three forms of experiments were performed to determine the effect of isothiocyanates on *Ditylenchus gigas* or *D. dipsaci.* The experiments were laid out in a completely randomised design with six replicates of each treatment and an average of twenty nematode individuals (mixed adults and juveniles) from nematode cultures (Section 2.2; Chapter 2) were placed in each experimental well. All experiments were performed using 6-well cell culture plates (Sarstedt, Germany) and each plate was sealed with parafilm

(Parafilm®) before being stored at 20ºC in the dark. Control treatments of distilled water and1% DMSO were used in assay 1 and 3 while only distilled water was used in assay 2 and 4. Stem nematodes were considered alive based on their response to stimuli from probing (up to 7 times) under a stereomicroscope at x40 magnification. For each treatment, stem nematode mortality counts were converted to percentage mortality.

Table 3.1. Characteristics and occurrence of synthetic isothiocyanates (ITC) used in experiments to determine stem nematode mortality

ITC	Chemical group	Parent glucosinolate	Molecular mass(g/mol)	Purity (%)	Common occurrence
Allyl	Aliphatic	Sinigrin	99.15	>98	*Brassica juncea*, *B. carinata*,
Sulforaphane	Aliphatic	Glucoraphanin	177.29	≥90	*B. rapa*, *Raphanus sativus*
Benzyl	Aromatic	Glucotropaeolin	149.20	≥97	*Sinapis alba* L.
2-phenethyl	Aromatic	Gluconasturtiin	163.23	>98	*B. campestris, B. juncea*

3.4.2.1 Effect of isothiocyanates on stem nematode survival

This stem nematode mortality assay was performed for both *Ditylenchus gigas* and *D. dipsaci*. Nematodes were exposed to one of several ITC solutions (benzyl, 2-phenethyl, allyl and sulforaphane) at initial concentrations (12.5, 25, 50, and 100 µg/ml) for 72 h. Over a period of 72 h, stem nematode mortality was assessed every 24 h. The plates were unsealed prior to assessment, and then resealed for further incubation. The mortality experiment was repeated for both species.

3.4.2.2 Stem nematode survival following isothiocyanates exposure for 48 and 72 h

Experiments were performed to determine the degree of stem nematode recovery after 48 and 72 h exposure to isothiocyanates. *Ditylenchus gigas* was exposed to benzyl, 2-phenethyl and allyl isothiocyanate concentrations (12.5, 25, 50, and 100 µg/ml). After a continuous exposure for 48 and 72 h, the nematodes were taken out of the ITC suspensions using a glass Pasteur pipette and placed into distilled water for 24 h to recover prior to nematode mortality counts.

3.4.2.3 Isothiocyanates toxicity against *Ditylenchus gigas* and *D. dipsaci* (LD_{50})

In the case of toxicity assessment involving dose-response, both stem nematodes *D. dipsaci* and *D. gigas* were exposed to benzyl, 2-phenethyl and allyl isothiocyanate concentrations (12.5-200 µg/ml) for 24 h. Afterwards, the nematodes were placed in distilled water for 24 h to recover before a further mortality assessment was conducted.

3.5 Data analysis

For this toxicity assays, a generalised linear regression analysis with binomial distribution was performed where the factors ITC x concentration were analysed in version 4.0.2 of R (R Core Team, 2021). In the following process, the package emmeans (function emmeans) was used to extract the respective contrast and estimated means. Afterwards, a Tukey post-hoc analyses for multiple comparisons was performed with significant differences at $P<0.05$ and compact letter display was generated using multcomp packages. Estimates of lethal dose (LD_{50}) values for isothiocyanates were generated using the drc package (Ritz *et al.*, 2015).

3.6 Results

3.6.1 Isothiocyanates and *Ditylenchus gigas* mortality

There was little difference in the mortality of *Ditylenchus gigas* recorded at 24, 48 and 72 h for each treatment (Figure 3.1 and Figure 3.2). Total *D. gigas* mortality recorded over a 72 h period differed significantly depending on the ITC and concentration applied (*P<0.001* for ITC, Concentration and ITC x Concentration interaction). When exposed to allyl ITC, benzyl ITC and 2-phenethyl ITC stem nematode mortality increased as the isothiocyanate concentrations increased. Concentrations of 50 µg/ml and 100 µg/ml of allyl ITC, 2-phenethyl ITC and benzyl ITC were extremely effective, causing significant *D. gigas* mortality compared to the controls; 100% mortality occurred after exposure to 100 µg/ml 2-phenethyl ITC and benzyl ITC while that of allyl ITC caused 80% (Assay 1, Figure 3.1) to 100% (Assay 2; Figure 3.2) mortality. Isothiocyanate concentrations of 25 µg /ml and 12.5 µg/ml failed to cause significant mortality compared to the control except 2-phenethyl ITC that resulted in a significant increase in mortality at these concentrations (Assay 1; Figure 3.1). While *D. gigas* mortality was observed following exposure to allyl ITC, 2-phenethyl ITC and benzyl ITC, no sulforaphane concentration (12.5 µg/ml to 100 µg/ml) caused any significant difference in *D. gigas* mortality compared to the controls.

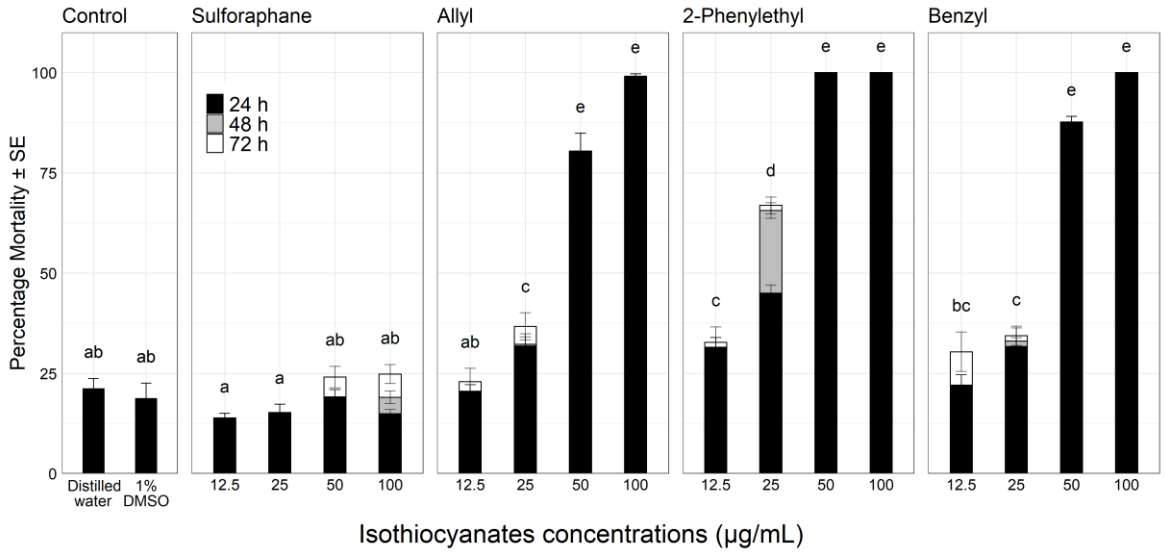

Figure 3.1. Assay 1-Mean mortality (%) of *Ditylenchus gigas* following exposure to sulforaphane, allyl, 2-phenylethyl and benzyl ITC for 24, 48 and 72 h. Error bars represent the standard error. Bars followed by a different letter are significantly different after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

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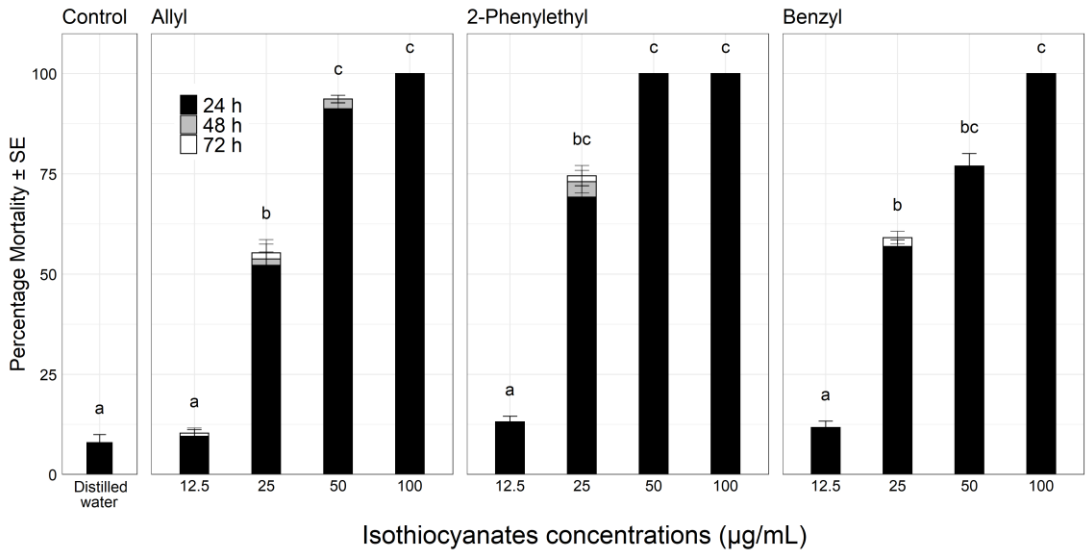

Figure 3.2. Assay 2- Mean mortality (%) of *Ditylenchus gigas* following exposure to allyl, 2-phenylethyl and benzyl ITC for 24,48 and 72 h. Error bars represent the standard error. Bars followed by a different letter are significantly different after 72 hrs according to Tukey's Honest Significant Difference test (P < 0.05)

3.6.2 Isothiocyanates and *Ditylenchus dipsaci* mortality

During exposure to ITC, the rate of *Ditylenchus dipsaci* mortality differed depending on the ITC and concentration applied (Figure 3.3; Figure 3.4). Mortality occurred in the first period of exposure 24 h and steadily increased with continuous exposure for up to 72 h period for allyl ITC, 2-phenethyl ITC and benzyl ITC isothiocyanates. After exposure to the four ITCs, total *D. dipsaci* mortality differed depending on the ITC and concentration applied (*P<0.001* for ITC, Concentration and ITC x Concentration, Figure 3.3 and Figure 3.4). Allyl ITC, 2-phenethyl ITC and benzyl ITC were extremely effective, leading to significant stem nematode mortality at 50 and 100 µg/ml concentrations after 24 h exposure. Complete mortality occurred after exposure to 100 µg/ml of allyl, 2-phenethyl and benzyl ITCs (Figure 3.3, Figure 3.4); and 50 µg/ml of 2-phenethyl ITC and benzyl ITC (Figure 3.3). However, there was a marked difference in mortality at different timepoints in assay 4 (Figure 3.4) compared to in assay 3 (Figure 3.3). Following exposure to sulforaphane, stem nematode mortality did not increase above that of the control for any of the concentrations tested (Figure 3.3).

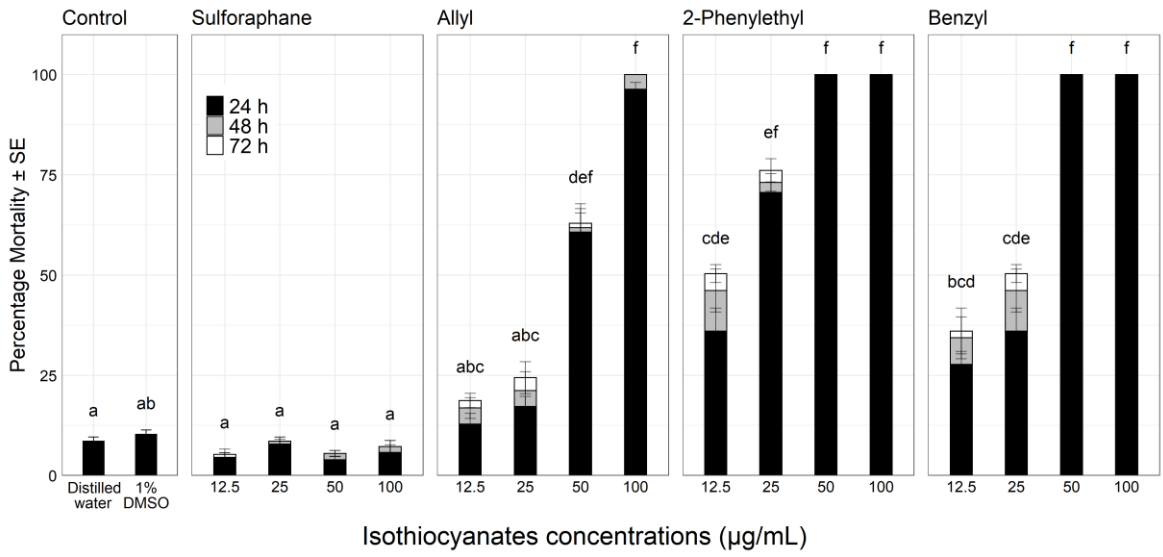

Figure 3.3. Assay 3- Mean mortality (%) of *Ditylenchus dipsaci* following exposure to sulforaphane, allyl, 2-phenylethyl and benzyl for 24,48 and 72 h. Error bars represent the standard error. Significant differences (P<0.05) compared to the control are indicated by differences in compact letter display

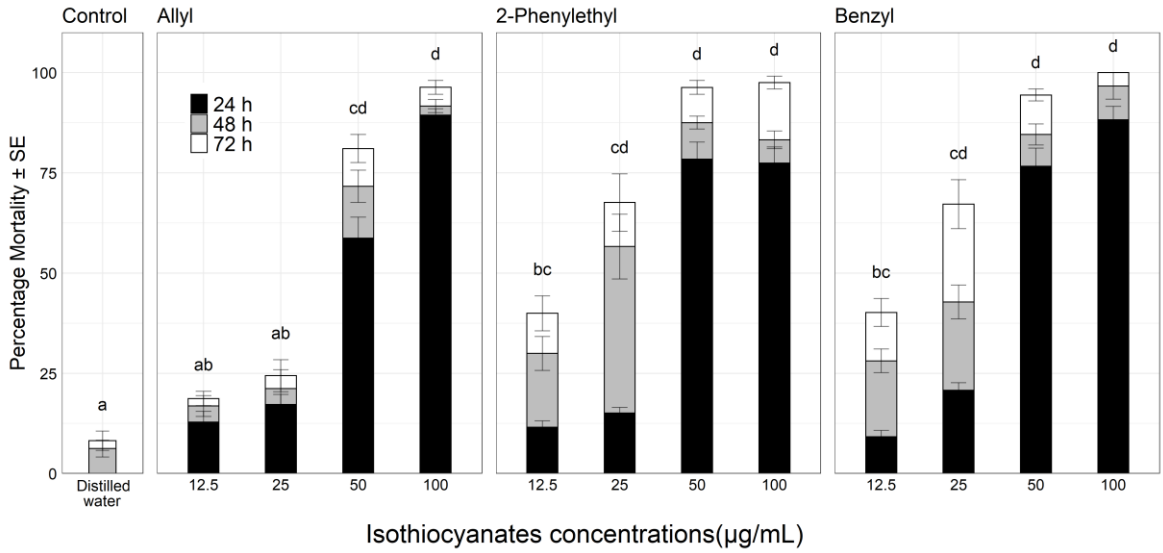

Figure 3.4. Assay 4- Mean mortality (%) of *Ditylenchus dipsaci* following exposure to allyl, 2-phenylethyl and benzyl ITC for 24,48 and 72h. Error bars represent the standard error. Significant differences (P<0.05) compared to the control are indicated by differences in control are indicated by differences in compact letter display

3.6.3 *Ditylenchus gigas* mortality following 48 and 72 h exposure to isothiocyanates

The mortality of *D. gigas* was assessed following continuous exposure to 12.5-100 µg/ml of allyl ITC, 2-phenethyl ITC and benzyl ITC, for 48 and 72h followed by a 24h period of recovery in distilled water. There were significant differences between treatment-concentrations with respect to the percentage of dead *D. gigas*; 50-100 µg/ml of benzyl ITC and 2-phenethyl ITC caused complete mortality of *D. gigas* after 48h exposure. Continuous exposure of *D. gigas* to 25-100 µg/ml and 50-100 µg/ml of 2-phenethyl ITC and benzyl ITC, respectively, for 72h followed by a 24h period of recovery in distilled water resulted in 100% mortality. Recovery of *D. gigas* after exposure to 12.5 µg/ml of 2-phenethyl ITC or benzyl ITC was lower after 72h compared to 48h exposure; mortality after 48h exposure to 2-phenethyl ITC and benzyl ITC and 24h recovery in water was 20% to 70% respectively, whereas a 72h exposure resulted in mortalities of 46% and 83% respectively.

On the other hand, the effect of pre-exposing *D. gigas* to allyl ITC at 12.5-100 µg/ml allyl ITC was similar when the nematodes were exposed for either 48 or 72h. With increasing allyl ITC concentration, the mortality of *D. gigas* increased. It can be seen from Figures 3.5 and 3.6, that the nematodes previously exposed to allyl ITC showed about a 50% recovery at the 100 µg/ml concentration. Despite the lower mortality recorded, allyl ITC-exposed-nematodes had limited movement and showed slow responses to the stimuli applied. In contrast, nematodes exposed to 2-phenethyl ITC and benzyl ITC were immobile.

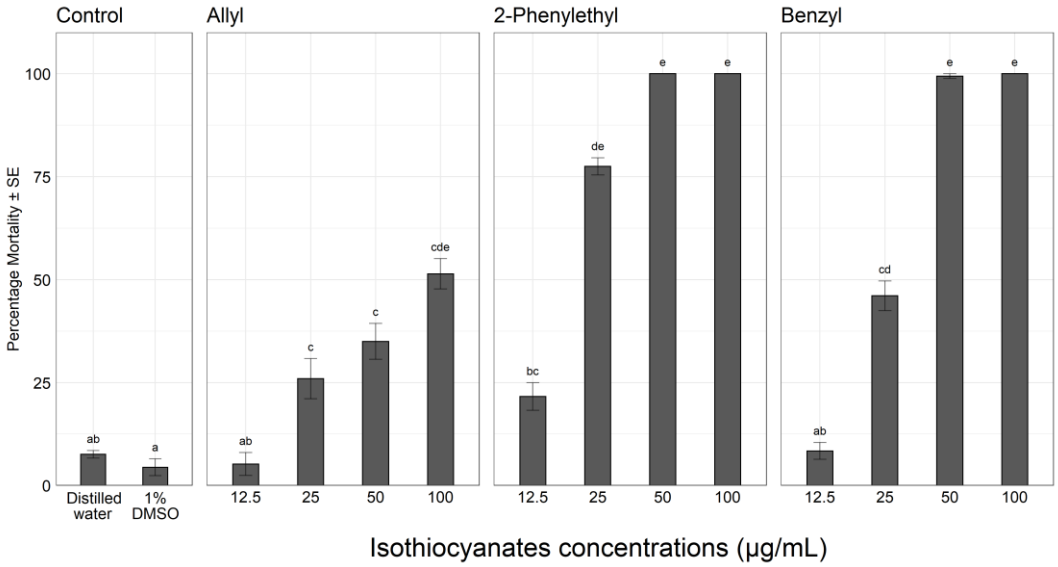

Figure 3.5. Mean mortality (%) of *Ditylenchus gigas* after following exposure to allyl, 2-phenylethyl and benzyl ITC for 48 h and recovery in water for 24 h. Error bars represent the standard error. Significant differences (P<0.05) compared to the control are indicated by differences in compact letter display

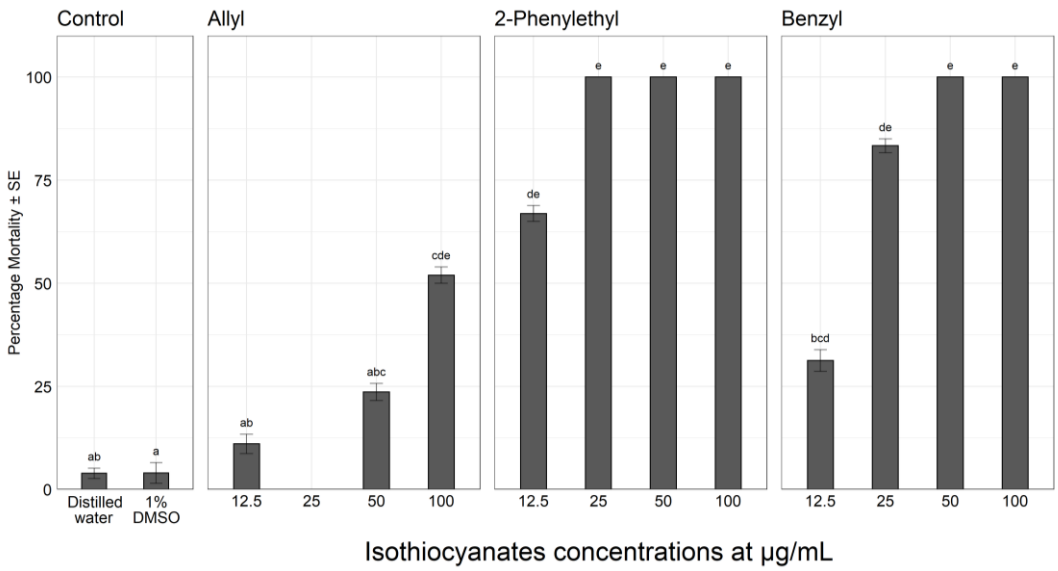

Figure 3.6. Mean mortality (%) of *Ditylenchus gigas* after following exposure to allyl, 2-phenylethyl and benzyl ITC for 72 h and recovery in water for 24 h. Error bars represent the standard error. Significant differences (P<0.05) compared to the control are indicated by differences in compact letter display

3.6.4 Isothiocyanates toxicity against *Ditylenchus gigas* and *D. dipsaci* (LD_{50})

The LD_{50} of isothiocyanates towards *Ditylenchus gigas* and *D. dipsaci* was calculated based on dose response curves (Figure 3.7 and 3.8). The LD_{50} for 2-phenethyl ITC, benzyl ITC and allyl ITC against *D. gigas* is shown in Table 3.2 with the LD_{50} estimated at 22 µg/ml, 24 µg/ml, and 79 µg/ml for 2-phenethyl ITC, benzyl ITC and allyl ITC respectively. *Ditylenchus gigas* was less sensitive to allyl ITC than 2-phenethyl ITC and benzyl ITC with only 200 µg/ml able to cause complete mortality (Figure 3.7). Isothiocyanate toxicity against *D. dipsaci* (LD_{50}) were similar to that recorded for *D. gigas*. *Ditylenchus dipsaci* was most sensitive to benzyl ITC followed by 2-phenethyl ITC and then allyl ITC; the LD_{50} were 24 µg/ml, 25 µg/ml, and 43 µg/ml respectively (Table 3.4). The pairwise comparison for the three isothiocyanates showed differences in the LD_{50} values with allyl ITC statistically less toxic (P<0.001; Tables 3.3 and 3.5) for both *D. dipsaci* and *D. gigas.* Whereas the LD_{50} values 2-phenethyl ITC and benzyl ITC differed for *D. gigas* (P=0.0256) and were statistically similar for *D. dipsaci* (P=0.564).

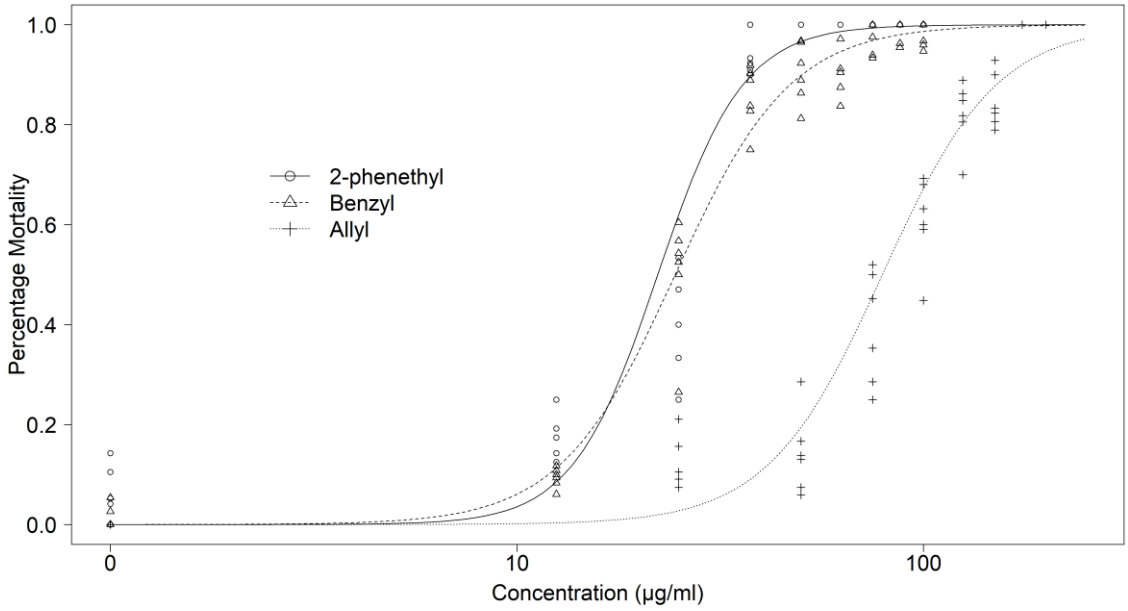

Figure 3.7. Percentage mortality of *Ditylenchus gigas* across a concentration range for 2-phenylethyl, allyl, and benzyl isothiocyanates

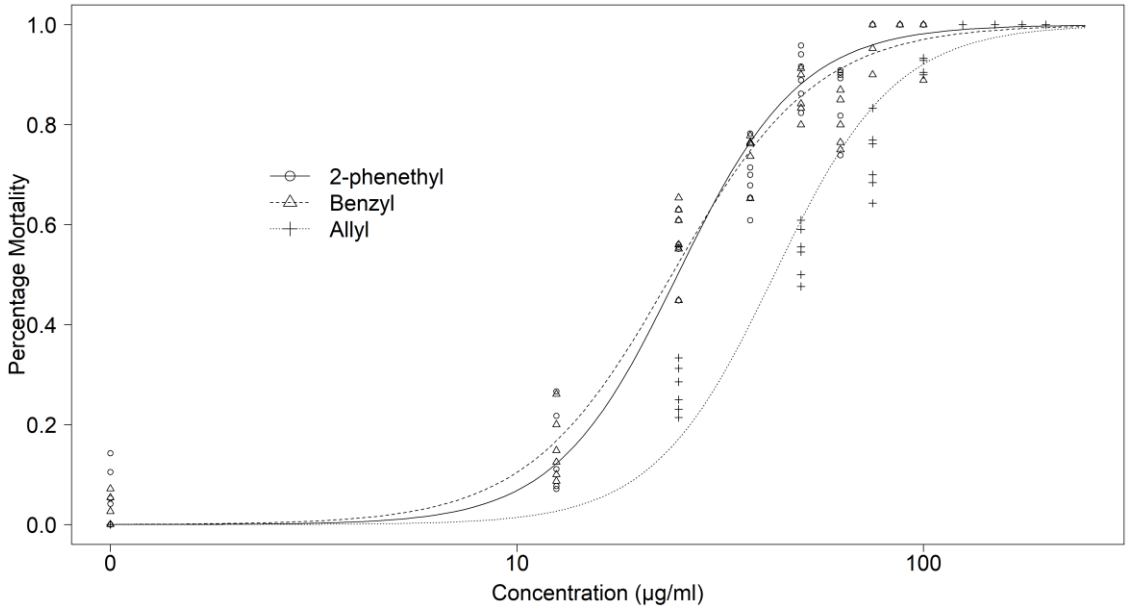

Figure 3.8. Plot of the relationship between the percentage mortality of *Ditylenchus dipsaci* and a concentration range for 2-phenylethyl, allyl, and benzyl isothiocyanates

Table 3.2. Lethal dose values (LD_{50}) for *Ditylenchus gigas* in relation to 2-phenylethyl, allyl and benzyl isothiocyanates (µg/ml) with upper and lower limit, and standard error

	Estimate	Std. Error	Lower	Upper
2-phenethyl	22.21	0.79	20.67	23.75
Allyl	79.25	2.26	74.82	83.67
Benzyl	24.51	0.73	23.07	25.94

Table 3.3. Pairwise comparison of the Lethal Dose values (LD_{50}) for *Ditylenchus gigas* for 2-phenylethyl, allyl and benzyl isothiocyanates

LD_{50} Ratio of ITC	***P value***
2-Phenethyl: Allyl	<0.001
Allyl: Benzyl	<0.001
2-Phenethyl: Benzyl	0.026

Table 3.4. Lethal dose values (LD_{50}) for *Ditylenchus dipsaci* for 2-phenylethyl, allyl, and benzyl isothiocyanates (µg/ml) with upper and lower limit, and standard error

	Estimate	Std. Error	Lower	Upper
2-phenethyl	24.86	0.91	23.07	26.63
Allyl	42.97	1.99	39.05	46.89
Benzyl	24.05	1.02	22.05	26.05

Table 3.5. Pairwise comparison of the Lethal Dose values (LD_{50}) for *Ditylenchus dipsaci* for 2-phenylethyl, allyl, and benzyl isothiocyanates

LD_{50} ratio of ITCs	***P value***
2-Phenethyl: Allyl	<0.001
Allyl: Benzyl	<0.001
2-Phenethyl: Benzyl	0.5638

3.7 Discussion

Different brassica species have different glucosinolate profiles, which reflect the isothiocyanate (ITC) derivatives that are toxic to nematodes. A substantial amount of biomass containing glucosinolate is required for ITC to successfully manage plant parasitic nematodes. To determine the effectiveness of potential biofumigants against plant parasitic nematodes, it is important to screen dominant ITC for their relative toxicities. The use of pure isothiocyanates provides a solid basis for a reliable and repeatable comparison of experiments against the use of crude isothiocyanates derived from the glucosinolate-containing brassica material. In fact, some synthetic nematicide formulations e.g. Metam Sodium have isothiocyanates as their active ingredients. There were no differences in stem nematode *Ditylenchus gigas* and *D. dipsaci* mortality between distilled water and 1% DMSO controls in all experiments. This indicates no negative effect of using DMSO as a solvent for assessing the synthetic ITC.

3.7.1 Non-toxicity of sulforaphane isothiocyanate

Glucoraphanin is the glucosinolate precursor for the isothiocyanate sulforaphane. Results from this study, highlight that sulforaphane does not have nematicidal or nematostatic activity on either *D. gigas* or *D. dipsaci* at the tested concentrations. Glucoraphanin is found in brassica species such as *Brassica oleracea* (Wathelet *et al*., 2004), *Eruca sativa* (Lord *et al*., 2011; Aissani *et al*., 2015; Ntalli, 2016) and *Raphanus sativus* (oilseed radish) (Ngala *et al*., 2015), which are known as hosts to *D. dipsaci* (Edwards and Taylor, 1963; Goodey *et al.*, 1965) (see Chapter 5). It could be hypothesised that stem nematodes have developed tolerance to sulforaphane. Alternatively, insensitivity of plant parasitic nematodes to sulforaphane have been repeatedly observed in previous studies (Aissani *et al*., 2015; Ntalli, 2016). When compared to benzyl ITC, sulforaphane's nematicidal activity (LD_{50}=152±35 µg/ml) against the root-knot nematode, *M. incognita,* was shown to be eighty times less toxic (Aissani *et al*., 2015; Ntalli, 2016). In addition, sulforaphane failed to cause mortality against *Caenorhabditis elegans* nematodes at concentrations up to 70 µg/ml (400 µM) (Qi *et al*., 2021). Rather, sulforaphane increased the survival of *C. elegans* at higher sulforaphane concentrations; hence the nematodes died faster at lower concentrations (Qi *et al*., 2021). This suggests that structural differences among isothiocyanates conferred different effects on nematodes which could be suppressive or possibly promoting survival. In contrast, Wood *et al*. (2017) reported 100% mortality after potato cyst nematode (*G. pallida*) juveniles were exposed to 50 µg/ml sulforaphane. While there are indications that higher doses of sulforaphane, than those used in this study, may affect the survival of *D. gigas* and *D. dipsaci,* such concentrations are unlikely to be achievable in field conditions. Leaf samples of *R. sativus* were reported to contain low concentrations of glucoraphanin —

25.4 µmol/g (Ngala *et al*., 2015), a concentration below 100 µg/ml, while another study showed that *Eruca sativa* (rocket) had <3 µmol of glucoraphanin g^{-1} dry weight (Lord *et al*., 2011). The relationship between host and pest appears to play a key role in the success of applying any selected biofumigant. Brassica plants with glucoraphanin may be suitable for the managing *Globodera* spp. but not for *D. gigas* and *D. dipsaci*.

3.7.2 Toxicity of isothiocyanates

It was observed that *D. gigas* and *D. dipsaci* showed differential physical responses to the type of ITC they were exposed to. Allyl ITC was found to cause both mortality and paralysis with reduced movement, while on the other hand 2-phenethyl ITC and benzyl ITC induced only mortality. The effect of 2-phenethyl ITC and benzyl ITC appeared to be irreversible as seen when the nematodes were placed in distilled water for 24 h after exposure to the ITCs. Allyl ITC has been previously reported to have nematotoxic effect against plant parasitic nematodes such as *G. pallida* (Wood *et al.*, 2017) and J2 of *M. javanica*, (Wu *et al.*, 2011) at similar concentrations used in this study. However, a nematostatic effect on *D. gigas* and *D. dipsaci* was recorded in this study. Some brassicaceous species predominantly produce 2-propenyl GSL (98-99% of total GSL), which is the precursor of allyl ITC. Specific examples include Ethiopian mustard (*Brassica carinata*) (93 µmol g^{-1}), Black mustard (*Brassica nigra*) (147 µmol g^{-1}) and Indian mustard (*B. juncea*) (90-154 µmol g^{-1}) (Zasada *et al*., 2009; Meyer *et al*., 2015; Ngala *et al*., 2015 and Curto *et al*., 2016).

Serra *et al*. (2002) recorded 17.3 % mortality of *Globodera rostochiensis* after 24 h exposure to 50 µg/ml of gluconasturtiin (2-phenylethyl GSL) following hydrolysis by myrosinase. At the same concentration and exposure time, ≈15% mortality was recorded when juveniles (J2) of *Globodera pallida* J2 were treated with 2-phenethyl ITC (Wood *et al.*, 2017). However, in the current study the same concentration and exposure time was sufficient to cause 100% mortality of *D. gigas* and *D. dipsaci*, whereas the LD_{50} for 2-phenethyl ITC was determined as 30 µg/ml. In another study, exposure of *Meloidogyne incognita* to gluconasturin and sinigrin, the precursors of 2-phenethyl ITC and allyl ITC respectively, showed that the LD_{50} of sinigrin was ≈3-fold less than that of gluconasturtiin (Lazzeri *et al*., 2004). The LD_{50} of allyl ITC against *D. gigas* and *D. dipsaci* was much greater than that calculated for 2-phenethyl ITC. Such large differences in LD_{50} were reported by Zasada *et al*., (2003) who showed an LD_{50} value of 0.10 mM (9.9 µg/ml) and 0.02 mM (3.3 µg/ml) for allyl ITC and 2-phenethyl ITC against *Meloidogyne javanica* respectively. While many plant-parasitic nematodes, including *D. gigas* and *D. dipsaci*, appear to be more susceptible to 2-phenethyl ITC than allyl ITC, the reverse is true for *Globodera* spp. This could suggest that the response of *Globodera* spp. to ITC differs from other nematode species. Buskov *et al*. (2002) observed that the mortality *G. rostochiensis* was >80% when exposed to 2 mM (818.8 µg/ml) glucotropaeolin (benzyl GSL) with myrosinase for 48 h. However, this study

showed that 100% mortality of *D. gigas* is achievable at much lower concentration of 0.38 mM (50 µg/ml) and at a shorter exposure time of 24 h. Although similar patterns of mortality were recorded for both 2-phenethyl ITC and benzyl ITC, lower concentrations such as 12.5 µg/ml 2-phenethyl ITC had a greater effect over benzyl ITC with 65% and 30% mortality after 72 h.

Finally, the structure of the isothiocyanates shows an interesting correlation with the observed effects on *D. gigas.* The aromatic isothiocyanates, 2-phenethyl ITC and benzyl ITC, had a greater effect on mortality over the aliphatic allyl ITC. Typically, aromatic GSL are produced in the roots while aliphatic GSL are mainly associated with the shoots (Van Dam *et al.*, 2009). It could be hypothesised that the aerial feeding *D. gigas* and *D. dipsaci* are more susceptible to the ITC produced from roots than the stem because they have not evolved tolerance to them. Moreover, since most brassicas plants used in biofumigation systems can release ≥50 µg/ml isothiocyanates concentrations under field conditions (Wood *et al.*, 2017), the results presented in this study suggest that brassica plants with associated ITC (allyl, 2-phenethyl and benzyl) may suppress field populations *D. gigas* and *D. dipsaci*. These prospects were the basis of selecting brassica plants used in field study (Chapter 6).

3.7.3 Isothiocyanates exposure time and mortality

Isothiocyanates are produced though the enzymatic decomposition of glucosinolates. The highest concentrations of isothiocyanates are likely to be released shortly after brassica crushing (2-5 h) (Gimsing and Kirkegaard, 2009). Their release from brassica tissue materials could continue for up to four days after incorporation (Morra and Kirkegaard, 2002). Hence, it was hypothesised that longer exposure to pure isothiocyanates will increase the percentage of nematode mortality. Longer exposure to generated isothiocyanates led to the increase in mortality of *M. incognita* nematodes as recorded by Buskov *et al*. (2002); these authors exposed the nematodes to brassica plant materials with the addition of myrosinase. Wood *et al*. (2017) also observed increased mortality of *G. pallida* every 24 h over a 72 h period following exposure to pure isothiocyanates. However, in this study, no increased mortality was observed when stem nematode mortality was assessed every 24 h (nematode-isothiocyanate system unsealed and resealed) over a period of 72 h except *D. dipsaci* (assay 2) mortality which showed difference over time. Increased mortality was only observed when the nematode-isothiocyanate system was continuously sealed. For example, when the *in-vitro* system was sealed for a prolonged period, a greater percentage mortality was recorded even at low concentration as was the case for 2-phenethyl ITC at 12.5 µg/ml leading to 100% *D. gigas* mortality after 72 h continuous exposure. However, there was little effect of longer exposure times when stem nematode mortality was assessed at 24 and 48 h, possibly

due to the loss of isothiocyanates when the testing system used was unsealed. Isothiocyanate are volatiles and can be lost from any system which is not airtight; their half-life could be as low as 1 h as estimated by Hanschen *et al*. (2015). Moreover, their loss can be facilitated by increasing temperature up to 30ºC (Gimsing and Kirkegaard, 2009).

3.7.4 Limitations of this study

The aim of this study was to determine the toxicity of isothiocyanates to the stem nematodes, *D. gigas* and *D. dipsaci.* Such studies are important for identifying potential brassica species in a biofumigation system. They are also important for comparing the performance of isothiocyanates against previous studies involving other plant-parasitic nematodes. However, there are a number of limitations to such *in-vitro* studies. In this study, stem nematodes were assessed based on their response to stimuli from a probing needle (up to 7 times) and were considered alive following any movements, however slight. This approach could underestimate the efficacy of the isothiocyanates, and the concentrations tested. This is particularly true for the bioactivity of allyl ITC where at higher concentrations, surviving species of stem nematodes tended to be nematostatic and showed very little response to probing but were considered alive. There may be more effective methods to evaluate the response of nematodes to compounds considered as nematotoxic or nematostatic and understand their sublethal effects.

The assessment of the sublethal effects of isothiocyanates on nematode biology is crucial because sublethal concentrations do not cause nematodes to die, but by interfering with biological functions they may reduce nematode fecundity. One such biological function is nematode host finding which is critical for their survival. The use of pluronic gel, an artificial system, to examine the plant parasitic nematodes migrations to host has been reported (Danquah, 2012). Hence it is hypothesised that sublethal concentrations of ITCs will influence the host finding ability of stem nematodes and this was tested in Chapter 4.

3.7.5 Conclusion

In summary, the application of plants with natural biocidal properties for crop protection could prove beneficial to crop production. Biofumigation appears to provide an environmentally friendly alternative to chemical fumigants. This study demonstrated the nematostatic effect of allyl ITC and nematotoxic effect of 2-phenethyl ITC and benzyl ITC, which are associated with some brassica plants used in biofumigation. It also confirms the biological activity of these ITC against the stem nematodes *Ditylenchus gigas* and *D. dipsaci* under concentrations obtainable in the field. Furthermore, structural differences among isothiocyanates can confer different effects on stem nematodes which can be suppressive (allyl, 2-phenethyl, and benzyl ITCs) and not sulforaphane. However, the use of mortality type assessments, a binary means of classification, has some limitations, which will be addressed in the Chapter 4.

4.0 Effect of sub-lethal isothiocyanate concentrations on the foraging activity and behavioural form of *Ditylenchus* spp.

4.1 Introduction

Isothiocyanates (ITC) are biocidal compounds that interfere with protein molecules by irreversibly reacting with nucleophiles such as the thiol and amine group of enzymes (Lazzeri *et al*., 1993; Brown and Hampton, 2011; Avato *et al*., 2013). These compounds inhibit nematode reproduction, thereby reducing number of eggs produced and the number of hatched juveniles. Additionally, ITC can inhibit nematode activity by affecting their mobility (Yu *et al*., 2007; Zasada *et al*., 2009). Ultimately, the various effects of ITC may lead to a reduction in the population of plant parasitic nematodes.

Previous *in-vitro* assays (Chapter 3) conducted on this project have investigated the mortality of *Ditylenchus gigas* and *D. dipsaci* following exposure to different concentrations and types of isothiocyanates. These experiments demonstrated the ability of 2-phenethyl, benzyl ITC to cause the death of *D. gigas* and *D. dipsaci* at a concentration of 50 µg/ml. On the other hand, 50% of the nematodes exposed to 100 µg/ml concentration of allyl ITC recovered following 24 h in distilled water. However, the activity (movement) of the nematodes following exposure to concentrations of 25 µg/ml or above allyl ITC was much reduced. The LD_{50} values of the various ITC were estimated after 24 h exposure; the LD_{50} of 2-phenethyl and benzyl was approximately 30 µg/ml.

It is known that the highest concentrations of ITC are released 30 mins to 2 h after brassica maceration and incorporation, while the concentration of ITC in soil declines by 90% after 24 h (Brown *et al*., 1991; Morra and Kirkegaard, 2002, Gimsing and Kirkegaard, 2006). This is because ITC are volatile, have limited half-life in soil, and interact with organic compounds in soil (Brown and Morra, 1997; Matthiessen and Shackleton, 2005; Gimsing *et al*., 2007). These factors minimize the concentration of free isothiocyanates and cause uneven distribution in soil (Zasada *et. al*, 2009), thus decreasing the availability of ITC to cause mortality of plant-parasitic nematodes. Moreover, aromatic isothiocyanates (2-phenethyl and benzyl) are generally more toxic than their aliphatic (allyl) counterparts but are quickly depleted from the soil environment (Gimsing and Kirkegaard, 2009). Hence understanding the effects of sub-lethal isothiocyanate concentrations on nematode biology could have implications for nematode management.

The motility of nematodes, following exposure to chemical treatments, can be reliably observed under axenic conditions such as pluronic gel as shown for *Globodera pallida* (Danquah, 2012), *Meloidogyne* spp. (Čepulytė *et al*., 2018) and *D. dipsaci* (Spiegel *et al*., 2002). In such systems, the response of nematodes to attractants or repellents, can be

observed and monitored in several ways. Previous studies have been undertaken on plant root exudates (Williamson and Čepulytė, 2017), synthetic chemicals such as glucose (Warnock *et al.*, 2016) and plant seedlings with roots (Čepulytė *et al.*, 2018).

Nematodes possess amphids in their anterior region – these are important organs that function as chemoreceptors, which receive signals (such as plant root leachates) from their host. Consequently, nematodes orientate towards or away from chemical stimuli in the soil and therefore propel themselves by undulating their body in response to the stimuli (Croll, 1970). Motility is essential for plant parasitic nematode survival, host invasion, mating and reproduction. Immobilised plant parasitic nematodes are unable to successfully complete these processes; some may starve to death, while others may be subjected to attack by predators.

Furthermore, nematodes attain some behavioural responses and forms in response to their environment. Active plant parasitic nematodes are motile with characterized sigmoid shape, however under adverse environmental conditions such as exposure to nematicides, they coil (Kearn *et al.*, 2017). Stem nematodes coiling/aggregating can be a strategy to withstand the effect of nematicides (Moens and Perry, 2009).

Therefore, a better understanding of the chemotactic behaviour of these nematodes when subjected to sub-lethal concentrations of isothiocyanates is important to appreciate the extent to which isothiocyanates may play a role in nematode management against plant-parasitic nematodes.

4.2 Objectives

a) Develop a suitable assay for assessing the host finding ability of *Ditylenchus* spp. when treated and untreated with isothiocyanates
b) Record the state of *Ditylenchus* spp. when treated and untreated with isothiocyanates
c) Assess the movement of stem nematodes towards their host when pre-exposed to ITC under *in-vitro* conditions.

4.3 Null hypotheses

Isothiocyanates have no effect on the ability of stem nematodes *Ditylenchus gigas* and *D. dipsaci* to migrate to their host *Vicia faba*

4.4 Materials and methods

4.4.1 Pluronic gel preparation

Pluronic gel was used in all experiments, throughout the development of this work. This medium allows nematodes to move in three dimensions, thus providing a greater chance for them to respond to plant roots, as opposed to agar, which only allows for surface movement. In addition, the transparency of pluronic gel allows nematode movement in the gel to be observed easily.

Pluronic gel was prepared following the method previously described by Danquah (2012). Up to 100 ml of distilled water was placed into a conical flask and twenty-three grams of Pluronic (PF-127) powder (Sigma Aldrich,UK) were added to the water, while stirring gently. Once dissolved the volume was topped up to 100 ml with distilled water and stored in a refrigerator at 4°C until use.

4.4.2 Field bean (*Vicia faba*) germination

Field bean *Vicia faba* (Feugo) seeds used in this study were provided by PGRO. The seeds were surface sterilised in sodium hypochlorite solution (1% available chlorine) for 1 min and rinsed in water three times for 2 min each. Seeds were germinated in flat-bottomed Petri dishes (8.5 cm in diameter) lined with Whatman® No. 1 filter papers and soaked with sterile distilled water for four days at room temperature (20°C). They were allowed to further develop to obtain roots (Sections 4.4.3.1 and 4. 4.3.4) or embryos (Section 4.4.3.2).

4.4.3 The development of a host finding assay for stem nematodes

Several assays were chosen from literature and tested to find the most suitable assay for the host finding assessment of stem nematodes. These included the use of plant root exudates (Williamson and Čepulytė, 2017), synthetic glucose (Warnock *et al*., 2016) and roots of plant seedlings (Čepulytė *et al*., 2018). The development of this protocol was conducted using *Ditylenchus gigas*, and each experiment was arranged following a completely randomised design and replicated three times.

4.4.3.1 The attractiveness of stem nematodes to roots of plants

In order to study the effect of isothiocyanates on the attraction of stem nematodes towards plant roots, the process of choosing suitable plants were conducted and examined. Field beans (*Vicia faba*), hairy vetch (*Vicia villosa*) and rocket (*Eruca sativa*) were selected based on their ability to host stem nematodes and/or their ability to produce suitable (slender) roots. Seedlings as prepared in Section 4.4.2 with primary roots approximately 1 cm in length were used.

Nematode suspension of approximately 300 *Ditylenchus gigas* per ml (Chapter 2) were gently mixed with 23% Pluronic gel (Section 4.4.1) and 3 ml was poured into the Petri dishes (60 x 5 mm) carefully to avoid the generation of bubbles. Immediately, the seedlings were place into the nematode-gel mix carefully and the set-up was allowed to set for 12 h before assessment. Assessments included the qualitative assessment of nematode movements to plant roots and the degree to which nematodes were visible.

4.4.3.2 Assessing the migration of stem nematodes to glucose and *Vicia faba* root diffusate

The attraction of stem nematodes to two test solutions: glucose and plant diffusate was conducted to determine their suitability in a bioassay. Test solution was prepared by dissolving glucose (Sigma Aldrich, UK) in distilled water to achieve a concentration range (1000mM, 100mM, 10mM, 1mM, 0mM) and a control (distilled water). These were prepared in 1.5 ml Eppendorf tubes sealed with Parafilm® sheet and stored at 4ºC before use. Plant root diffusate or "plant water" was obtained from three 4-day old field beans seedlings by placing them in a 25 ml falcon tube containing 1 ml of water and stored in the dark at 15ºC for 24 h after which the seedlings were removed. The "plant water" was passed through a 0.2 µm cellulose acetate membrane syringe filter to obtained pure plant diffusate suspension free of cells.

Slits (depth 0.1-0.2 mm) into the nematode-gel mix (As per Section 4.4.3.1) were made with Pasteur pipette when dispensing 2 µl of the respective glucose concentrations or plant diffusate. The set-up was allowed to set for 12 h before assessment. Assessments included the counts of migrated nematodes within 1 cm around the slits "central point" for the two test solutions.

4.4.3.3 Assessing nematode migration towards the embryos of *Vicia faba*

Sprouted seeds of field beans (Section 4.4.2) with a visible plumule and/or radicle were selected, before seed coats and cotyledons were gently separated from the embryos using dissecting needles and a scalpel. Stem nematodes (≈1000) were exposed to three concentrations (12.5, 25 and 50 µg/ml) of allyl, 2-phenylethyl and benzyl isothiocyanates (Sigma-Aldrich, UK) for 24 h. Two controls were included: nematodes heated at 60ºC for 1 h were dead and unheated nematodes in distilled water. After exposure to the treatments, stem nematodes were allowed to recover in distilled water for 24h. Finally, they were mixed with pluronic gel and 3 ml of the nematode-gel mix was poured into Petri dishes (60 x 5 mm) marked with a grid (Figures 4.1 and 4.2). The embryos of *V. faba* were introduced to the centre of the dish and kept at 23ºC for 24 h. Afterwards, the counts of nematodes within the 1.5 cm marked diameter (Figure 4.2) around the embryo for each treatment was recorded. The net number of migrated nematodes were calculated as

shown in Equation 4.1. For each treatment, the numbers of invaded stem nematodes within *V. faba* embryos were quantified using the acid fuchsin staining method which involves the use of a Waring blender as described by Hooper (1986).

$$Net\ migration = Nematode\ count\ (treatment) - \ Nematode\ counts\ (heated\ control)$$

Equation 4.1: The net number of nematodes obtained for each treatment after exposing *Ditylenchus gigas* to concentrations of isothiocyanates

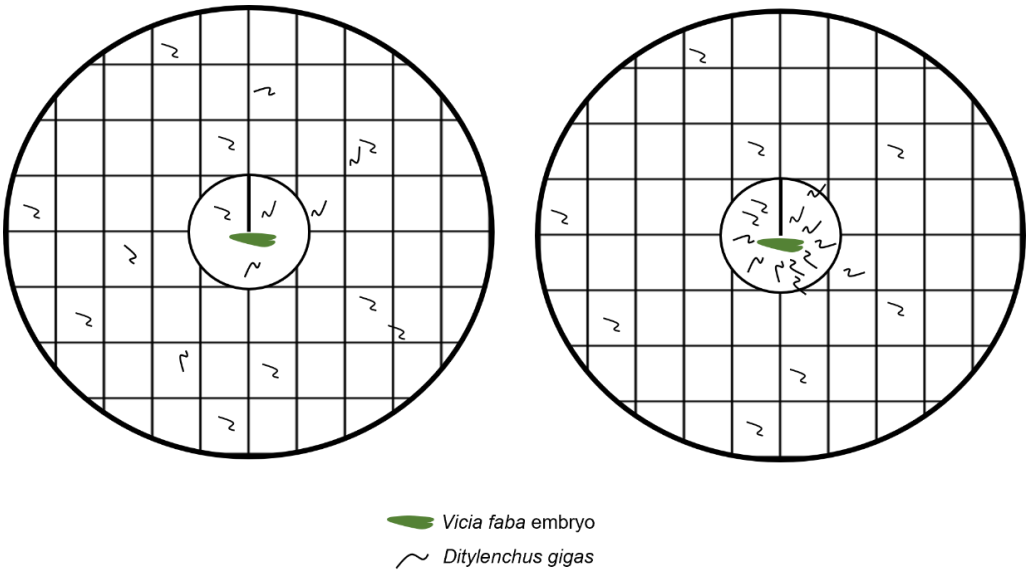

Figure 4.1. A schematic diagram showing the experimental set up for a plate at the start (a) and after 4 or 24 h (b) of the host finding bioassay

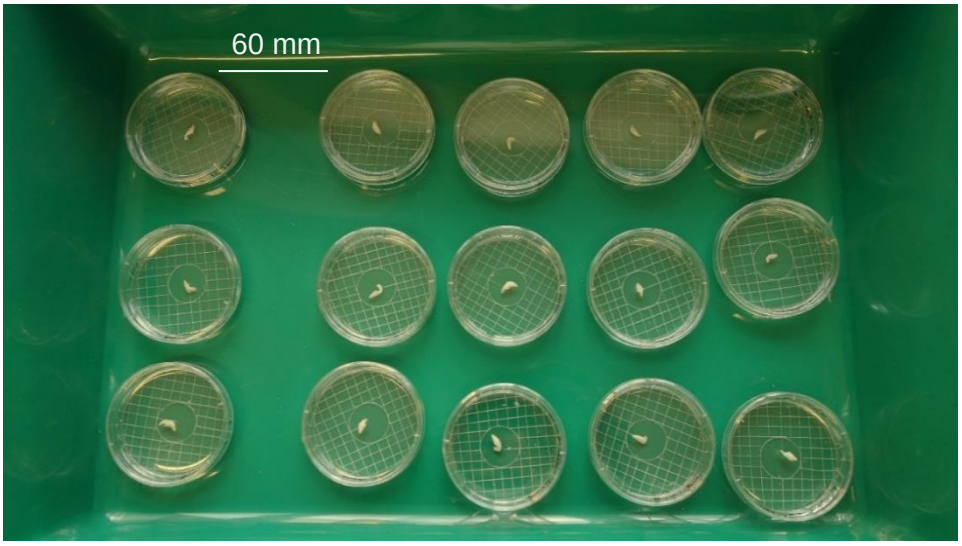

Figure 4.2. Experimental set-up used to assess the attraction of stem nematodes (*D. gigas*) to the embryos of Vicia faba in the centre of each experimental plate

4.4.3.4 Assessing nematode migration towards the roots of *Vicia faba*

Sprouted seeds of *Vicia faba* (Section 4.4.2) were placed in a 1 L beaker lined with tissue paper (70 x 100 m) as shown in Figure 4.3. Two-three weeks after germination, lateral roots tips (1-2 cm) were excised using a pair of scissors for use in a prototype bioassay. The set up described in Section 4.4.3.3 was used to determine the attraction of *D. gigas* to the roots of *V. faba*. Since the prototype allowed satisfactory visualisation of nematode attraction it was used as a basis of the experiments described in Section 4.4.4.

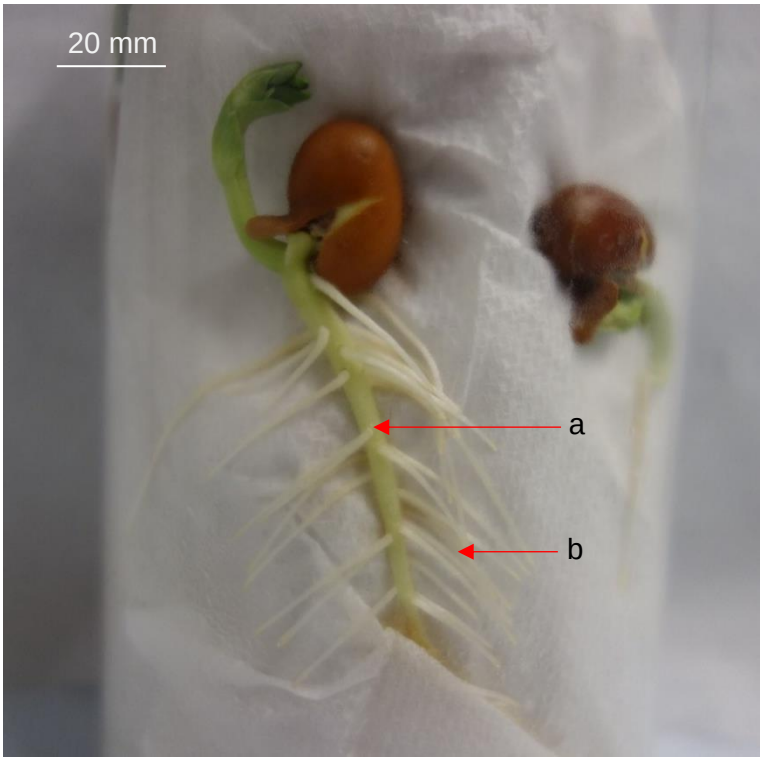

Figure 4.3. Germinating seedling of *Vicia faba* with a primary (a) and lateral roots(b)

4.4.4 The effect of isothiocyanates on the movement *Ditylenchus* spp. to the root of *Vicia faba* and their behavioural response

4.4.4.1 Experimental design

Based on the method in Section 4.4.3.4 involving the roots of *V. faba*, four experiments were conducted to determine the mobility of isothiocyanates pre-exposed nematodes to its host. The following nematode states/behavioural forms were also considered: straight (paralysis), coiled (affected) and sigmoid (normal active state).

The experiment was laid out in a completely randomised design. The design consisted of four isothiocyanates: sulforaphane, allyl, benzyl and 2-phenethyl at concentrations ranging from 3.125 to 100 µg/ml (Table 4.1) and two controls (heated and unheated nematode suspension). Treatment-concentration was replicated six times (Experiment 1 and Experiment 3) and four times (Experiment 2 and Experiment 4). All experiments were performed in 6-well culture plates (Sarstedt, Germany) with each well having 4.2 cm diameter. Stem nematode assessment were made using a Leica M80 stereomicroscope (Leica microsystems Milton Keynes, UK) at x40 magnification.

4.4.4.2 Bioassay procedure

Mixed life stages of *D. gigas* or *D. dipsaci* previously obtained from cultures (Chapter 2) were used in this assay. Each well of the 6-well plates received 2 ml of pluronic gel containing approximately 2000 nematode individuals which had either been pre-exposed to isothiocyanates concentrations for 24 h, heated at 60°C for 1 h or left untreated. One piece of *Vicia faba* root (length 1-2 cm) (as per Section 4.4.3.4) was placed in the centre of each well, and the gel was allowed to solidify at 23°C. The nematodes within ≈1cm diameter of roots were counted every 24 h over a 72 h period.

In addition, three body forms (Section 4.4.4.1) of stem nematodes in the same diameter were counted and converted to their percentage proportion.

4.4.4.3 Data analysis

For the host finding assays, a generalised linear model (GLM) with Poisson distribution was performed for the nematodes counts while a GLM with binomial distribution was utilised for assessing the proportion of nematode behaviours/body form under the influence of isothiocyanates-concentrations. A Tukey post-hoc analyses for multiple comparisons was performed with significant differences at ($P<0.05$) for treatments-concentrations and the controls (unheated and heated) using emmeans and compact letter display was generated using multcomp packages. All packages and analysis used were in R programming language (R Studio 4.0.2).

Table 4.1. The list of isothiocyanates and their respective concentrations, and the controls (heated at 60oC for 1 h and unheated) used for this study. Patterns corresponds to concentrations considered for experiments 1 and 3 (vertical), and experiments 2 and 4 (horizontal).

		Isothiocyanate concentrations µg/ml																						**Controls**	
		Allyl						**2-phethyl**						**Benzyl**						**Sulforaphane**						
		3.1	6.2	12.5	25	50	100	3.1	6.2	12.5	25	50	100	3.1	6.2	12.5	25	50	100	3.1	6.2	12.5	25	50	100	H	UH
Exp. 1	*D. gigas*																										
Exp. 2																											
Exp. 3	*D. dipsaci*																										
Exp. 4																											

4.5 Results

4.5.1 The development of host finding assay

4.5.1.1 The attractiveness of stem nematodes to roots of plants

The aim of the study was to identify the most suitable plants for a host finding assay. A plant was qualified if it attracted the nematodes and the degree to which nematodes were visible was high. The results show that twelve hours after exposure, stem nematodes were more attracted to the roots of *V. faba*, aggregating around the root tips (primary root) (Figure 4.4) than *V. villosa* (Figure 4.5) or *E. sativa* (Figure 4.6). Plant roots were most attractive to stem nematodes in this order: *V. faba* > *V. villosa* > *E. sativa*. Stem nematodes were most visible in the gel with roots of *E. sativa* followed by *V. faba* then *V. villosa*.

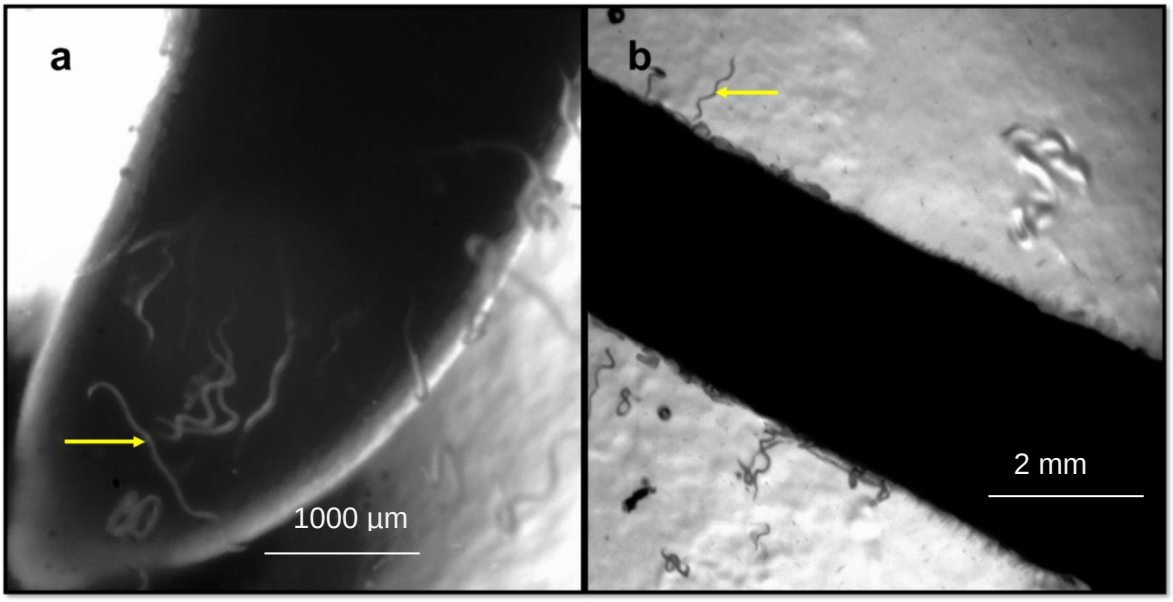

Figure 4.4. Response of stem nematodes *Ditylenchus gigas* (yellow arrow) to the roots of *Vicia faba* in a pluronic gel. Photographs were obtained 12 hours after set-up and taken at x100 (a) and x40 (b) magnification

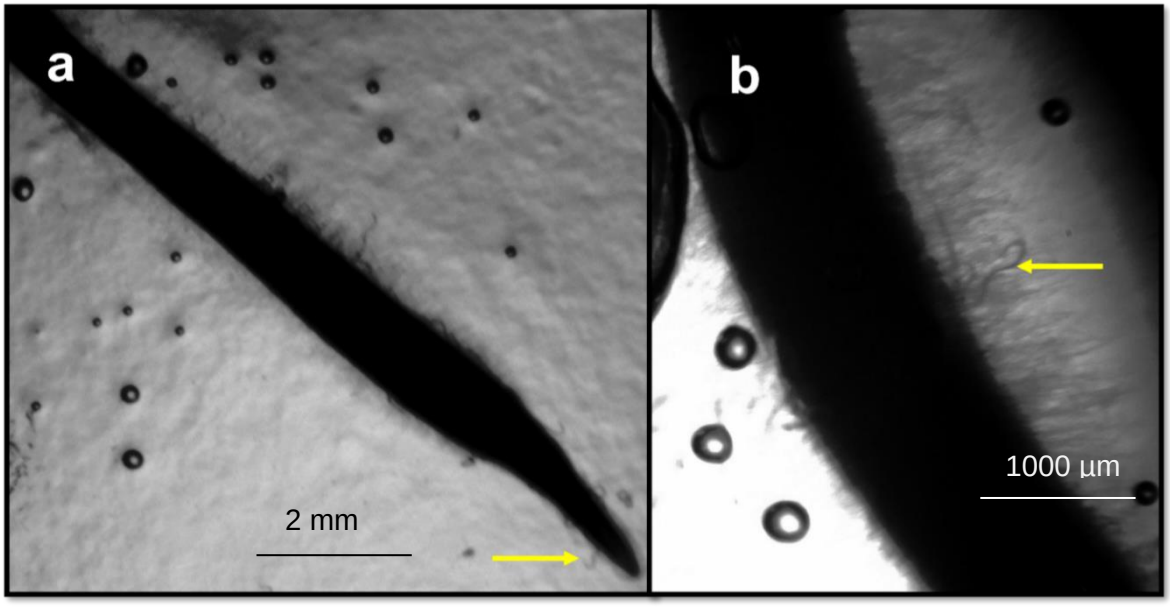

Figure 4.5. Response of stem nematode *Ditylenchus gigas* (yellow arrow) to the roots of *Vicia villosa* in a pluronic gel. Photographs were obtained 12 hours after set-up and taken at x40 (a) and x100 (b) magnification

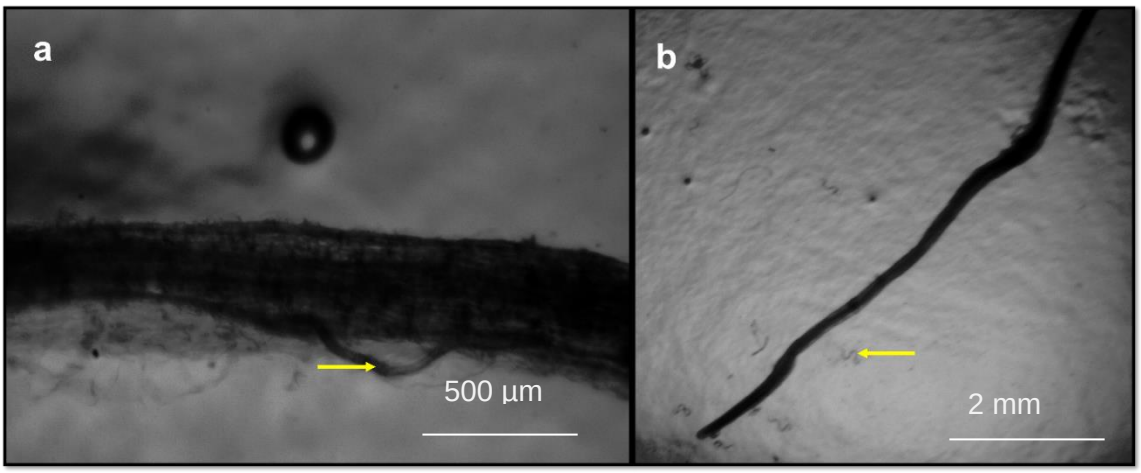

Figure 4.6. Response of stem nematodes *Ditylenchus gigas* (yellow arrow) to the root of *Eruca sativa* in a pluronic gel. Photographs were obtained after 12 hours after set-up and taken at x100 (a) and x40 magnification (b)

4.5.1.2 Migration of stem nematodes to glucose and *Vicia faba* root diffusate

The counts of stem nematodes within 1 cm around the slit "central point" containing either glucose or *Vicia faba* root diffusate were recorded. Nematode counts for slits containing glucose at all concentrations tested were not different from the counts of nematodes recorded for distilled water control (data not shown). Figure 4.7 shows the lack of response of stem nematodes to the glucose concentration at 1000 mM. Similarly, there was no stem nematode response to the root diffusates introduced in the nematode-pluronic gel set up when compared to nematode response to distilled water (data not shown).

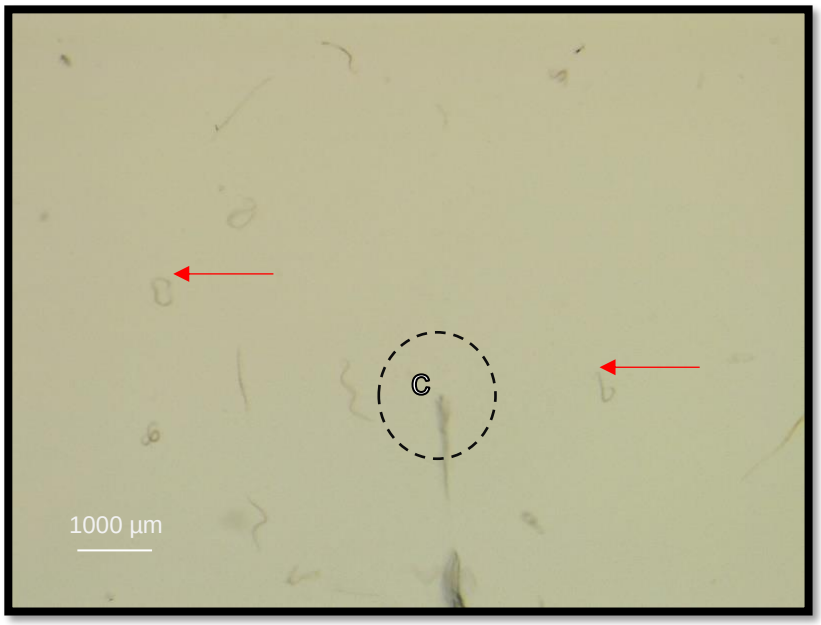

Figure 4.7. Lack of response of stem nematodes, *Ditylenchus gigas* (red arrow), to glucose concentrations (1000mM) dispensed at the slit "central point" (c) of the nematode-pluronic gel set up

4.5.1.3 The migration of stem nematodes towards *Vicia faba* embryos

There was a positive stem nematode response towards the embryos of *V. faba*. The result of the net counts of nematodes within the 1.5 cm marked Petri dish diameter is shown in Figure 4.8. There was no difference in the net number of counts obtained for the tested isothiocyanate concentrations with allyl, benzyl and 2-phenethyl at 50 µg/ml and the distilled water control. Moreover, at 12.5 µg/ml the net nematode count for benzyl and 2-phenethyl were five and ten times over the net counts in the distilled water, respectively. Stem nematodes were seen to have migrated into the radicle of *V. faba* embryo (Figure 4.9 a, b). Further assessment involving the blending of the embryo resulted in disintegrated nematodes (Figure 4.9 c). Nematode counts after blending could not be determined.

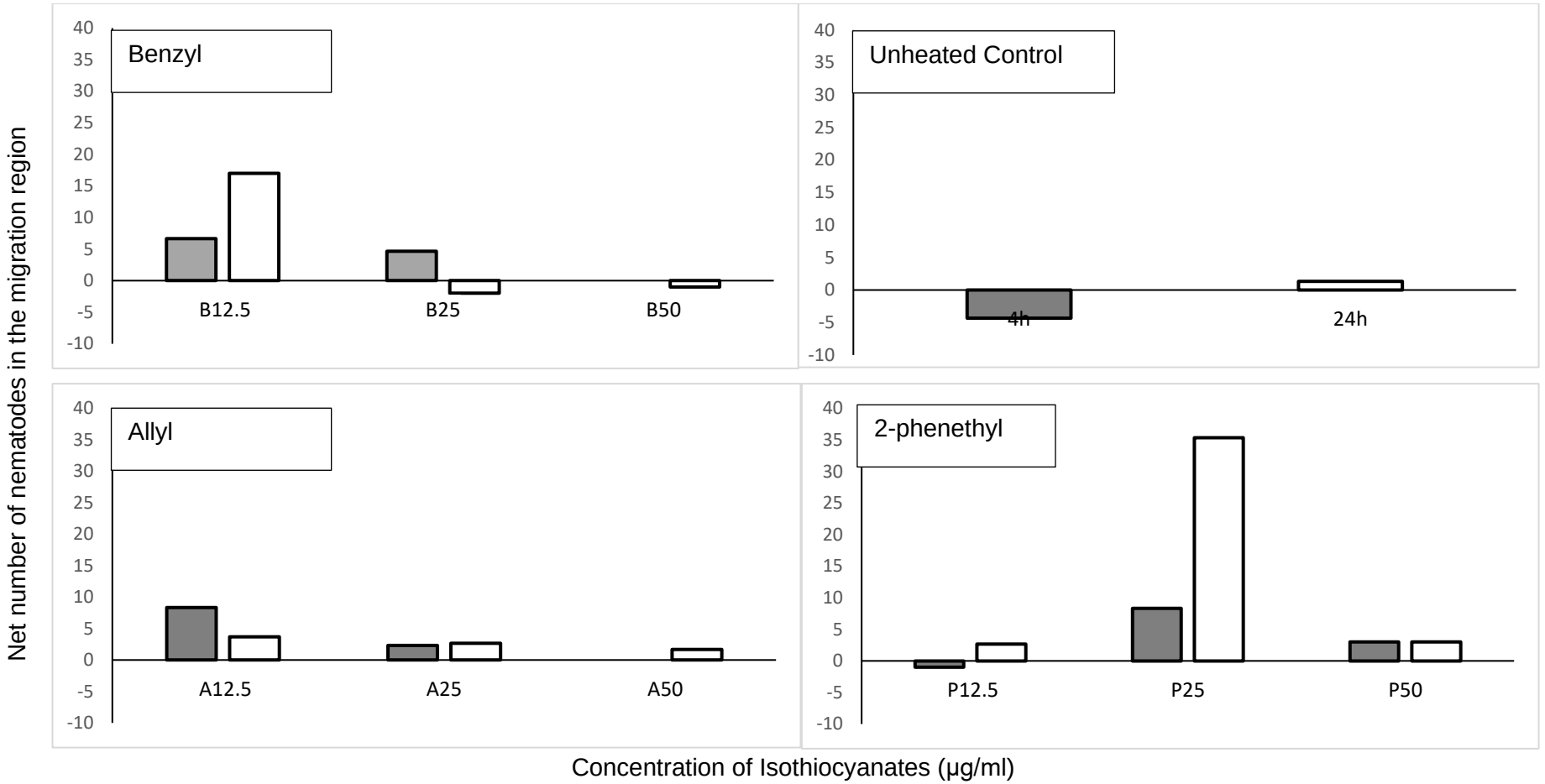

Figure 4.8. The net migration of stem nematodes (*Ditylenchus gigas*) towards the embryo of *Vicia faba* following pre-exposure to benzyl, allyl, 2-penethyl isothiocyanates at concentrations 12.5, 25, and 50 µg/ml or the water control

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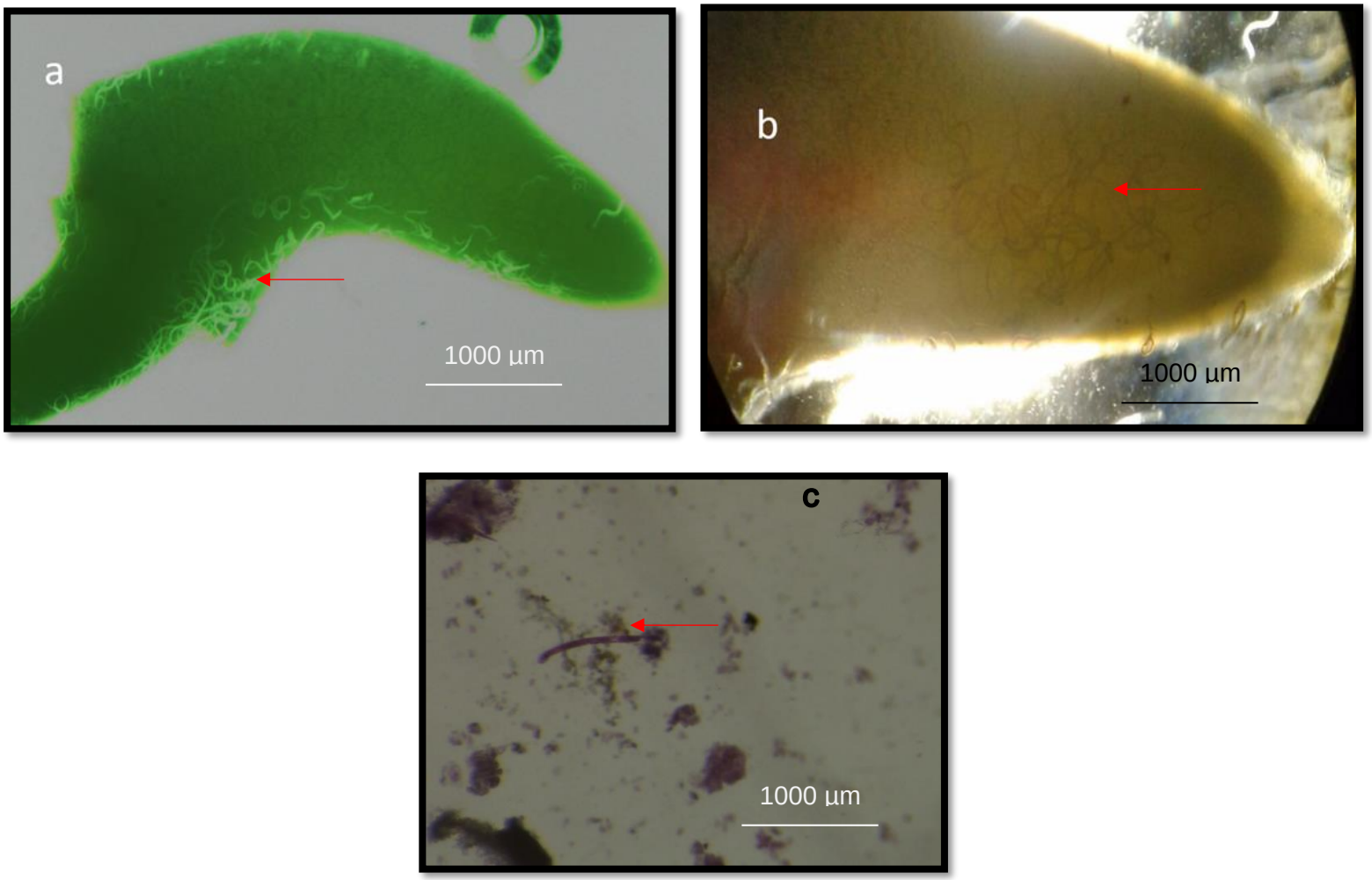

Figure 4.9. (a) Attraction of stem nematodes (*Ditylenchus gigas*) (red arrow) to the embryo of *Vicia faba*. (b) Stem nematodes successfully penetrated the embryo of *Vicia faba*. (c) Damaged stem nematodes recovered after maceration of the *Vicia faba* embryo using a Waring blender

4.5.1.4 The migration of stem nematodes to *Vicia faba* root

The stem nematode responded positively to the root of *V. faba* and the nematodes in the nematode-gel mix were visible (Figure 4.10). The movement of the nematodes could be monitored for 1-72 h after experimental set up and the nematode numbers migrating to within 1 mm of the root were counted. The result of the nematode count was approximately 250 after 24 h.

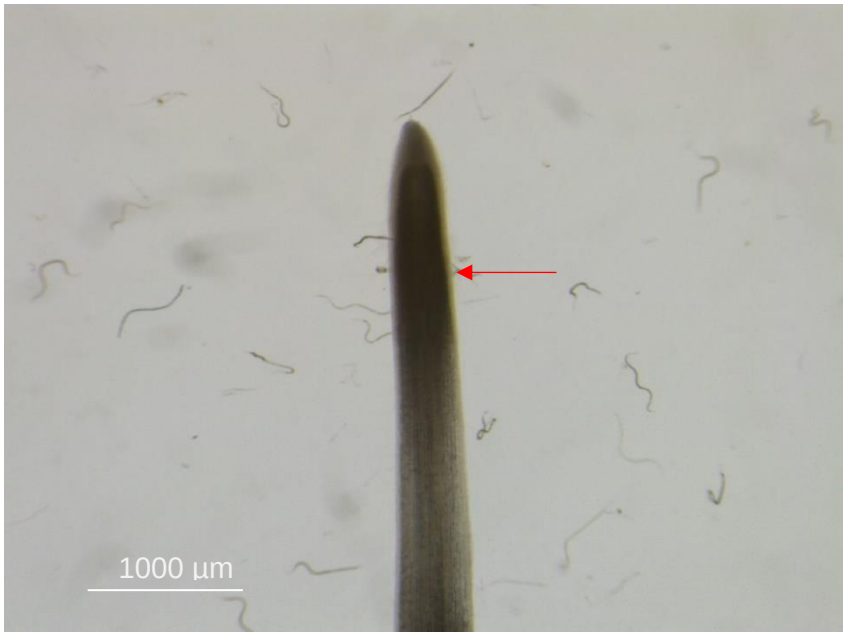

Figure 4.10. The attraction of stem nematodes *Ditylenchus gigas* (red arrow) to the root of *Vicia faba*

4.5.2 The effect of isothiocyanates on the movement *Ditylenchus* spp. to the root of *Vicia faba* and their behavioural response

4.5.2.1 The effect of isothiocyanates on the ability of *Ditylenchus* spp. to move towards the roots of *Vicia faba*

The two stem nematodes *Ditylenchus gigas* and *D. dipsaci* untreated with isothiocyanates had migrated to *V. faba* roots in all four experiments at 24 h after assay initiation. When nematodes reached the surface of *V*. *faba* roots, they were seen to aggregate near the root tip. With additional time, a greater number of nematodes responded to the root, particularly in the unheated control. There was a significant difference in the attraction between heat treated and unheated nematodes at all times checked 24-72h post-exposure ($P<0.05$). There was no

significant difference between the number of both stem nematode species which were pre-exposed to sulforaphane concentrations 3.1- 100 µg/ml and their respective unheated control

The effect of isothiocyanates on the movement of *Ditylenchus gigas*

The results of *Ditylenchus gigas* migration after exposure to isothiocyanates are shown in Figures 4.11 (Experiment 1) and 4.12 (Experiment 2). It was found that *D. gigas* moved to the roots of *Vicia faba* even though they had previously been exposed to isothiocyanates at low concentrations. The isothiocyanates (allyl, 2-phenethyl, and benzyl) had no effect on the migration of nematodes to the roots at concentrations of 3.1 and 6.25 g/ml and the numbers recorded were not statistically different from the unheated control ($P>0.05$) (Experiment 2; Figures 4.12). A slightly stronger ITC effect was seen in Experiment 1 for allyl and 2-phenethyl ITC at 6.25 µg/ml with reduced number of migrating stem nematodes, but the level of reductions were not similar to that recorded for the heated control ($P<0.05$). There was a decrease in the migration of nematodes when isothiocyanate concentrations increased. Despite the significant reduction of migrating stem nematodes following exposure to 2-phenethyl and benzyl ITC at 12.5 µg/ml, the numbers were still higher than in the heated control except for allyl ITC at the same concentration (Figure 4.11). Moreover, at concentrations ≥ 25 µg/ml for the isothiocyanates (allyl, 2-phenethyl and benzyl) the nematode numbers recorded within ≈1 cm diameter of the root were not statistically different from the heated control (Figures 4.11 and 4.12).

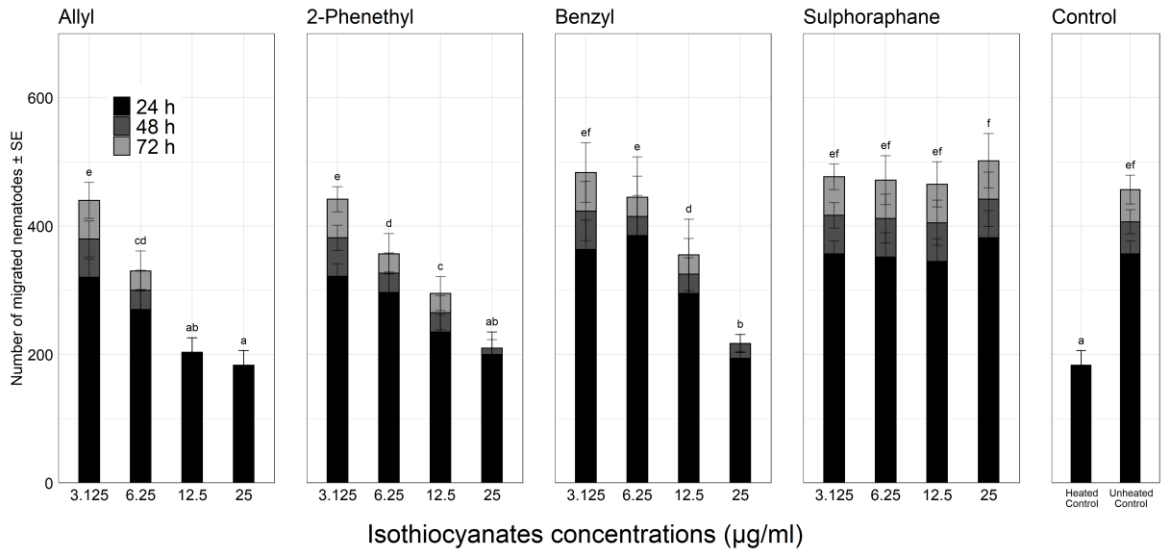

Figure 4.11. Experiment 1- Migration of *Ditylenchus gigas* through pluronic gel for 24-72 h after pre-exposure to a concentrations range 3.1 to 25 µg/ml of allyl, benzyl, 2-phethyl, sulforaphane and distilled water controls (heated and unheated). Error bars represent the standard error of the mean of six replicates. Bars followed by a different letter are significantly different after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

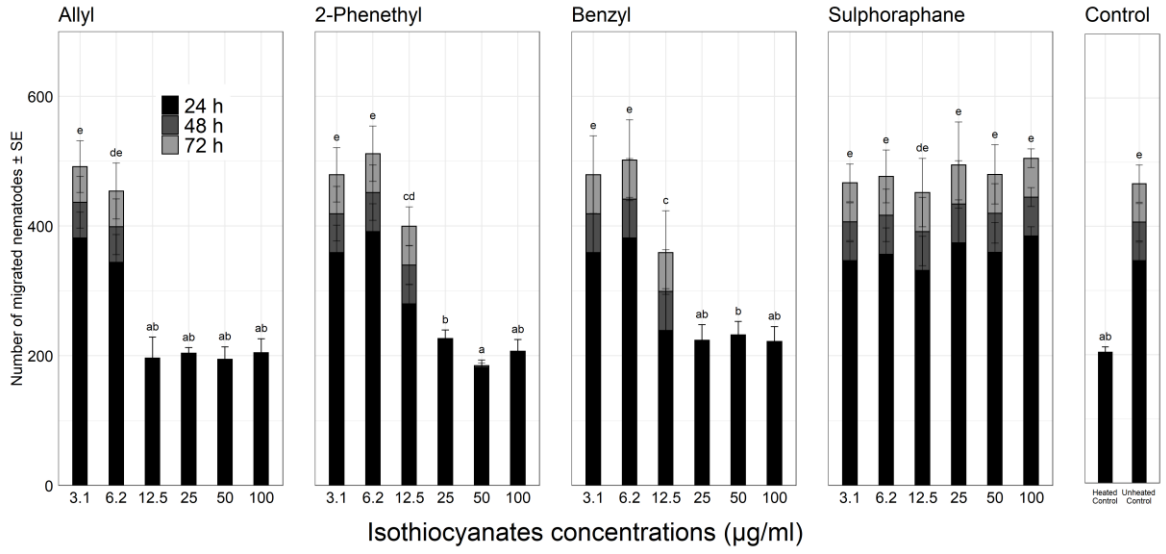

Figure 4.12. Experiment 2- Migration of *Ditylenchus gigas* through pluronic gel for 24-72 h after pre-exposure a concentrations range 3.1 to 100 µg/ml of allyl, benzyl, 2-phethyl, sulforaphane and distilled water controls (heated and unheated). Error bars represent the standard error of the mean of four replicates. Bars followed by a different letter are significantly different after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

The effect of isothiocyanates on the movement of Ditylenchus dipsaci

In Figures 4.13 (Experiment 3) and 4.14 (Experiment 4), the effects of isothiocyanates on *Ditylenchus dipsaci* migration are shown. Upon pre-exposure to low isothiocyanates concentrations, *D. dipsaci* could still move to the roots of *Vicia faba*. The isothiocyanates (allyl, 2-phenethyl, and benzyl) had no effect on the migration of nematodes to the roots at concentrations of 3.1 µg/ml and the numbers recorded were not statistically different from unheated control (P>0.05) (Figures 4.13 and 4.14). Despite increase in isothiocyanates concentrations to 6.2 and 12.5 µg/ml for benzyl ITC, and 6.2 µg/ml for 2-phenethyl ITC, the number of stem nematodes migrating to the roots were statistically similar (P>0.05) to the number in unheated control (Experiment 4; Figure 4.14). The attraction of *D. dipsaci* nematodes to the ≈1 cm diameter of *V. faba* root was significantly inhibited at 6.2 and 12.5 µg/ml for allyl ITC, 12.5 and 25 µg/ml for 2-phenetheyl ITC, and 25 µg/ml for benzyl ITC (Experiment 4; Figure 4.14); however, the number of stem nematodes were not statistically similar to the heated control. At concentrations ≥ 25 µg/ml for allyl ITC and ≥50 µg/ml for both 2-phenethyl and benzyl ITC, the nematodes were not seen to be attracted to *V. faba* root as they were statistically similar to the heated control (Figure 4.14).

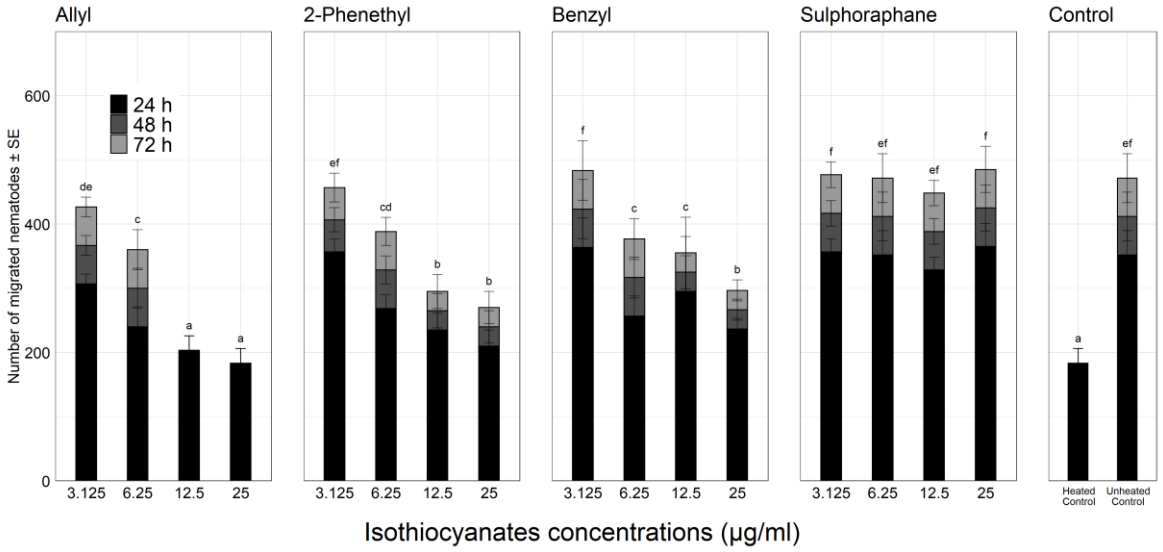

Figure 4.13. Experiment 3- Migration of individuals of *Ditylenchus dipsaci* through pluronic gel for 24-72 h after pre-exposure concentrations range 3.1 to 25 µg/ml of allyl, benzyl, 2-phethyl, sulforaphane and distilled water controls (heated and unheated). Error bars represent the standard error of the mean of six replicates. Bars followed by a different letter are significantly different after 72 hrs according to Tukey's Honest Significant Difference test (P < 0.05)

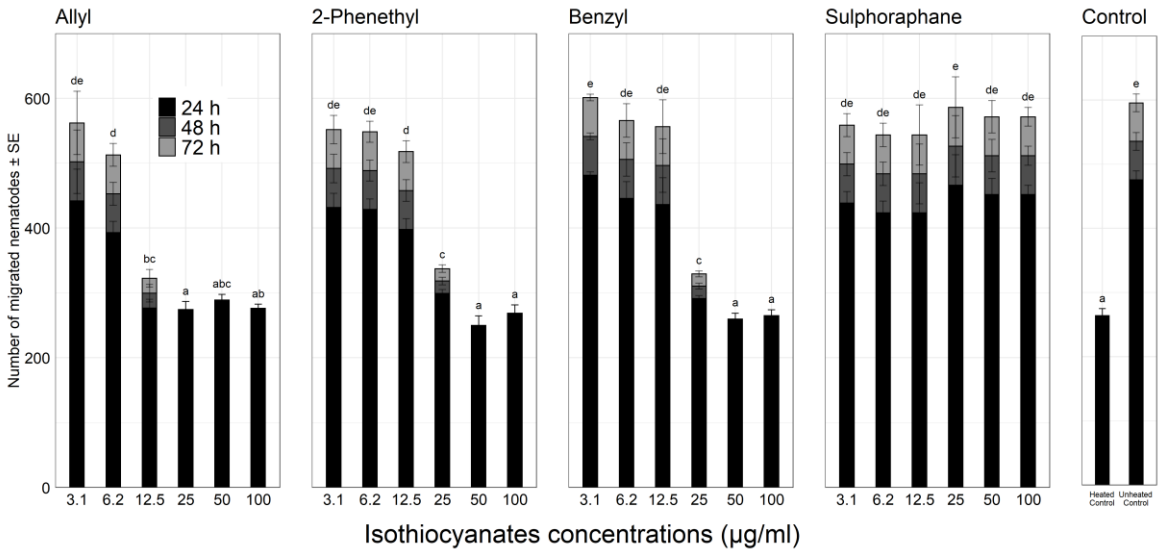

Figure 4.14. Experiment 4-Migration of *Ditylenchus dipsaci* through pluronic gel for 24-72 h after pre-exposure a concentrations range 3.1 to 100 µg/ml of allyl, benzyl, 2-phethyl, sulforaphane and distilled water controls (heated and unheated). Error bars represent the standard error of the mean of four replicates. Bars followed by a different letter are significantly different after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

4.5.2.2 The effect of isothiocyanates on the behaviour and form of *Ditylenchus* spp.

The two stem nematodes *Ditylenchus gigas* and *D. dipsaci* untreated with isothiocyanates were active in the presence of *V. faba* roots in all four experiments. There was a significant difference in the body form between heat treated and unheated nematodes at 72h post-exposure ($P<0.05$). There was no significant difference between the body form and behaviour of both stem nematode species which were pre-exposed to sulforaphane concentrations 3.1-100 µg/ml and their respective unheated control. Generally, the stem nematodes coiled more despite being unheated (controls) in Experiments (4.15) and (4.17) than they were in Experiments (4.16) and (4.18).

The effect of isothiocyanates on the behaviour of *Ditylenchus gigas*

The results of *Ditylenchus gigas* behaviour and body form after exposure to isothiocyanates are shown in Figures 4.15 (Experiment 1) and 4.16 (Experiment 2). The concentrations 3.1 and 6.25 µg/ml for allyl, benzyl and 2-phenethyl ITC failed to affect the body form of the nematodes as they had statistically similar ($P>0.05$) active nematodes as the unheated control (Figures 15 and 16). Moreover, at 12.5 µg/ml the similar active stem nematodes were

recorded for benzyl and 2-phenethyl ITC except allyl ITC which had a similar proportion of inactive nematodes to that of heated control (Experiment 1). Isothiocyanates concentrations greater than 25 µg/ml resulted in less active stem nematodes. However, the proportion of straight nematodes were significantly similar to the heated control for only 2-phenethyl ITC at 100 µg/ml, benzyl ITC at 50-100 µg/ml and allyl ITC at 25-100 µg/ml (Figure 4.16). Furthermore, coiling was mostly observed for the stem nematodes at 12.5 µg/ml isothiocyanates (ally, benzyl and 2-phenethyl) concentrations (Figure 4.16).

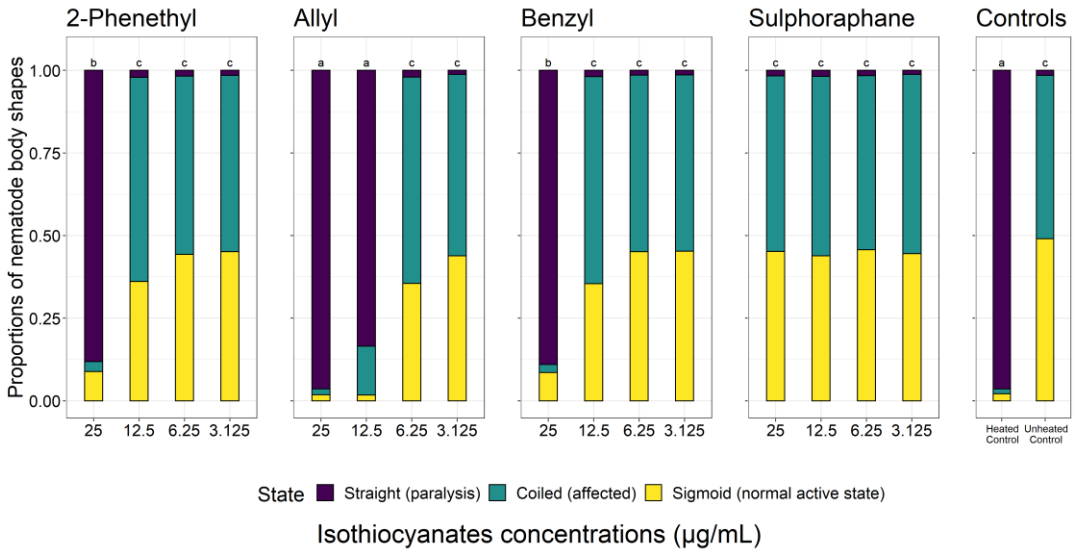

Figure 4.15. Experiment 1 - The proportion of *Ditylenchus gigas* individuals expressing different body forms in a pluronic gel assay following pre-exposure to allyl, benzyl, 2-phethyl, sulforaphane isothiocyanates at a range 3.1 to 25 µg/ml. The graph shows nematodes incubated in distilled water with and without heating at 60°C for 1 h (negative and positive controls respectively). Bars followed by a different letter are significantly different for the proportion of sigmoid nematodes after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

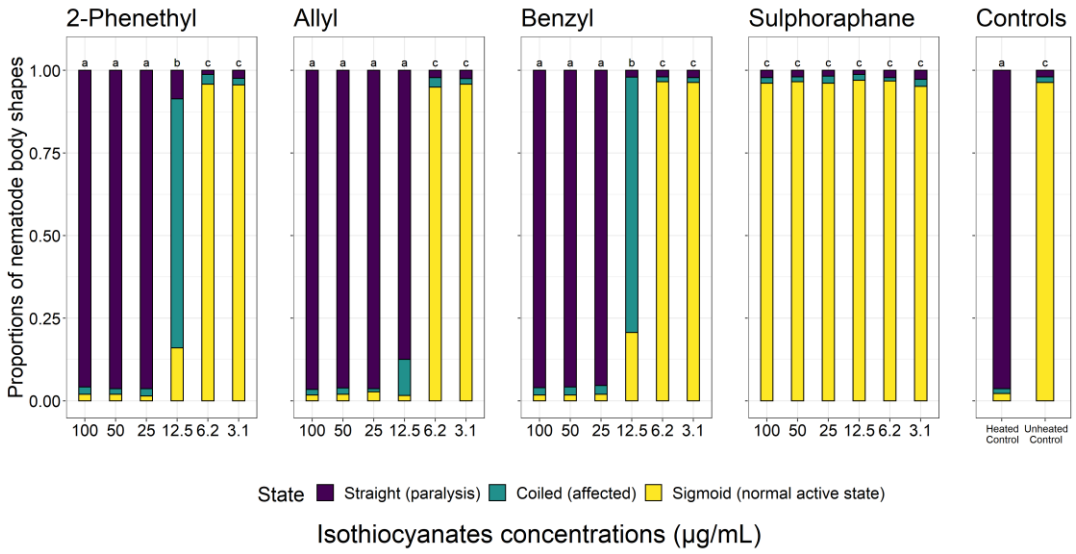

Figure 4.16. Experiment 2 - The proportion of *Ditylenchus gigas* individuals expressing different body forms in a pluronic gel assay following pre-exposure to allyl, benzyl, 2-phethyl, sulforaphane isothiocyanates at a range 3.1 to 25 µg/ml. The graph shows nematodes incubated in distilled water with and without heating at 60°C for 1 h (negative and positive controls respectively). Bars followed by a different letter are significantly different for the proportion of sigmoid nematodes after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

The effect of isothiocyanates on the behaviour of *Ditylenchus dipsaci*

The behaviour of the *D. dipsaci* under the influence of isothiocyanates were seen in Figures 4.17 and 4.18. The proportion of active nematodes were statistically similar ($P>0.05$) for all the isothiocyanates concentrations (3.1-25 µg/ml) and the unheated control except allyl ITC at 12.5 and 25 µg/ml (Experiment 3). Whereas pre-exposure to allyl ITC at 12.5 and 25 µg/ml resulted in greater than 50% straight nematodes and 10-15% active nematodes; this proportion in 25 µg/ml allyl ITC was statistically similar to the heated control. Between 70-95% of *D. dipsaci* individuals were largely straight in shape, 72 hours after pre-exposure (for 24 hours) to 50 and 100 µg/ml of allyl, benzyl, and 2-phenethyl (Figure 4.18) and they were statistically similar to the heated control.

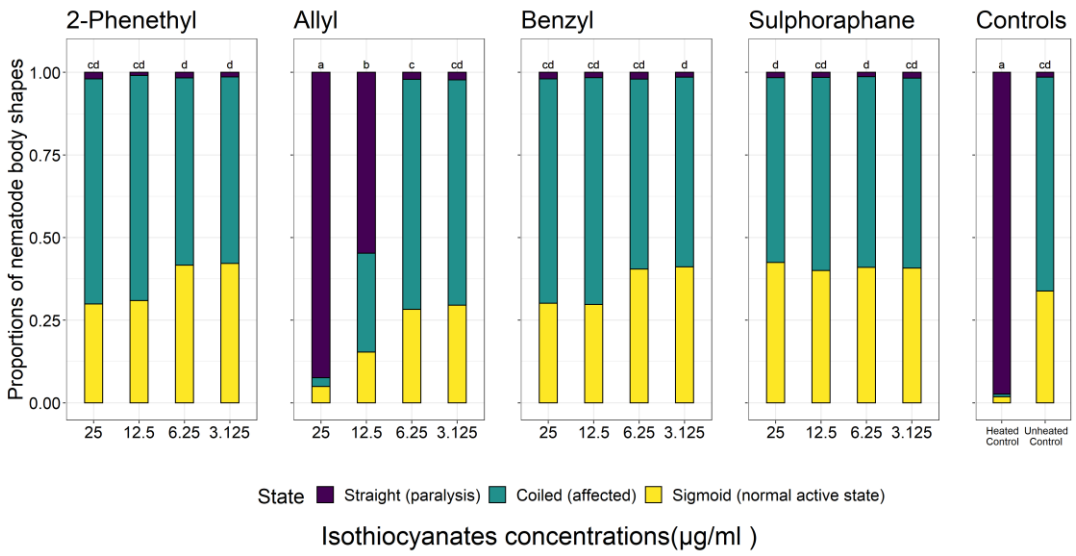

Figure 4.17. Experiment 3 - The proportion of *Ditylenchus dipsaci* individuals expressing different body forms in a pluronic gel assay following pre-exposure to allyl, benzyl, 2-phethyl, sulforaphane isothiocyanates at a range 3.1 to 25 µg/ml. The graph shows nematodes incubated in distilled water with and without heating at 60°C for 1 h (negative and positive controls respectively). Bars followed by a different letter are significantly different for the proportion of sigmoid nematodes after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

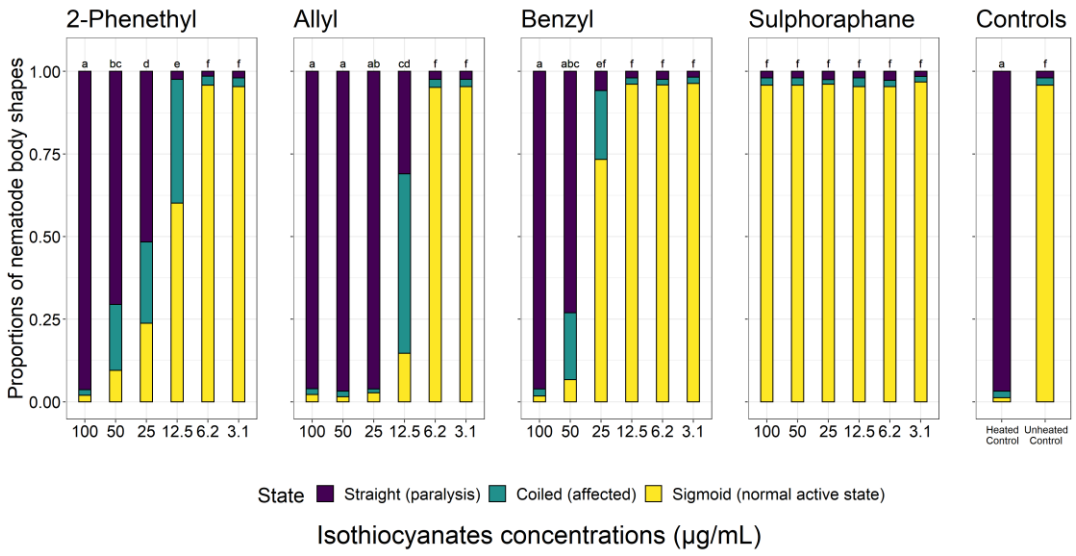

Figure 4.18. Experiment 4- The proportion of *Ditylenchus dipsaci* individuals expressing different body forms in a pluronic gel assay following pre-exposure to allyl, benzyl, 2-phethyl, sulforaphane isothiocyanates at a range 3.1 to 25 µg/ml. The graph shows nematodes incubated in distilled water with and without heating at 60°C for 1 h (negative and positive controls respectively). Bars followed by a different letter are significantly different for the proportion of sigmoid nematodes after 72 hrs according to Tukey's Honest Significant Difference test ($P < 0.05$)

4.6 Discussion

It is vital to the life cycle of nematodes that they move in response to cues from their host. Chemical and physical cues play a significant role in the orientation of plant parasitic nematodes towards their host plants (Perry, Roland N. and Aumann, 1998; Bais *et al.*, 2006; Rasmann *et al.*, 2012). Hence, inhibition of the chemosensory function of plant parasitic nematodes has long been considered a potential route to the control of these economically important parasites (Perry, 1996).

The first objective of this chapter was to develop suitable experimental methods for evaluating the effect of isothiocyanates on the motility and behaviour of stem nematodes under *in-vitro* conditions. As plant parasitic nematodes move through the rhizosphere, they appear to follow the gradients of secretions produced by their host plants, but elsewhere in soil they move randomly (Blake, 1962). However, the attraction of the stem nematodes *D. gigas* and *D. dipsaci* to field beans is largely unclear. A few investigations conducted on stem nematode attractants/preference include chemical and physical cues in their environment such as temperature (Wallace, 1961) and carbon dioxide (Klingler, 1963). Although *Ditylenchus* spp. can be attracted to carbon dioxide, this compound is also produced by decaying plant tissues, therefore other plant attractants are likely to have a role. This study examined glucose and the root diffusates of *Vicia faba* as attractants, but stem nematodes failed to show any attraction to the concentrations tested. In order to further understand the lifecycle of stem nematodes, more work is needed on the role of field bean root diffusates in the attraction of stem nematodes.
Stem nematodes showed positive response and attraction to the root and embryo of *V. faba* when assessed in the *in-vitro* assays. Of the three methods tested, with different forms of bean roots (primary root, embryo, and lateral roots), the one with cut-lateral roots was the best suited for the assay because the roots were translucent, allowing nematodes to be easily distinguished and counted. *Ditylenchus dipsaci* have been previously reported to be attracted to the roots of oats (*Avena sativa*), whereby they cluster around the root-stem joints of oat seedlings (Blake, 1962) before gaining access to the cortex. A similar attraction was seen in this study, but nematodes were found to cluster near the tip of the root.

There are a lack of studies investigating the impact of isothiocyanates on plant-parasitic nematodes, especially on the ability of nematodes to locate their hosts. This study is one of the first to investigate how sub-lethal levels of different isothiocyanates can influence nematode behaviour and adds relevant information about brassica based biofumigation. One critical step is screening various isothiocyanates for their effect against stem nematode

migration to their host. In this study, the isothiocyanate, sulforaphane failed to inhibit the host finding ability of *Ditylenchus gigas* or *D. dipsaci* at the concentrations tested. These stem nematodes moved to *V. faba* roots at similar rate as the unheated control. This further confirms the non-toxicity of sulforaphane on the two species as reported in the previous chapter (Chapter 3). Similarly, the movement of *D. gigas* and *D. dipsaci* to *V. faba* roots was not hindered at the lowest concentration (3.1 µg/ml) for benzyl, allyl and 2-phetheyl isothiocyanate concentrations. The lack of effect at this concentration may be attributed to the length of nematode exposure to ITC. In this study stem nematodes were exposed to ITC for 24 h whereas a previous study found that exposing root-knot nematode *Meloidogyne javanica* to allyl ITC less than 3 µg/ml for 72 h will immobilise the nematode juveniles (Wu *et al*., 2011).

Higher isothiocyanate concentrations caused inhibition/reduction in the number of stem nematodes that could find their host roots. There was lack of movement for *D. dipsaci* and *D. gigas* at 50 µg/ml and 100 µg/ml isothiocyanates (allyl, 2-phenethyl and benzyl) even when provided with their host for 72 h. The absence of mobility at these concentrations could be attributed to stem nematodes paralysis with 70-90% of their form being straight. This result is in accordance with the finding in Chapter 3 where mortality (lethal effects) was recorded for stem nematodes under the same concentrations of allyl, 2-phenethyl and benzyl ITC. The sub-lethal concentrations, 12.5 and 25 µg/ml of the isothiocyanates (allyl, 2-phenethyl and benzyl) suppressed the activity (behaviour) of the two species of stem nematodes and reduced their migration to their host. The report by Zasada *et al*. (2009) showed similar significant effect on the behaviour of *M. incognita* J2 at sub-lethal concentrations of benzyl ITC. It is also possible that excessive energy loss resulting from isothiocyanate-induced nematode heat shock proteins could result in nematode inactivity (Brolsma *et al*., 2014).

This study provides evidence that the isothiocyanates interfere with nematode function particularly during the early stages of parasitism. Even though isothiocyanates (allyl, benzyl and 2-phenethyl) at a concentration of 12.5 µg/ml showed lower nematicidal activity against *D. gigas* and *D. dipsaci* (surviving nematodes were active/ coiled – see Chapter 3), they were able to affect host finding behaviour and mobility. Thus, while not immediately killed, ITC treated stem nematodes may not be capable of feeding or further development. Such isothiocyanate-paralysed nematodes are likely to die due to starvation or become food for nematode predators.

It is likely that there would be an even greater effect of isothiocyanates if penetration and development of stem nematodes pre-exposed to sub-lethal concentrations at less than 12.5 µg/ml were assessed. In a previous study, benzyl isothiocyanate decreased *Heterodera*

glycine juveniles' reproduction on soybeans which had multiple effects on the nematode, affecting both infective juvenile movement and embryonic development, and decreasing reproduction (Wu, HaiYan *et al.*, 2014). There can be long-term effects on the offspring of nematodes that are not directly exposed to the compound, such as those recoded for *M. incognita* (Masler *et al.*, 2010). Furthermore, hatching of nematodes from eggs could be strongly inhibited, as seen for *H. glycines* eggs, which were exposed to allyl isothiocyanate (Yu *et al.*, 2005).

The fact that lower doses of certain ITC affect the behaviour of nematodes is important because concentrations of ITC in soil post biofumigation may not be sufficiently high enough to induce mortality. Gimsing *et al*. (2007) reported that soil-dependent half-lives of glucosinolate and isothiocyanates can be as short as 18 and less than 8 h respectively. In addition, loss of isothiocyanates in soil may be accelerated by other factors including sorption and volatilization. Isothiocyanates are hydrophobic and are adsorbed mainly by organic matter in soil. The toxicity of isothiocyanates in soils rich in organic matter was lower than in those with low organic matter (Brown and Morra, 1997; Matthiessen and Shackleton, 2005). Such factors reduce the availability of isothiocyanates in soil and limit the chance of nematodes being exposed to lethal concentrations.

This study provides reliable insights for practical issues associated with biofumigation experiments in glasshouses and fields. Since sub-lethal concentrations of isothiocyanates have significant effects on nematode behaviour, infectivity, and reproduction, how can biofumigants be applied to achieve consistent and predictable control of nematodes of crop plants? And how can partial-biofumigation be exploited for the control of plant-parasitic nematodes? Partial-biofumigation involves the release of glucosinolates (and eventually hydrolysed to ITC) into soil from the roots of growing brassica plants. Brassica species such as mustard *Brassica juncea*, oil radish *Raphanus sativus*, and rocket *Eruca sativa*, have been shown to suppress potato cyst nematodes *Globodera pallida* during their growing period (Ngala *et al*. 2015). Although the concentrations of ITC released in a partial-biofumigation system have not been quantified; it can be hypothesised that the ITC concentrations released are lower than the concentrations that cause mortality of plant-parasitic nematodes during full-biofumigation.

4.7 Conclusion

This experimental work has shown that allyl, 2-phenethyl and benzyl isothiocyanates affect motility and orientation of *Ditylenchus* spp. towards the roots of *Vicia faba* at sub-lethal concentrations of 12.5 and 25 µg/ml. Further work should be undertaken to examine the effect of ITC on nematode sensory organs such as amphids.

5.0 Host status of selected brassica plants for *Ditylenchus gigas* and *D. dipsaci*

5.1 Introduction

The genus *Ditylenchus* has been recorded on all continents where agricultural activities are performed (Sturhan and Brzeski, 1991). The species *D. gigas, D. dipsaci, D. destructor* and *D. angustus* are important plant parasites causing losses in a range of crops (Sturhan and Brzeski, 1991); host preference varies for each species, and some species share plant hosts (Vovlas, Nicola *et al.*, 2016). Plant parasitic *Ditylenchus* spp. typically infect and parasitise the aerial regions of their host plants, reproducing in large numbers in less than 21 days (Yuksel, 1960). Such nematode infestations inhibit photosynthesis, leading to stunted growth and yield loss. *Ditylenchus* spp. can cause quantitative and qualitative crop losses of up to 60% on field beans (*Vicia faba*) (Sturhan and Brzeski, 1991; Andres and Lopez-Fando, 1996) . The most important species in field bean production are the stem nematodes *D. gigas* and *D. dipsaci*. Symptoms associated with heavy infestations may include browning of the stems and pods with blistering that turns brown to black as the infection becomes more severe.

Managing *D. gigas* and *D. dipsaci* in infested fields is challenging, due to the absence of nematicides and because of the polyphagous feeding habit of these species, which limits crop rotation options. *Ditylenchus gigas* and *D. dipsaci* share hosts such as field beans, but *D. dipsaci* can infect a wider range of plant hosts including carrots, narcissus, garlic and 500 other plant species. The polyphagous pest *D. dipsaci* pose a huge threat in the UK (FERA, 2019). The two species have been designated as regulated non-quarantine pests by the European and Mediterranean Plant Protection Organization following the evaluation of the plant health regime (EPPO, 2019)

In recent years, biofumigation, the process of releasing volatile biocidal compounds into soil by incorporating brassica plants, is being recommended to suppress agricultural pests and diseases. However, there are concerns that growing brassica species for biofumigation could increase populations of plant parasitic nematodes, especially those species that are polyphagous (Viaene and Abawi, 1998; Edwards, 2014; Ntalli and Caboni, 2017). Several brassica plants, for instance, have been classified as hosts for root-knot nematodes (*Meloidogyne* spp.) (Edwards and Ploeg, 2014; Daneel *et al.*, 2018). Brassica plants that support plant-parasitic nematode populations are not suitable for biofumigation as this increases the population which may damage subsequent crops (Dutta *et al.*, 2019).

There is limited information regarding the ability of stem nematodes such as *D. dipsaci* and *D. gigas* to infect and reproduce on brassica plants. Erstwhile, several members of the Brassicaceae were identified as hosts for *D*. *dipsaci,* including *Brassica nigra* and *B. campestris* (Edwards and Taylor, 1963; Hesling, 1972). This indicates that more brassica species could act as hosts for stem nematodes.

5.2 Objectives

The aims of this study are to:

a) Determine the host suitability of selected brassica species for *D. dipsaci* and *D. gigas* infection.

b) Describe the symptoms related to *D. dipsaci* and *D. gigas* infection on brassica plants

c) Assess the lifecycle of *D. dipsaci* and *D. gigas* on selected brassica species.

5.3 Null hypothesis

Brassica plants will fail to support the reproduction of stem nematodes *Ditylenchus dipsaci* and *D. gigas*

5.4 Materials and Methods

5.4.1 Nematode Inoculation

The two stem nematodes were cultured as described in Section 2.2 Chapter 2 and extracted from plant tissues using the Whitehead tray method as described in Section 2.3.1 Chapter 2. Both nematode cultures yielded 50-98% fourth stage juveniles (J4). The resulting nematode suspensions were stored in a 50-ml centrifuge tube at 4°C before inoculation. Prior to plant inoculation, the nematodes were either mixed with 1% carboxymethyl cellulose (CMC) suspension (Experiment 2) or not (Experiments 1 and 3).

For Experiment 1, the soil around each seed were inoculated with ca. 500 individuals of *D. gigas*. While Experiments 2 and 3, the experimental plants were inoculated two weeks after sowing with ca. 500 individuals of *D. gigas* or *D. dipsaci* (Experiments 2) and 100 individuals of *D. dipsaci* (Experiment 3). The controls received nematode free suspension (water or 1% CMC accordingly).

5.4.2 Test plants used for host status assessment

The experiments in this chapter used the plants listed in Table 5.1. Experiments were conducted September – November 2018 (Experiment 1); February – April 2019 (Experiment 2); October – November 2019 (Experiment 3).

Table 5.1. Legume and brassica plants investigated for their host status to *Ditylenchus dipsaci* and *D. gigas* in three separate experiments

Treatment	Variety	Experiment 1	Experiment 2	Experiment 3
Vicia faba	Feugo	*Tested*	*Tested*	*Tested*
Brassica juncea	Brons	*Tested*	*Tested*	*Tested*
Eruca sativa	Trio	*Tested*	*Tested*	*Tested*
Rhaphanus sativus	Terranova	*Tested*	*Tested*	*Tested*
Brassica carinata	Cappucchino	*Tested*	*Tested*	*Tested*
Sinapis alba	Architect	*Tested*	*Tested*	*Tested*
Vicia villosa	Villana	*Tested*	*Tested*	*Not tested*

5.4.2.1 Experiment 1: Reproductive performance of *Ditylenchus gigas* on brassica plants

This experiment was setup to investigate the host status of brassica plants (Table 5.1) for the stem nematode *Ditylenchus gigas*. Using a randomised complete block design, seven blocks were blocked along a gradient from the source of ventilation in the glasshouse. This experiment only considered the reproductive performance of this nematode on the brassica plants. Experimental plants were grown in a mixture of John Innes No. 2 sterilised loam-based compost and horticultural sand at a ratio of 3:1 in 25 cm diameter pots (n = 49). Seven seeds of all plant types were sown into their respective pots to depth of ≈1.5 cm and immediately inoculated with 500 individuals of *D. gigas* using a pipette. Each pot was covered with a perforated polythene bag for a week (Figure 5.1), to create a humid microclimate to facilitate nematode infection of the plants (Griffin, 1987). Ten weeks after sowing, each plant (seven plants per pot) was cut from its base to a height of 10 cm to give in the region of 10-15 g of plant material. These cut-stems were then cut into 1-2 cm lengths and placed in modified Whitehead tray (Whitehead and Hemming, 1965) to extract the nematodes without blending plant parts in a waring blender (EPPO, 2013). The set-up was left for 24 h before the resultant suspension was passed through a 60 µm sieve. The number of stem nematodes recovered per treatment were counted under a Leica MC170 HD microscope at a

magnification of 40X. The number of stem nematodes in the soils were also evaluated. The nematodes were extracted using a two-flask method described in Section 2.3.2 Chapter 2.

Figure 5.1. Pots (25 cm diameter) covered with a perforated polythene bag in order to generate a humid climate for stem nematode infection

5.4.2.2 Experiment 2: Reproductive performance of *Ditylenchus dipsaci* and *D gigas* on brassica plants

The experiment was laid out in a completely randomised design. The design considered sources of variation in the glasshouse, including ventilation source, bay entrance, light and other growing plants in the bay of the glasshouse. The experimental design had seven types of test plants as the main factor and three sub-factors (the two nematode species and a non-inoculated negative control). Each treatment was replicated three times for each combination of plant and nematode species (21 treatments). Experimental plants were grown in a mixture of John Innes No. 2 sterilised loam-based compost and horticultural sand at a ratio of 2:1 and placed into 20 cm diameter pots (n = 63). Five seeds of all plant types (Table 5.1) were sown into their respective pots. Two weeks post-sowing, plants were thinned to three plants per pot. Following that, the respective inoculum (500 individuals of stem nematodes or nematode free water) was applied to the plants in similar procedure highlighted in Figure 5.2.

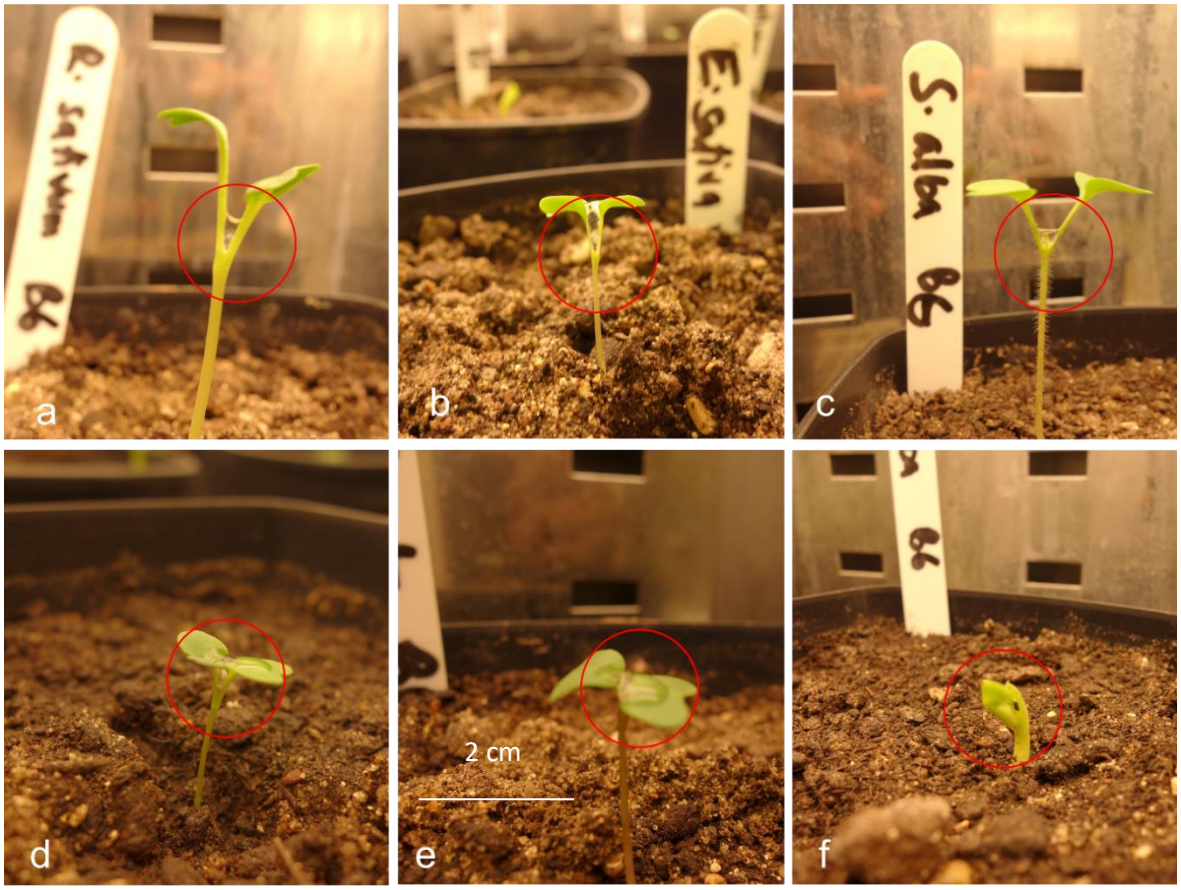

Figure 5.2. Seedlings of brassica plants (a) *Raphanus sativus* (b) *Eruca sativa* (c) *Sinapis alba* (d) *Brassica juncea* (e) *Brassica carinata* and a legume (f) *Vicia faba* inoculated with *Ditylenchus dipsaci*. The red circles indicate the point of inoculation on the plants.

The experiment was conducted during a 16-hour photoperiod with day-time and night-time temperatures of 15-20 and 10-5°C, respectively. The moisture content was maintained at field capacity by filling saucers with water. At four weeks after sowing, N-K Sulphur at a rate of 185 kg/ha was applied to the soil and watered immediately. Plants were free of insect infestation until a week to terminating the experiment when black bean aphids (*Aphis fabae*) were seen on *Vicia faba*. No insecticide spray was applied.

The experiment was terminated ten weeks post-sowing and the following data were collected: plant fresh weight (shoot and root), number of tillers, plant height, number of un-senesced and senesced leaves on each plant. Plant samples were separated into root and shoot parts before they were cut into ca. 2-3 cm pieces and divided into two sub-samples. One part for molecular nematode assessment (Chapter 7) and the second part was used for a traditional nematode count.

The sub-samples for traditional nematode assessment were extracted using a modified Whitehead tray method (Whitehead and Hemming, 1965) with prior blending of plant parts in

a Waring blender for 10 seconds. The set-up was left for 24 h after which the suspension was washed over 60 µm sieve. The number of stem nematodes recovered per treatment were counted under a Leica MC170 HD microscope at a magnification of 40X.

5.4.2.3 Experiment 3: Reproductive performance of *Ditylenchus dipsaci* on brassica plants

A third experiment focused on the reproductive performance of *Ditylenchus dipsaci* on brassica plants. The experiment was undertaken in a controlled environment cabinet (Fitotron® Modular Plant Growth Chambers, Type HGC) with eight blocks that were organised in a randomised complete block design. Experimental plants were grown in a mixture of John Innes No. 2 sterilised loam-based compost and horticultural sand at a ratio of 2:1 and placed into 12 cm pots (n = 48). Two seeds of each plant type (Table 5.1) were sown into their respective pots. Two weeks post-sowing, plants were thinned to one plant per pot. Following that, a suspension containing 200 mixed life stages of *D. dipsaci* was applied to the aerial region of the young seedling using a pipette.

The experimental area had a 16 h photoperiod with temperatures of 18 and 10°C day and night, respectively. Water and nutrients were administered as described for Experiment 2. The experiment was terminated ten weeks post-sowing. Plants were blended before extraction as described previously. The suspension obtained from the nematodes were frozen at -80°C before freeze drying to proceed with the molecular assessment, as detailed in Chapter 7.

5.5 Data analysis

The analysis of the data obtained in this study depended on their nature/distribution. For Experiment 2, a generalised linear regression analysis was used with a negative binomial distribution recommended for count data with overdispersion (function: glmer.nb, library lme4; Bates *et al*. 2015). The plant measurements in Experiment 2 were analysed using ANOVA. In the case of Experiment 3, the total counts of nematodes were transformed to Log10 before performing an ANOVA test. In all cases of analysis conducted, assessing whether means across treatments were significantly different ($P<0.05$), a post hoc comparison of means by computing estimated marginal means (function: emmeans, library: emmeans; Lenth 2021) was performed and compact letter display was generated using multcomp packages in R programming language (R Studio 4.0.2).

5.6 Results

5.6.1 Experiment 1

5.6.1.1 Number of stem nematodes recovered from the shoot of brassica plants and soils

Figure 5.3 shows the number of stem nematodes *Ditylenchus gigas* that were recovered from brassica plants and legumes from the experiment. The shoots of brassica and legume species except *Vicia faba* did not contain any *D. gigas*. The soils had low numbers for all the test species as well. There was a maximum recovery of 11% of the initial number from the soil of *Brassica juncea*, and only 1% of the initial number for *Sinapis alba*. *Vicia faba* showed the greatest number of nematodes in the soil and on the shoots, with 22% of the number initially introduced.

Figure 5.3. The mean number of nematodes obtained for the shoot and soil of two leguminous and five brassica species 10 weeks after introducing stem nematodes *Ditylenchus gigas*.

5.6.2 Experiment 2

5.6.2.1 Number of stem nematodes recovered from the shoot of brassica plants

In this experiment, a low number of nematodes were recovered after terminating the experiment. The number of nematodes recovered from plant tissue at 10 weeks after planting (WAP) was just 10% of the initial number introduced to the plants at 2 WAP for all plant and nematode species combinations.

Figure 5.4 displays the number of stem nematodes recovered from each treatment 10 WAP. *Ditylenchus gigas* was found in far greater numbers in the tissues of *V. faba*, compared to *V. villosa* and the brassica species in the experiment. The number of *D. gigas* recovered from *V. faba* was 100-fold higher than other plant species and were statistically different ($P < 0.05$). As a comparison, the number of *D. dipsaci* isolated from the different plants was either similar to or different from the amount isolated from *V. faba*. The result shows the number of *D. dipsaci* recovered from *V. faba* were significantly greater than numbers from *Brassica carinata*, *B. juncea, Raphanus sativus* and *Sinapis alba* ($P < 0.05$). In contrast, there were no differences between the number of *D. dipsaci* found in *Eruca sativa* and *V. villosa* when compared to *V. faba*.

In general, there were differences between the infection of *V. faba* and *V. villosa* by the two nematode species. Higher numbers of *D. gigas* compared to *D. dipsaci* were found on *V. faba* ($P<0.05$), while *V. villosa* supported a greater number of *D. dipsaci* over *D. gigas* ($P<0.05$). In this experiment, infection of brassica species by *D. gigas* and *D. dipsaci* was statistically similar. Hence both stem nematode species showed similar ability to infect each brassica species tested.

5.6.2.2 The developmental stages of *Ditylenchus gigas* and *D. dipsaci* recovered from brassica plants

In order to assess the development of stem nematodes introduced to the experimental plants, the development stages of nematodes recovered were categorised as adults (male and female) or juvenile stages (mostly J4) (Table 5.2). Generally, the proportion of juveniles of both stem nematodes was greater than that of adults for all plant species. There was an absence of adults of both *D. dipsaci* and *D. gigas* in *B. carinata* and *B. juncea*. In addition, no adult stages were seen for *D. dipsaci* and *D. gigas* on *Eruca sativa* and *Raphanus sativus* respectively.

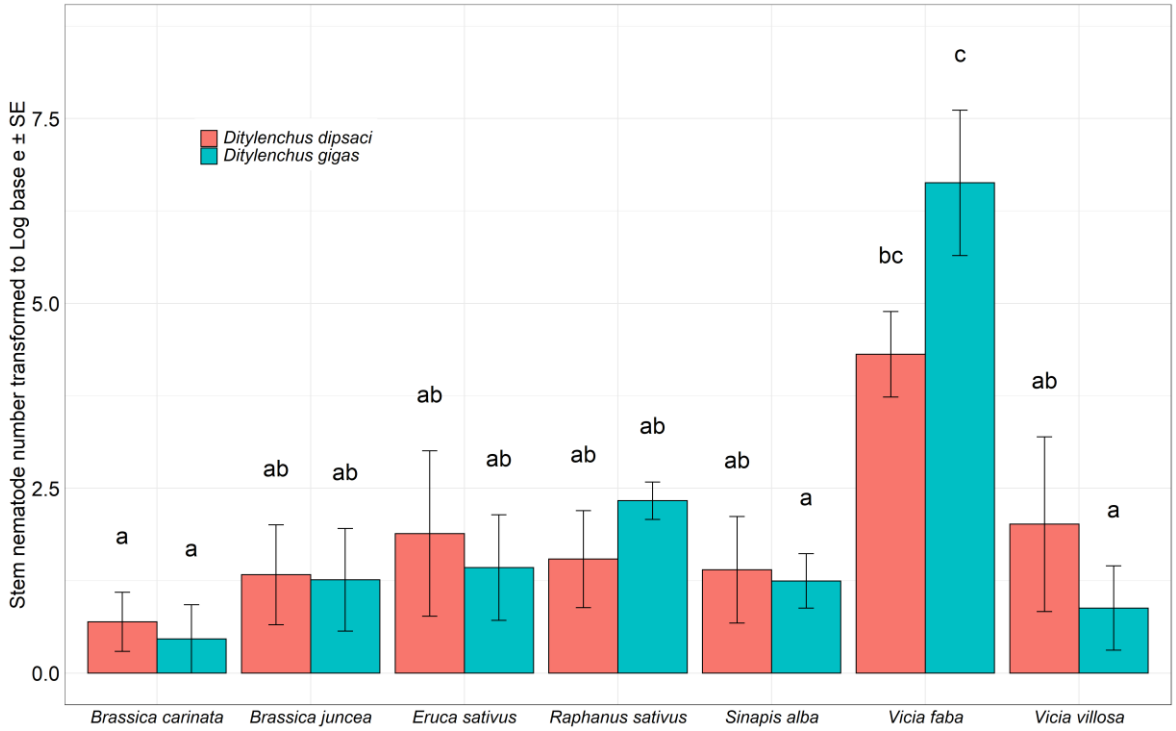

Figure 5.4. The log number (base e) of stem nematodes obtained 10 weeks after introducing stem nematodes *Ditylenchus dipsaci* and *D. gigas* to two leguminous plants and five brassica plants. Error bars show the standard error of the mean. Bars followed by a different letter are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$)

Table 5.2. The presence (✓) and absence (×) of adults and juveniles of *Ditylenchus gigas* and *D. dipsaci* recovered from leguminous and brassica plants 10 weeks post inoculation

Plant group	Treatment plant	*Ditylenchus gigas*		*Ditylenchus dipsaci*	
		Adult	Juvenile	Adult	Juvenile
Legumes	*Vicia faba*	✓	✓	✓	✓
	Vicia villosa	✓	✓	✓	✓
Brassicas	*Sinapis alba*	✓	✓	✓	✓
	Eruca sativa	✓	✓	×	✓
	Raphanus sativus	×	✓	✓	✓
	Brassica juncea	×	✓	×	✓
	Brassica carinata	×	✓	×	✓

5.6.2.3 The growth measurements of brassica plants inoculated with *Ditylenchus gigas* and *D. dipsaci*

Each plant was cut off at the soil surface and the growth parameters of above and below ground biomass was recorded. There was no influence of the stem nematodes on the growth parameter tested. The two stem nematodes failed to reduce the biomass (shoot and root) of the brassica and legumes varieties tested when compared to the corresponding non-inoculated controls (P>0.05) (Table 5.3). Similarly, no reduction in the height of the brassica and legume plants was observed except for the *V. faba* plants that received *D. dipsaci* which appeared to have promoted the plant height by ≈12 cm. The number of un-senesced leaves were greater for plants exposed to the two stem nematodes *D. dipsaci* and *D. gigas* than the non-inoculated control.

Symptoms of stem nematode infection with sweeling and distortion of stems was observed on the leguminous plants *V. faba* and *V. villosa* (Figure 5.5).

Figure 5.5. Suspected symptom of *Ditylenchus dipsaci* infection on *Vicia villosa* with swelling and distortion of stem

Table 5.3. Plant growth measurements (treatment means) obtained after inoculation with stem nematodes *Ditylenchus gigas* and *D. dipsaci*, 10 weeks after planting. Mean data ± standard error. Means with the same letter are not significantly different from each other according to Tukey's Honest Significant Difference test ($P < 0.05$)

Plant Species	Treatment	Tillers	Un-senesced leaves	Senesced leaves	Plant height (cm)	Shoot fresh weight (g)	Root fresh weight (g)
V. faba	*D. gigas*	1.67 ± 0.88	32.22 ± 2.61	1.67 ± 1.67	32.78 ± 2.55[a]	71.60 ± 9.41	56.82 ± 9.81
	D. dipsaci	0.67 ± 0.67	37.67 ± 5.10	2.33 ± 2.33	45.91 ± 0.87[b]	90.69 ± 7.85	75.71 ± 16.1
	Control	1.83 ± 0.93	29.33 ± 1.50	1.33 ± 1.33	33.57 ± 2.35[a]	70.99 ± 5.89	48.58 ± 6.64
V. villosa	*D. gigas*	4.72 ± 0.54	>100	0.00	118.61 ± 4.94	48.05 ± 3.52	29.74 ± 2.14
	D. dipsaci	5.67 ± 0.19	>100	0.00	118.44 ± 12.22	51.53 ± 3.82	33.25 ± 5.05
	Control	4.56 ± 0.68	>100	0.00	102.33 ± 5.81	47.64 ± 4.45	31.01 ± 4.01
B. juncea	*D. gigas*	0.00	4.11 ± 0.11	10.00 ± 0.00	3.14 ± 0.59	69.50 ± 16.89	7.43 ± 1.27
	D. dipsaci	0.00	5.00 ± 0.67	10.00 ± 1.15	2.97 ± 0.56	64.61 ± 18.10	7.66 ± 2.67
	Control	0.00	4.67 ± 0.38	5.67 ± 1.33	2.94 ± 0.49	82.52 ± 9.42	10.37 ± 2.17
B. carinata	*D. gigas*	0.00	7.89 ± 1.16	12.67 ± 1.45	21.33 ± 1.59	73.94 ± 13.59	4.20 ± 0.42
	D. dipsaci	0.00	9.11 ± 1.68	7.33 ± 3.71	18.42 ± 5.18	72.42 ± 4.06	3.99 ± 0.52
	Control	0.00	9.89 ± 0.58	8.33 ± 1.67	16.44 ± 1.67	90.64 ± 15.94	6.19 ± 0.60
S. alba	*D. gigas*	1.33 ± 0.67	12.22 ± 1.49[b]	5.33 ± 2.73	24.84 ± 2.59	58.64 ± 20.58	9.73 ± 6.29
	D. dipsaci	0.00	10.67 ± 0.38[ab]	12.00 ± 1.00	27.86 ± 0.55	70.36 ± 9.22	6.05 ± 0.61
	Control	0.00	7.78 ± 0.77[a]	9.33 ± 1.20	21.61 ± 2.42	48.16 ± 4.02	4.33 ± 1.52
E. sativa	*D. gigas*	0.00	16.33 ± 0.93	0.00	4.62 ± 3.82	41.59 ± 20.31	1.57 ± 0.83
	D. dipsaci	0.00	18.5 ± 0.76	0.00	5.9 ± 2.72	40.86 ± 20.55	1.68 ± 0.92
	Control	0.00	16.67 ± 2.04	1.00 ± 1.00	6.7 ± 4.68	48.81 ± 5.27	2.21 ± 0.20
R. sativus	*D. gigas*	0.00	7.33 ± 0.51	10.33 ± 1.86	3.23 ± 0.90	93.03 ± 13.21	23.37 ± 4.40
	D. dipsaci	0.00	7.11 ± 0.62	8.67 ± 1.33	2.57 ± 0.10	75.68 ± 12.22	10.23 ± 2.90
	Control	0.00	7.89 ± 0.78	8.00 ± 1.73	3.08 ± 0.17	88.37 ± 6.80	18.93 ± 0.98

5.6.3 Experiment 3

5.6.3.1 Number of stem nematodes recovered from the shoot of brassica plants

Total nematodes (juveniles, adult males, and adult females) recovered from the aerial portion of the legume (*Vicia faba*) and the different brassica plants are shown in Table 5.4 below. Overall, nematodes were 10 times higher in the stem tissue of *V. faba* than the rest of the plant species considered in this experiment. The reproductive factor (Rf: final density/initial density ratio) in *V. faba* (18) highlights its suitability as a host. Among the brassica plants, *Sinapis alba* appeared to be relatively conducive for *D. dipsaci* multiplication followed by *Eruca sativa*. The reproductive factor on these plants were 2.5 and 1.5 respectively. However, these values are not significantly different ($P>0.05$) for the two brassica plants. The remaining brassica plants, *Brassica carinata*, *B. juncea*, and *Raphanus sativus* had reproductive factors ≤ 1 indicating that they were generally poor hosts for nematode reproduction.

The percentage of juveniles (mostly J4) recovered from the plants were between 75.3% for *V. faba* and 89.1% for *B. carinata*. Adult males and females of *D. dipsaci* were generally low <25% of the numbers recovered across the brassica species and *V. faba* plants.

Table 5.4. Reproductive factor values (Rf: final density/initial density ratio) of *Ditylenchus dipsaci* on the legume *Vicia faba* and five brassica species used in biofumigation, and the proportion of adults and juveniles recovered. All plants were inoculated with an initial density of 100 mostly fourth-stage juveniles/plant *D. dipsaci*. Mean number of *D. dipsaci* ± standard error. Means with the same letter are not significantly different from each other according to Tukey's Honest Significant Difference test ($P < 0.05$).

	Mean number of *Ditylenchus dipsaci*				**Proportion**	
	Total	**Adult**	**Juvenile**	**Rf**	**Adults**	**Juveniles**
Vicia faba	1803 (±347.38) [d]	444.88(±137.62)	1358.5 (±254.99)	18.0	24.7%	75.3%
Sinapis alba	246 (±171.47) [c]	28 (±11.94)	217.5 (±165.40)	2.5	11.4%	88.6%
Eruca sativa	152 (±76.65) [c]	27.8 (±8.99)	123.75 (±71.53)	1.5	18.3%	81.7%
Brassica carinata	97.5 (±79.85) [bc]	10.62 (±11.03)	86.88 (±75.42)	1.0	10.9%	89.1%
Brassica juncea	29.8 (±17.93) [ab]	4.38 (±5.70)	25.38 (±13.34)	0.30	14.7%	85.3%
Raphanus sativus	19.6 (±13.23) [a]	3.50 (±4.60)	16.13 (±10.64)	0.2	17.8%	82.2%
CV	**169.7%**	**195.7%**	**164.2%**			

5.7 Discussion

Biofumigation involves the incorporation of fresh brassica biomass into field soil to suppress target pests, weeds, and pathogens. The process principally involves the release of isothiocyanates and other volatile compounds from hydrolysed glucosinolates. In theory, biofumigation of land infested with stem nematodes will succeed if the selected brassicas do not support reproduction of plant parasitic nematodes (Ntalli and Caboni, 2017). Three separate experiments determined the reproductive ability of *D. gigas* and *D. dipsaci* on the brassicas, *B. carinata, B*. *juncea, Raphanus sativus, Sinapis alba,* and *Eruca sativa* and the legumes *V. villosa and V. faba*. In addition, the symptoms and damage of stem nematode infection was assessed on these plants.

Typical symptoms of *D. dipsaci and D. gigas* infection of field beans (*V. faba*) include swellings and brown discoloration on stems. These indicate the positive development and reproduction of the stem nematodes on their host. Such symptoms were only seen on field beans in Experiments 2 and 3. The vetch, *Vicia villosa*, had one record of such symptoms with *D. dipsaci* in Experiment 2. The brassica plants, however, did not show any typical symptoms associated with stem nematode infection. Symptoms which are accompanied with plant damage occur at certain nematode thresholds which are yet to be determined for many hosts of *Ditylenchus* spp. (Castillo *et al.*, 2007). Hence, the inability of low nematode numbers recorded in these cases may explain the absence of any symptoms. In many cases, the presence of nematodes colonising a plant is evident with symptoms. But a few studies, such as (Whitehead *et al.*, 1987), have recorded symptomless plants even when *D. dipsaci* was reproducing successfully. In this respect, symptoms on host plants remain a poor indicator of the plant host status for plant parasitic nematodes.

Following inoculation, stem nematode (*D. dipsaci* and *D. gigas*) infection of legume and brassica species was determined by examining the density of nematodes recovered from stem tissue. In general, *D. gigas*, has a better reproduction rate on *V. faba* than any of the other plants tested. The low numbers of *D. gigas* recovered from the brassicas tested and the legume *V. villosa* strongly confirms the findings from other reports, that *D. gigas* has a very limited host range (Sturhan and Brzeski, 1991; Vovlas *et al.*, 2011). In comparison, the second nematode species evaluated, *D. dipsaci*, infects a far greater range of plant species (Hesling, 1972; Sturhan and Brzeski, 1991). In experiments 2 and 3, successful *D. dipsaci* reproduction was observed in *V. faba, V. villosa*, *Sinapis alba,* and *Eruca sativa*.

The two legumes, *V. faba* and *V. villosa,* are considered good hosts for *D. dipsaci* (Goodey, 1951; Sturhan and Brzeski, 1991; Caubel *et al*., 1998; Rajan and Lal, 2005). Similarly, wild mustard *Sinapis arvensis* and white mustard *S*. *alba* (cv Concerta and Hohenheimer) also support the reproduction of *D*. *dipsaci* (Knuth, 2006). The present study indicates that rocket (*E. sativa*) can act as a host for *D. dipsaci*, which was previously unknown.

Experiment 3 showed that both *D. gigas* and *D. dipsaci* reproduced poorly on *Brassica juncea, B. carinata* and *Raphanus sativus* with reproduction factor ≤1. Similar findings were observed when four populations of *D. dipsaci* isolated from Quebec and Ontario failed to multiply on mustard *Brassica juncea* cv. Caliente 119 (Poirier *et al.*, 2019) and *B. napus* (Hajihassani *et al.*, 2016), while a *D. dipsaci* population from sugar beet (*Beta vulgaris*) also failed to develop on *B. juncea* and *R*. *sativus* (Knuth, 2006).

Plant defence responses to stem nematodes *D. gigas* and *D. dipsaci* may resemble that seen for other plant parasitic nematodes. Every plant cell possesses rigid cell walls, which helps provide a barrier against pest and pathogen attack. But this barrier is less effective against plant parasitic nematodes including stem nematodes, as they can penetrate plant cell walls of a host and non-host through the mechanical activity of their stylets (Kaplan and Keen, 1980) and release a mix of active enzymes that degrade cell walls (Rai *et al.*, 2015). After being punctured by nematodes, plants can either succumb to the invasion or prevent its progression, so that they become non-hosts. This nematode hindrance can be accomplished by releasing chemical metabolites that are nematotoxic or repellent (Kaplan and Keen, 1980; Chitwood, 2002). For example, *Pratylenchus penetrans* are killed through thiophene (α-terthienyl) toxicity when the nematodes penetrate the roots of Marigold (*Tagetes* spp.) (Viaene and Abawi, 1998; Korthals *et al*., 2014).

Resistance to plant-parasitic nematodes is defined as the capability of the host to inhibit nematode reproduction (Trudgill, 1991). Such plants have resistant (*R*) genes that can provide complete resistance to a specific plant-parasitic nematode population. Brassica plants are known to have genetic resistance against nematodes. Such brassica plants include oil radish (*Raphanus sativus*) that have on its chromosome D the genetic factor(s) controlling resistance against beet cyst nematode, *Heterodera schachtii*. However, there are no reports of genetic resistance in brassica plants against stem nematodes. Albeit it is known that brassica plants such as *B. juncea,* and *B. carinata* release biocidal isothiocyanates e.g. allyl isothiocyanates when their cells are ruptured (Mayton *et al*., 1996; Morra and Kirkegaard, 2002). The lack of stem nematodes recovered from such brassica species in the current

experiments may relate to the release of toxic isothiocyanates during the invasion of their tissues. Moreover, *in-vivo* studies are needed to verify this claim.

A number of authors suggest that stem nematodes, utilise water films to travel upwards on susceptible plant stems, before infecting plants aerial structures (Wallace and Doncaster, 1964; Griffith, G. S. *et al.*, 1997). Movement of stem nematodes on stems and foliage requires high humidity (> 80%) and an optimum temperature range 15-23°C (Griffith *et al.*, 1997). Each of the three studies performed, followed a sequence of modifications to provide optimum conditions. In Experiment 1, the nematodes were inoculated into the soil at time of sowing. The approach in Experiment 1 was based upon the fact that the soil borne J4 are likely to initiate infection under field conditions. But there is little published on how stem nematodes infect the aerial parts of the plant. However, it could be a result of rain splash transmission (Griffith *et al.*, 1997), which is absent in glasshouses. In the absence of knowledge regarding this part of the lifecycle, the inoculated soil approach was reasonable. The approach has been shown to be successful in previous studies on root infecting nematodes such as *Meloidogyne* spp. (Teklu *et al*., 2014), but failed in this study for stem nematodes that colonized the aerial region of most of its host plants. A modified soil inoculation method is being described for a successful infection of sugar beet *Beta vulgaris* with *Ditylenchus* spp. (Storelli *et al.*, 2021).
For Experiments 2 and 3, the aerial region of seedlings were inoculated with a suspension of stem nematodes. The aerial portion the young plants differ in their morphology and hence their ability to retain inoculum. Field beans with their stipules, better provided support for the nematode suspension and limited nematode desiccation, which is likely to favour nematode infection (Figure 5.2). The other plant species tested, do not possess stipules or other suitable appendages, and therefore, the nematode suspension was placed in between the first true leaves of these plants (*Brassica juncea*, *Brassica carinata*, *Sinapis alba*, *Eruca sativa* and *Raphanus sativus*) or onto the nodes of young seedlings (*Vicia villosa*). In these areas, there is a risk of nematode disturbance and desiccation. Stem nematode infection of host plants is generally low following artificial inoculation. The environment (especially relative humidity and temperature) strongly affects the development of *Ditylenchus* spp. As a result, over 75% of inoculum can be lost during host invasion (Mercer and Grant, 1995). Additionally, injecting plants using a hypodermic syringe to dispense nematode suspensions is limited to 5 µl and hence not a feasible alternative (Kühnhold, 2011; Cook and Evans, 1988).

Literature reports over 500 plant species including carrot (*Daucus carota*), garlic (*Allium sativum*) and onion (*Allium cepa*) as hosts for *D. dipsaci* (Sturhan and Brzeski, 1991) but

these plants also show varying degree for supporting the development and reproduction of different populations of *D. dipsaci* (Douda, 2005). For example, four populations of *D. dipsaci* retrieved from infested garlic fields were tested by Poirier *et al*., (2019); the four populations reproduced on garlic and onion but failed to multiply on carrot (*Daucus carota* subsp. sativus cv. Enterprise). Such reproductive failure was also observed on spinach (*Spinacia oleracea*) and leek (*Allium ampeloprasum*) when similar garlic populations of *D. dipsaci* were used (Douda, 2005).

Sometimes stem nematodes host preference is very specific with no cross infection to other hosts. For example, a *D. dipsaci* population from lucerne (*Medicago sativa*) only reproduced on *M. sativa* and not on any of the following host plants; oat (*Avena sativa*), red clover (*Trifolium pratense*), white clover (*Trifolium repens*), narcissus (*Narcissus* spp.) tulip (*Tulipa* spp.) and onion whereas *D. dipsaci* population from onion could reproduce on lucerne (Webster, 1967). The populations of *D. dipsaci* and *D. gigas* used in this experiment were originally isolated from *Narcissus* spp. and *V. faba* respectively. It is possible that the poor nematode reproduction on these non-host plants (*R sativus*, *B. juncea*, *B. carinata*) is solely the result of the populations tested and different populations may perform differently on these plants.

Furthermore, caution is needed when dealing with a specific population/race because intra-population variation exists. According to Edwards and Taylor (1964), an “onion race” population of *D. dipsaci* reproduces on soybean and tomato, while another “onion race” population studied by Sayre and Mountain (1962) could not reproduce on these plants. While *D. dipsaci* populations can infest a wide host range, *D. gigas* on the other hand have limited host range. Of the plants they attack, most are weeds. Their attack on economic plants have however only been reported on *Vicia faba* (Vovlas *et al.*, 2011).

Plant growth and development are usually affected by nematode pest establishment. These effects are often negative resulting in poor plant biomass and quality damage. Plant features including weight and height are primary indicators of a plant’s response to pest attack. In this study, no plant was affected by the stem nematodes inoculated. Each plant that received either *D. gigas* or *D. dipsaci* maintained every measured feature just as the uninfected plants. The trend was also observed in garlic infected with 100 individual of *D. dipsaci* per plant with its weight unaffected (Hajihassani *et al.*, 2016). The number of tillers and amount of dried leaves recorded from the plants also do not reflect nematode damage. In addition, the application of fertilizer can mask the damaging effect of the plant pest including stem nematode (Moussart *et al.*, 2007).

Furthermore, stem nematodes do not always act solely to cause damage on plants, their interaction with pathogens causing secondary infection is crucial. Stem nematodes, *D. dipsaci*, are known to promote the spread of *Clavibacter michiganensis* subsp. Insidiosum (Manzanilla López and Marbán Mendoza, 2012) and *Fusarium oxysporum* f. sp. Medicaginis (Griffin, 1990) on alfalfa *Medicago sativa*, and *R. solani* on sugar beet (Hillnhütter *et al*., 2011). Due to their role in allowing secondary infection, such interactions could harm plants even at low stem nematode numbers.

5.8 Conclusion

These experiments confirm the species delimitation of *D. dipsaci* and *D. gigas*, based on their host preference. *Ditylenchus dipsaci* clearly has a wider range of hosts over *D. gigas*.
The inclusion of brassica plants, which are poor hosts of *D. gigas* and *D. dipsaci*, and their subsequent biofumigation effects may be able to alleviate stem nematode problems of field beans and simultaneously improve soil quality in agricultural systems. Three Brassicas, *Brassica juncea*, *B. carinata*, and *Raphanus sativus*, evaluated, showed potential in the management of *Ditylenchus* spp.

6.0 Investigating selected brassica plants for application in biofumigation systems against stem nematodes

6.1 Introduction

Plant-parasitic nematodes are burdensome pests to crop production across the globe causing huge economic losses to the industry. Global crop losses caused by plant-parasitic nematodes including stem nematodes were estimated at 14.6% in tropical and subtropical climates and 8.8% for temperate climates (Nicol *et al.*, 2011). Several plant families such as Liliaceae and Fabaceae are negatively affected by the stem nematodes, *Ditylenchus gigas* and *D. dipsaci*. One member of the Fabaceae, field beans (*Vicia faba*), is an important food crop used in several different parts of the world (Crépon *et al.*, 2010). Members of the *Ditylenchus* genus cause up to 50% loss to agricultural crops (Venter *et al.*, 1992). In field bean production, *Ditylenchus dipsaci* and *D. gigas* pose a significant threat (Sikora, Richard A. *et al.*, 2018; Thompson *et al.*, 2000). Previously, management of these pests has included soil solarization (Greco, N., 1993), a long rotation for up to 8 years (Caubel *et al.*, 1998) and application of chemical fumigants such as methyl bromide to infested fields (Powell, 1974; Gray and Soh, 1989). However, the use of synthetic fumigants has been banned in crop production; following their application, synthetic chemicals show strong negative impact to soil environments and human health (Zasada, Inga A. *et al.*, 2010). This has brought stem nematodes to the forefront as they become a major threat to crops (Mouttet *et al.*, 2014). In addition, there are no field bean varieties resistant to these pests (Stawniak, 2011). As such, there are currently no recommended control measures against stem nematodes of field beans (Dr Becky Howard, Research and Development manager at PGRO, personal comm.)

Alternative plant-parasitic control measures, which are safe to humans and the environment have long been investigated (Halbrendt, 1996). Many such options are derived from plants that possess nematicidal compounds in their homogenates, leachates, and decomposing residues. Members of the Brassicaceae family, possessing secondary metabolites known as glucosinolates, have drawn particular interest to researchers and their application in crop protection is termed biofumigation (Kirkegaard and Matthiessen, 2004). Biofumigation involves growing glucosinolate containing plants and incorporating them in soil to produce biocidal isothiocyanates. Isothiocyanates produced during biofumigation can provide a similar effect to synthetic pesticides such as metam sodium and dazomet; these compounds liberate methyl isothiocyanate (Gimsing and Kirkegaard, 2009). Moreover, biofumigation is

considered to be safer for the environment and soil organisms than synthetic soil fumigants due to no accumulation of isothiocyanates (Gimsing and Kirkegaard, 2009).
Field studies have shown that this approach is capable of controlling plant-parasitic nematodes in different cropping systems, including *Meloidogyne javanica* in vineyards (Rahman *et al.*, 2011), *Meloidogyne incognita* in courgette production (Lazzeri *et al.*, 2009) and *Globodera pallida* in potato production (Ngala *et al.*, 2015) . The reason for their success was linked to the release of isothiocyanates (allyl and 2-phenethyl) which had toxic effects on the plant-parasitic nematodes.
The sensitivity of *D. gigas* and *D. dipsaci* to isothiocyanates under *in-vitro* conditions was recorded in previous chapters (Chapters 3 and 4) with three isothiocyanates – allyl, 2-phethethyl and benzyl- having negative impact against stem nematodes survival and motility. Their associated brassica plants are known to produce of isothiocyanates at toxic concentrations under field conditions (Wood *et al.*, 2017). Moreover, results from the controlled environmental conditions (Chapter 5) revealed that some brassica plants including *Brassica juncea*, *B. carinata*, and *Raphanus sativus* are poor/non-host for both *D. gigas* and *D. dipsaci*. It is recommended that for a successful biofumigation effect, the inclusion of brassica plants which are poor hosts of target plant-parasitic nematodes is essential (Ntalli and Caboni, 2017). Therefore, these results (Chapters 3, 4 and 5) were a bases for selecting brassica plants to investigate the effect of biofumigation on field populations of stem nematodes.

6.1.1 Objectives

Two field experiments were undertaken with following objectives:

a) Assess the ability of growing and incorporating brassicaceous cover crops to suppress stem nematodes (*D. gigas* and *D. dipsaci*) under field conditions
b) Investigate a mixture of vetch (*Vicia villosa*) and brassica biofumigants for the suppression of stem nematodes (*D. gigas* and *D. dipsaci*).
c) Compare the effect of soil disturbance caused by the equipment used in biofumigant destruction and incorporation in the presence or absence of brassica biofumigants.
d) Prior to brassica incorporation, analyse the glucosinolate content in the shoots and roots of sown brassicas

6.2 Null hypotheses:

a) Biofumigation using brassica cover crops does not influence soil populations of stem nematode (*D. gigas* and *D. dipsaci*) under field conditions.

b) There is no effect of mixing vetch (*Vicia villosa*) and brassica cover crops on the soil populations of stem nematode (*D. gigas* and *D. dipsaci*) under field conditions.

c) The type and concentration of glucosinolates derived from brassica plants does not determine the effect of brassica on stem nematode populations

6.3 Materials and Methods

6.3.1 Field Experiment 1 (Malvern, Worcestershire, UK; August 2017)

6.3.1.1 Choice of brassicas, cover crop cultivars and experimental site

For the first field experiment, the choice of brassica species was based on two considerations; (i) brassica cultivars reported to have nematicidal effects against field population of other plant parasitic nematodes and (ii) the potential of brassica species or cultivars to produce high concentration of glucosinolates under temperate field conditions. All of the brassicas selected in Table 6.1 were previously shown to have nematicidal effects against potato cyst nematodes *Globodera pallida* in replicated field experiments (Ngala *et al.*, 2015). In addition, the isothiocyanates associated with these brassicas were shown to have a strong suppressive effect against stem nematodes under *in-vitro* conditions (Chapter 3).

Field experiment 1 was conducted at a site in Malvern, Worcestershire, UK (52.122897, -2.242475). This field has a history of stem nematodes infecting field beans (Dr Becky Howard, Research and Development manager at PGRO, personal comm.). Using an aerial view of the field bean crop (taken in 2016 growing season), an area that was thought to have higher populations was carefully chosen (indicated by damage) for nematode soil sampling; and six sampling points were selected. In June 2017, six soil samples from this stem nematode infested site were collected with a spade to a depth of 20 - 30 cm, each sample weighed approximately 7 kg. The density of stem nematodes in these soil samples was determined, to establish the position of stem nematode hot spot on this field site. The sample point with eight individuals of stem nematodes (*Ditylenchus* spp.) per 200 grams of soil was selected as the central point for the positioning of the experiment as it contained the highest stem nematode number. Prior to the experiment the previous crop was winter wheat.

Table 6.1. Treatments used in field experiments 1 and 2 to assess the effect of biofumigation of brassica cover crops on field populations of *Ditylenchus dipsaci* and *D. gigas*. Seeds were supplied by RAGT and Joordens Zaden and sown at the recommended seed rates in the two field experiments.

Common name	Species	Cultivar	Field experiment 1	Field experiment 2
Sole crop				
Hairy vetch	*Vicia villosa*	Villana		
Rocket	*Eruca sativus*	Trio		
Oilseed radish	*Raphanus sativus*	Terranova		
Indian mustard	*Brassica juncea*	Brons		
White mustard	*Sinapis alba*	Architect		
Ethiopian mustard	*Brassica carinata*	Cappucchino		
Crop mixtures				
Hairy vetch + Rocket				
Hairy vetch + Oilseed radish				
Hairy vetch + Indian mustard				
Controls				
Fallow (cultivated)				
Fallow (uncultivated)				

Field experiment 1 was designed in a randomised complete block design (RCB) and replicated four times with nine treatments that consisted of three brassica cultivars and their mixtures with hairy vetch (Table 6.1). Seed-rate for mixtures was half of that used for treatments sown alone. Each experimental unit was demarcated into plots of 3 x 3 m. All treatments were planted on 23/08/17 (Figure 6.1) along side two fallow controls (cultivated and uncultivated). Nine cores of soils samples were obtained from the experimental plots for assesment of initial number of stem nematodes. The aerial view of the nine treatments across four blocks at three weeks after planting is shown in Figure 6.2. The plants were subsequently allowed to mature for a total of 12 weeks.

6.3.1.2 Plant and soil samples assessment

For each plant assessment carried out, only the shoot and leaves of plant samples were harvested using secateurs. The fresh and dry plant weights of two sub-samples of the plants in each experimental plot were determined for areas of 50 x 50 cm (sub-plot). Plants were dried at 60ºC overnight prior to dry weight measurement. The number of plants per square meter for each plot was calculated using the average of the total plant counts from the sub-samples.

In addition, brassica plants for glucosinolate analysis were obtained from one sub-plot and transported to the laboratory. These plants were stored at -80ºC, then frozen using liquid nitrogen before they were freeze dried (GVD6/13 MKI freeze dryer, GIROVAC Ltd, North 12 Walsham, UK) for a week.

Soil samples (nine cores of 4 cm diameter and 20 cm depth) from each experimental plot were obtained for nematode analysis to investigate the following: the effect of partial-biofumigation prior to brassica incorporation and the full biofumigation effect two weeks post brassica incorporation. Nematodes from each soil sample was extracted using the two-flask method described in Section 2.3.2 (Chapter 2).

The experimental plots were marked out using GPS (RTK) (Figure 6.3). Afterwards, the experimental plants left on the field were macerated using a flail mower RMU290, Agrimaster and incorporated (Figure 6.4).

Figure 6.1. The process of sowing experimental seeds at Malvern, Worcestershire using tractor and planter to a depth of 10 mm

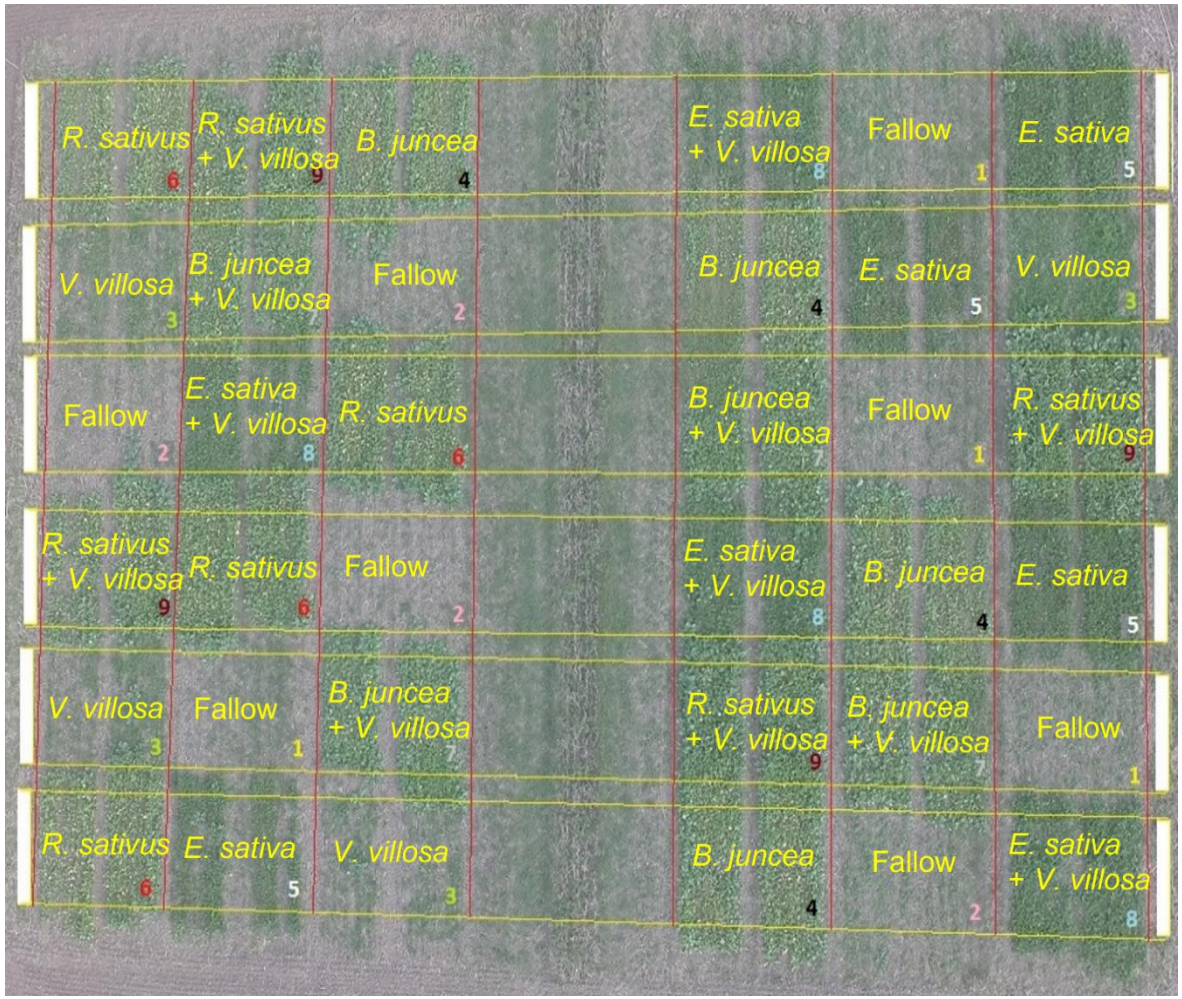

Figure 6.2. Field experiment 1 (Malvern, Worcestershire, UK) from an aerial view showing the distribution of nine treatments across four blocks. Photograph taken on 14/09/17

Figure 6.3. Marking respective experimental plots using a GPS coordinate system prior to brassica incorporation in field experiment 1 (Malvern, Worcestershire, UK)

Figure 6.4. Case IH 5150 tractor operating a flail mower (RMU 290, Agrimaster) to macerate brassica plants in field experiment 1 (Malvern, Worcestershire, UK)

6.3.2 Field Experiment 2 (Newport, Shropshire, UK; August 2018).

6.3.2.1 Choice of brassica cultivars and experimental site

The choice of brassica cultivars for field experiment 2 was informed by the sensitivity of *D. gigas* and *D. dipsaci* to isothiocyanates under *in-vitro* conditions and their ability to produce high amounts of glucosinolates. Three isothiocyanates – allyl, 2-phethethyl and benzyl–had negative impact against stem nematodes survival and motility (Chapters 3 and 4). This experiment took place from 02/08/18 to 19/11/18 at Large Marsh field, Harper Adams University (52.783316, -2.429342). The field had previously been used for the cultivation of winter field beans from late 2017 and symptoms of stem and bulb nematode infestation were observed; over 200 plants showed symptoms of *Ditylenchus* spp. and these were randomly distributed across the site. The field beans on the area identified with most stem nematodes symptoms were chopped and incorporated to maintain a high population of stem nematodes for the experiment. The experiment was arranged in a RCB design, and each treatment (Table 6.1) was replicated six times with plots of 6 x 3 m (Figure 6.5). Initial soil samples for nematode assessment were taken by 12 soil cores (of 4 cm diameter and 20 cm depth) per plot. Following the planting of the biofumigants, all plots received granulated sulphur 'N' fertiliser (Sulphur N 26N 35SO_3, Origin Fertilizers UK Ltd) at a rate of 120 kg ha^{-1} nitrogen (N) and 64 kg ha^{-1} sulphur on the 28/08/18.

Two weeks after establishing the experiment, a high population of volunteer beans were found across in the experimental plots, as shown in Figure 6.6. The herbicide Dow Shield at 500 ml/ha was applied on the 14/09/18 in order to control the volunteer beans (Figures 6.6 and 6.7). During incorporation, brassica tissue was flailed with a tractor-driven flail mower (Spearhead Q18S, Figure 6.8) and immediately followed by incorporation with a Howard 300 rotavator within the top 30 cm of soil. A soil roller was used right after brassica tissues were incorporated into the soil to reduce soil porosity.

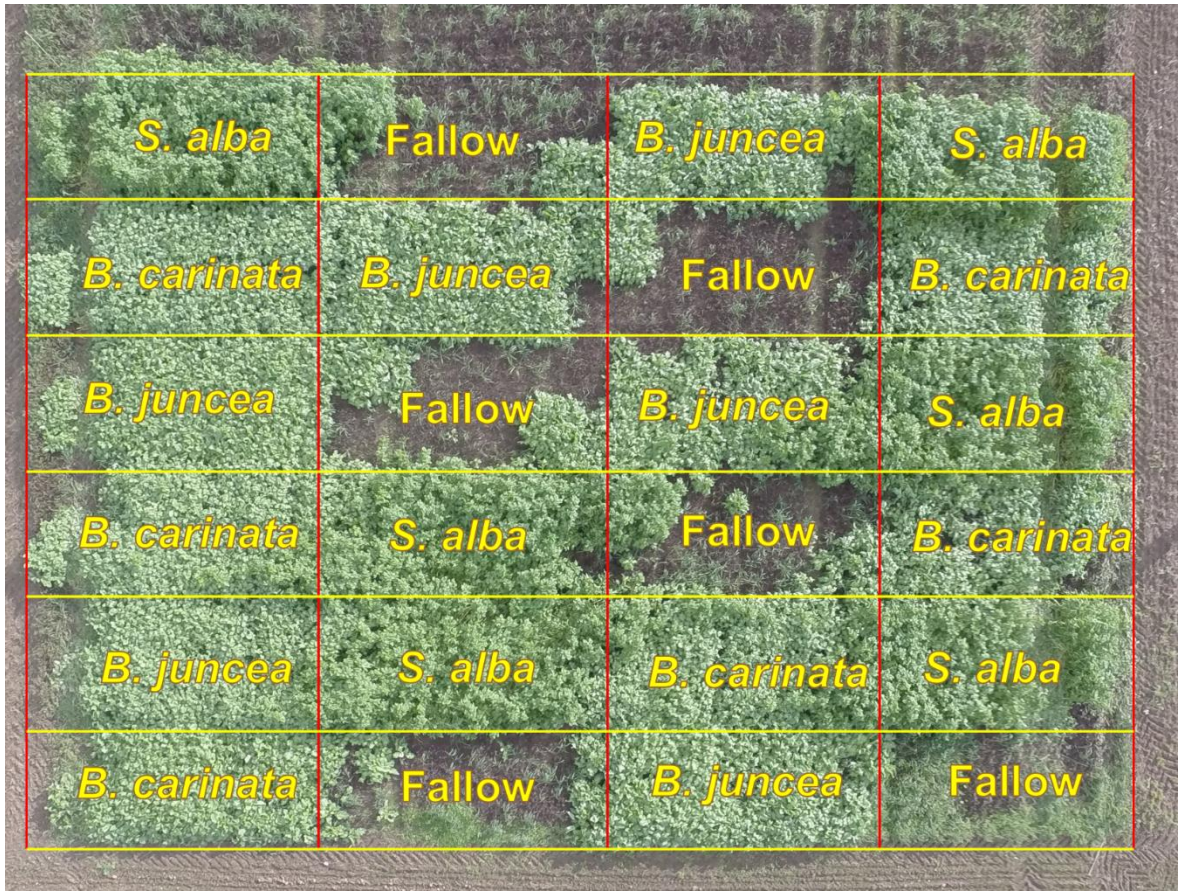

Figure 6.5. Field experiment 2 (Newport, Shropshire, UK) from an aerial view showing the distribution of four treatments across six blocks. Photograph taken on 28/10/2018

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Figure 6.6. Volunteer field beans interfering with the development of brassica plants in field experiment 2 (Newport, Shropshire, UK).

Figure 6.7. Volunteer field bean plants dying off while brassica plants unaffected after the application of herbicide Dow Shield at 500ml/ha field experiment 2 (Newport, Shropshire, UK)

Figure 6.8. Operation 1: New Holland T6040 tractor operating a flail mower (Spearhead Q18S) and a rotavator (Howard 300 Rotavator); Operation 2: Kubota M135GX-IV tractor operating a roller on field experiment 2 (Newport, Shropshire, UK).

6.3.2.2 Plant and soil samples assessment

The procedure for obtaining plant samples were similar to that in Section 6.3.1.2. However, the following differences are highlighted: the plants sub-samples were obtained from an area of 330 x 330 cm for plant assessment; and both shoots and roots of brassica plants were processed for glucosinolate analysis.

Soil samples from each experimental plot were obtained for nematode analysis as described in Section 6.3.1.2 except that 12-cores were taken for each experimental plot. The soil temperature was measured for each experimental plot using a digital thermometer (Digitron T206TC, UK) while soil moisture values were measured by using a soil moisture meter (Field Scout TDR 100) at a depth of 0–12 cm.

6.3.3 Data analysis

The analysis of the data obtained in this study depended on their nature/distribution. The nematode data for field experiment 1 was analysed using an analysis of variance (ANOVA). In addition, a paired t-test was conducted for nematode numbers recorded for each treatment prior to brassica establishment and post brassica incorporation. For field experiment 2, a generalised linear mixed-effects model from the lme4 package was used for nematode counts. The remaining data including the fresh and dry weights of brassica shoots and roots from the two experiments; soil moisture and temperature; and glucosinolate concentration data from field experiment 2 were analysed by ANOVA. In all cases of analysis conducted, a Tukey post-hoc analyses for multiple comparisons for means across treatments were significantly different ($P<0.05$), a post hoc comparison of means by computing estimated marginal means (emmeans) (Lenth 2021) was performed and compact letter display was generated using multcomp packages in R programming language (R Studio 4.0.2).

6.4 Results

6.4.1 The outcome of growing brassicaceous plants

Brassicaceous crops produced a much greater crop biomass in the second field experiment conducted at Harper Adams University. For *B. juncea* cv. Brons, a cultivar used in both experiments, the average crop biomass produced in field experiment 2 was approximately nine-fold more than those produced without fertilizer (N and S) in field experiment 1 (Figures 6.2 and 6.5; Tables 6.4 and 6.5). Brassica biofumigants grown in field experiment 1 were severely affected by a lack of nutrition (Figure 6.2) leading to yellowing of the leaves and premature senescence.

Generally, the biomass (fresh weight) for the cover crop treatments of field experiment 1 was below 4 t/ha. An analysis of covariance test carried out to compare the biomass of brassica species as sole crops or in legume-mixtures indicated that no statistical differences of biomass performances occurred for any species (Table 6.2). However, brassicas sown in a mixture with hairy vetch (*Vicia villosa*) appeared to have performed better, considering that their seed-rate was half of that used for treatments where brassicas were sown alone. A comparison of brassica species showed that there were no significant differences ($P > 0.05$) in their biomass when they were sown without *V. villosa*. While in a mixture, *Raphanus sativus* produced a significantly greater biomass than that of *Brassica juncea* ($P = 0.001$) (Table 6.3). The total biomass was calculated for all plants in each experimental plot and compared across treatments. The result show that only the mixture of *R. sativus* and *V. villosa* provided significantly more total biomass compared to other treatments ($P < 0.05$) (Table 6.4).

In field experiment 2, there was no significant difference ($P > 0.05$) in the fresh weight of the brassica biomass for both shoots and roots across the three brassica treatments (Table 6.5). When their dried biomass was considered, ANOVA revealed a significant difference in the root biomass for *Sinapis alba* in comparison to *Brassica juncea* ($P < 0.05$). However, no significant differences were seen in the dry weight for shoots.

The soil environmental factors measured at the time of brassica incorporation were temperature and moisture. The result showed that soil temperatures recorded for all treatments including fallow control were not statistically different ($P= 0.264$). However, there was differences ($P<0.05$) in the soil water content in the soils; with fallow control been highest at 33.95% of field capacity and soils of *S. alba* the lowest at 16.73% of field capacity. The soils of *B. carinata* and *B. juncea* were not different from each other with 28.07 and 28.16% field capacity respectively (Table 6.6).

Table 6.2. Fresh weights (t/ha) of hairy vetch (*Vicia villosa*) when grown alone or in a mixture with *Brassica juncea*, *Raphanus sativus*, and *Eruca sativa* in field experiment 1 (Malvern, Worcestershire, UK). Different superscript letters represent significant differences in biomass between treatments according to Tukey's multiple range test ($P < 0.05$). Numbers in parentheses show the standard error of the mean. Mixtures received half the seed rates of hairy vetch and respective brassica.

	Treatments				***P-value***	**CV%**
	Brassica juncea + *V. villosa*	*Raphanus sativus* + *V. villosa*	*Eruca sativa* + *V. villosa*	*V. villosa* alone		
Fresh Weight of *Vicia villosa* (t/ha)	0.66 (±0.19) [a]	0.50 (±0.03) [a]	0.96 (±0.17) [a]	2.08 (±0.49) [b]	0.001	74.40

Table 6.3. Fresh weights (t/ha) of *Brassica juncea*, *Raphanus sativus*, and *Eruca sativa* as sole crop and in mixture with hairy vetch in field experiment 1 (Malvern, Worcestershire, UK). Different superscript letters represent significant differences in biomass between treatments according to Tukey's multiple range test ($P < 0.05$). Numbers in parentheses show the standard error of the mean. Mixtures received half the seed rates of hairy vetch and respective brassica.

	Treatments						***P-value***	**CV%**
	B. juncea	*R. sativus*	*E. sativa*	*B.j /V. villosa*	*R.s /V. villosa*	*E.s /V. villosa*		
Brassica Fresh Weight (t/ha)	1.97 (±0.21) [ab]	2.04 (±0.23) [ab]	2.95 (±0.40) [ab]	1.48 (±0.16) [a]	3.24 (±0.50) [b]	2.11 (±0.45) [ab]	0.008	42.95

Table 6.4. Total fresh weights (t/ha) of *Brassica juncea*, *Raphanus sativus*, and *Eruca sativa* as sole crop and in mixture with Hairy vetch in field experiment 1 (Malvern, Worcestershire, UK). Different superscript letters represent significant differences in biomass between treatments according to Tukey's multiple range test (P < 0.05). Numbers in parentheses show the standard error of the mean. Mixtures received half the seed rates of hairy vetch and respective brassica.

	Treatments								
	B. juncea	*R. sativus*	*E. sativa*	*B.j /V. villosa*	*R.s /V. villosa*	*E.s /V. villosa*	*V. villosa*	***P-value***	**CV%**
Total Fresh Weight (t/ha)	1.97 (±0.21) [a]	2.04 (±0.23) [a]	2.95 (±0.40) [ab]	2.14 (±0.25) [ab]	3.75 (±0.50) [b]	2.14 (±0.25) [ab]	2.08 (±0.49) [ab]	0.009	42.32

Table 6.5. Shoot and root fresh and dry weights (t/ha) of *Brassica juncea*, *Brassica carinata*, and *Sinapis alba* in field experiment 2 (Newport, Shropshire, UK). Different superscript letters represent significant differences in biomass between treatments according to Tukey's multiple range test (P < 0.05). Numbers in parentheses show the standard error of the mean.

	Fresh weight		**Dry weight**	
Treatment	***Shoot***	***Root***	***Shoot***	***Root***
Brassica juncea	17.37 (±1.28)	1.23 (±0.14)	1.42 (±0.07)	0.23 (±0.03) [a]
Brassica carinata	17.56 (±2.63)	1.14 (±0.14)	1.49 (±0.28)	0.26 (±0.03) [ab]
Sinapis alba	20.53 (±1.84)	1.38 (±0.15)	1.79 (±0.15)	0.34 (±0.03) [b]
P value	0.4622	0.4942	0.3387	0.0469
%CV	37.13	39.15	41.31	40.89

Table 6.6. Soil moisture and soil temperature values for the soils in field experiment 2 (Newport, Shropshire, UK). Means (±SE) with the same letter are not significantly different according to Tukey's multiple range test (P < 0.05).

Treatment	Soil moisture	Soil temperature
Brassica juncea	28.07 (±0.86) [b]	7.84 (±0.14)
Brassica carinata	28.16 (±0.87) [b]	7.80 (±0.13)
Sinapis alba	16.73 (±0.58) [a]	7.53 (±0.13)
Fallow control	33.95 (±0.53) [c]	7.80 (±0.09)
P value	<0.001	0.264
%CV	25.3%	5.48%

6.4.2 Effect of growing brassicaceous plants on the populations of *Ditylenchus dipsaci* and *D. gigas*

For most of the treatment plots and blocks in Field Experiment 1, stem nematode populations (*Ditylenchus dipsaci* and *D. gigas*) were not significantly different before the establishment of Brassicas (Table 6.7). In the case of *Ditylenchus dipsaci*, there were no significant differences between treatment plots (P = 0.831) or blocks (P = 0.220). Despite no significant differences in *D. gigas* population densities between treatments (P = 0.5788), differences were still found between block 1 and block 2 at the start of the experiment. The number of stem nematodes in the soil varied from 0-27 per 200 g across the experimental area.

There was no statistical difference in the number of stem nematodes recorded for each treatment when comparing the numbers prior to brassica establishment and post brassica incorporation (Table 6.7). The numbers of stem nematodes also did not differ statistically among treatments that received brassica alone or in combination with vetch and those in fallow.

Field experiment 2 (Table 6.8) showed no significant difference in stem nematode numbers between treatment plots and blocks before brassica establishment. The density of *Ditylenchus dipsaci* did not differ between treatments (P = 0.839) or blocks (P = 0.293) and the density of *D. gigas* produced no significant difference between treatments (P = 0.887) or blocks (P = 0.281). Following brassica incorporation, stem nematodes population density was lower in plots treated with brassicas than in plots left fallow. There was a statistically lower number of *D. gigas* in soils in which the three brassicas (*Brassica juncea*, *B. carinata*, *Sinapis alba*) were incorporated when compared to the fallow control. However, the numbers of *D.*

dipsaci recovered from the soils that were incorporated with the three brassicas were not significantly different from those from the fallow control ($P>0.05$).

Table 6.7. The mean number of stem nematodes (*Ditylenchus gigas* and *D. dipsaci*) recovered from field plots pre-establishment and post incorporation of brassica biofumigants grown alone or in mixture with hairy vetch (*Vicia villosa*) in field experiment 1 (Malvern, Worcestershire, UK). Different superscript letters represent significant differences in nematode numbers between treatments according to Tukey's multiple range test ($P < 0.05$). Numbers in parentheses show the standard error of the mean.

Mean number of *Ditylenchus* spp./200 g soil (±SE)				
	Ditylenchus dipsaci		***Ditylenchus gigas***	
Treatment	**Pre-establishment**	**Post incorporation**	**Pre-establishment**	**Post incorporation**
Eruca sativa	6.75 (±2.84)	19.25 (±12.02)	8.00 (±1.87)	13.25 (±4.21)
Brassica juncea	4.00 (±1.68)	5.25 (2.78)	11.75 (±5.65)	9.00 (±2.30)
Raphanus sativus	2.50 (±1.66)	2.50 (1.26)	3.75 (±2.46)	6.50 (±3.71)
Vicia villosa	6.50 (±3.97)	5.00 (±1.47)	8.25 (±5.20)	8.75 (±1.55)
E. sativa /V. villosa	6.00 (±5.67)	15.75 (±7.62)	8.25 (±5.45)	19.50 (±10.19)
B. juncea /V. villosa	2.75 (±1.80)	8.25 (±2.02)	3.25 (±1.80)	11.00 (±2.27)
R. sativa /V. villosa	1.00 (±1.00)	3.25 (±1.10)	4.75 (±2.95)	6.50 (±3.43)
Fallow One	4.00 (±0.82)	5.50 (±1.71)	4.75 (±1.11)	9.00 (±2.38)
Fallow two	4.00 (±1.96)	5.25 (±2.78)	4.25 (±1.65)	7.50 (±3.12)
P value	0.831	0.215	0.5788	0.3405
% CV	126.02	107.7	91.1	134.2

Table 6.8. The mean number of *Ditylenchus gigas* and *D. dipsaci* and their standard errors in brackets for pre-establishment and post incorporation of Brassica juncea, Brassica carinata, Sinapis alba and a fallow control in field experiment 2 (Newport, Shropshire, UK). Different superscript letters represent significant differences in nematode numbers between treatments according to Tukey's multiple range test ($P < 0.05$)

		Mean number of *Ditylenchus* spp./200 g soil(±SE)	
Nematode species	**Treatments**	**Pre establishment**	**Post incorporation**
Ditylenchus dipsaci	*Brassica juncea*	6.00 (±3.88)	2.17 (±0.60)
	Brassica carinata	4.50 (±2.99)	1.33 (±0.21)
	Sinapis alba	2.83 (±1.25)	1.67 (±0.21)
	Fallow control	3.33 (±1.74)	1.33 (±0.80)
	P value	0.8411	0.6432
	% CV	150	76.6
Ditylenchus gigas	*Brassica juncea*	17.2 (±4.09)	2.33 (±0.21) [a]
	Brassica carinata	17.00 (±3.40)	2.17(±0.48) [a]
	Sinapis alba	14.30 (±3.71)	2.17 (±0.17) [a]
	Fallow control	20.3 (±8.56)	7.5 (±1.67) [b]
	P value	0.8890	0.0001
	% CV	72.4	86.9

6.4.3 Field Experiment-2: Brassica glucosinolate profiles and their concentration

There were ten GSLs found in the brassica cultivars used in field experiment 2, with the occurrence and concentration of each differing by cultivar (Table 6.9). *Sinapis alba* had the highest GSL variation, with eight different GSLs found. *Brassica juncea* and *Brassica carinata* were found to have seven and six GSLs, respectively. The total GSL concentration in the brassica cultivars varied from 7.09 to 16.81 µmol/g dry weight (Table 6.9). When total GSL concentrations for the cultivars were compared, there were significant differences (P = 0.035). The glucosinolates glucoberin, progoitrin, and neoglucobrassicin were found in all of the brassica species (*Brassica juncea*, *Brassica carinata*, and *Sinapis alba*) and their concentrations were not statistically different between these species. On the other hand, the concentration of the glucosinolates sinigrin and gluconasturtiin, differed significantly between brassica species. *Brassica juncea* had higher concentrations of sinigrin and gluconasturtiin compared to *Brassica carinata* and *Sinapis alba*.

Table 6.9. The mean GSL concentrations (µmol/g dry weight) found in *Brassica juncea*, *Brassica carinata* and *Sinapis alba* used in field experiment 2 (Newport, Shropshire, UK). For each GSL, the means that have the same letter are not statistically different according to Tukey's multiple range test (P < 0.05). Standard error of the means are shown in parentheses.

Glucosinolates (GSL)	**Brassica species**				
	Brassica carinata	***Brassica juncea***	***Sinapis alba***	***P value***	**%CV**
Glucoberin	1.40 (±0.11)	1.54 (±0.08)	1.35 (±0.09)	0.327	15.8
Progoitrin	0.13 (±0.03)	0.12 (±0.02)	0.12 (±0.03)	0.928	51.8
Sinigrin	6.19 (±1.29) ^{ab}	13.12 (±3.08) ^{b}	0.21 (±0.21) ^{a}	0.001	108.0
Glucobrassicin	0.32 (±0.08) ^{b}	0.07 (±0.02) ^{a}	*ND*	0.029	96.9
Gluconasturtiin	0.10 (±0.25) ^{ab}	1.74 (±0.53) ^{b}	0.42 (±0.05) ^{a}	0.046	91.4
Neoglucobrassicin	0.05 (±0.01)	0.09 (±0.02)	0.04 (±0.01)	0.139	69.0
Gluconapin	*ND*	0.12 (±0.03)	*ND*	*ND*	54.4
Glucoraphanin	*ND*	*ND*	0.25 (±0.04)	*ND*	37.1
Glucosinalbin	*ND*	*ND*	2.34 (±0.53)	*ND*	54.1
Glucotropaeolin	*ND*	*ND*	2.95 (±0.59)	*ND*	49.4
Total GSLs	9.09 (±1.37) ^{ab}	16.81(±3.71) ^{b}	7.31 (±1.56) ^{a}	0.035	63.8

ND-Not detected

6.5 Discussion

The suppression of field populations of *Ditylenchus dipsaci* and *D. gigas* was examined after macerating and incorporating various brassica cover crops in two experiments performed between 2017 and 2018. Field experiment 1 involved mixing brassica species cover crops with the leguminous hairy vetch (*Vicia villosa*) while field experiment 2 focused on the effect of three different brassica species. In field experiment 1, the selected brassicas and their mixtures failed to suppress the field population of *D. dipsaci* and *D. gigas*. However, *Brassica juncea*, *Brassica carinata* and *Sinapis alba* all showed a suppressive effect against *D. gigas*, but not *D. dipsaci* in the second experiment.

6.5.1 The impact of brassicas for suppressing stem nematodes

In the second field experiment, all the brassicas investigated including *Sinapis alba*, *Brassica juncea* and *Brassica carrinata* suppressed the numbers of *Ditylenchus gigas* by up to 30% after incorporation of brassica plant biomass. However, such suppression was not observed against *D. dipsaci*. This may have been due to the variability in the distribution of the nematodes and the low numbers present. Biofumigant cultivars have been shown in previous experiments to improve plant parasitic nematode mortality prior to incorporation and post incorporation (Ngala *et al.*, 2015).

This implies that nematotoxic compounds including isothiocyanates produced are formed during brassica development and brassica incorporation by the result of glucosinolate hydrolysis. Hydrolysis is possible as glucosinolates released form the roots of brassica are broken down by myrosinase-producing soil microorganisms (Borek and McCaffrey, 1996; Sakorn *et al.*, 1999; Rakariyatham *et al.*, 2005) and at incorporation by brassica produced myrosinase. The effect of growing brassicas alone without incorporation was not investigated in this study, therefore the result obtained in this study is a combination of both effects involving brassicas as partial-biofumigants and biofumigants. However, these processes are strongly dependent on glucosinolate levels. In Section 1.5 (Chapter 1), GSL concentrations and their impact in a biofumigation system was discussed. Based on the expected 1% conversion of GSLs to ITCs (Morra and Kirkegaard, 2002), the ITC dose would be unlikely to supress stem nematodes in soil (Morra and Kirkegaard, 2002).
There is an indication that the reduction in the number of *Ditylenchus* spp. observed in the current study was not solely due to the glucosinolate hydrolysis process involving isothiocyanate release. The primary reason is based on the glucosinolate concentrations

identified after the examination of the all the brassica GSL profiles. While the cultivars used in this study have the potential to generate sufficient isothiocyanates to affect nematode populations during brassica development and subsequent incorporation, the conditions, particularly the low atmospheric and soil temperature in the second field experiment, were not optimal for their performance. Furthermore, the numbers of *D. gigas* and *D. dipsaci* post-brassica-incorporation were generally low, perhaps attributable to large numbers of volunteer field beans present in the early part of the experiment, before the application of the selective herbicide. Hence the most likely suppressive effect is attributed to organic or green manuring effect accompanied with brassica biomass incorporation. Vervoort *et al*. (2014) ascribed the impacts of biofumigation on the nematode population to tillage and green manuring rather than isothiocyanates release. Green manuring adds benefits to the soil-health including suppression of soil-borne pest. This can be achieved by promoting beneficial antagonistic bacteria such as *Bacillus*, *Pseudomonas* (Hollister *et al.*, 2013; Wang *et al.*, 2014) which are known to harm plant-parasitic nematodes (Topalović *et al.*, 2020). In addition, some compounds such as dimethyl disulfide and methylsulfide are released at high concentrations after the incorporation of Brassica (e.g. *B. juncea*) biomass into the soil (Bending and Lincoln, 1999) and such compounds have been reported to have pest/pathogen suppressive effect (Wang *et al*., 2009). There is therefore room for further investigation on whether the effect of incorporating solely brassica cultivars with higher GSL will act against *D. gigas* and *D. dipsaci* or not. Clearly, no assumption can be made that the suppressive the effect recorded in the experiment was solely that related to isothiocyanates.

6.5.2 Trap cropping effect on stem nematode populations

In the previous section, the glucosinolate-based effect was clearly not the only factor that led to the suppression of the two stem nematodes. Another possible reason behind the suppression seen in *D. gigas* is the trap crop effect. Trap crops are plant species that promote plant parasitic nematodes attraction and root invasion while preventing the life cycle from being completed. Trap crops are used to reduce nematode population densities in the soil prior to growing the target crop. The trap crop technique makes use of host plants either resistant or susceptible. The susceptible trap crops are mulched before the nematodes contained inside the root tissues mature and begin reproducing (Vestergård, 2019). Even weeds, for instance, have been used as trap crops and successfully improved for this purpose. Populations of nematodes can multiply exponentially throughout the growing season and their reproductive rate increases in warmer soils than they do in colder soil. Hence for trap crops to be effective, the time for trap crop termination is crucial. In this study, the

experiment site had volunteer field beans growing alongside the experimental brassicas plant. These volunteer plants may have acted as trap crops and hence reducing the numbers of *D. gigas* and *D. dipsaci* in the experiment overall. Trapping plant parasitic nematode using brassica plants have been reported. For instance, white mustard (*Sinapis alba*) has been used as trap plants successfully against sugar beet cyst nematodes *Heterodera schachtii* and incorporated as green manures prior to sugar beet cultivation (Caubel and Chaubet, 1985; Lelivelt and Hoogendoorn, 1993). Oilseed radish 'Adagio' (*Raphanus sativus*) and white mustard 'Luna' (*Sinapis alba*) trapped *H*. *schachtii* and their subsequent soil incorporation resulted in sugar beet root yield increase compared with fallow treatment (Hemayati *et al.*, 2017). Brassica plants in a biofumigation system can trap nematodes and therefore adding to the multifaceted effects involved in such systems.

Moreover, such nematode trapping brassica crops could equally play a key role in biofumigation systems involving cover crop mixtures. Mixing nematode trap cover-crop and a biofumigant could improve the efficiency of biofumigation management against parasitic nematodes. Growing cover crop mixtures has been shown to be successful in reducing damage linked to plant-parasitic nematodes such as root-knot nematode (Desaeger and Rao, 2001). Hence investigating what cover-crop mixtures are suitable against plant parasitic nematodes could be beneficial.

6.5.3 The impact of environmental and soil conditions on the growth and development of brassicas for suppressing stem nematodes

There are several reasons for the differences observed in the success of growing biofumigants for the suppression of stem nematodes. These include factors such as the availability of soil nutrients and the soil moisture content during the growth of the crop and at biomass incorporation. Additionally, warmer temperatures, longer day lengths, and increased radiation are essential for the efficient generation of brassica biomass with high concentrations of GSLs (Björkman *et al.*, 2011).

For field experiment 1, *Brassica juncea*, *Raphanus sativus* and *Eruca sativa* were chosen based on their potential glucosinolate content and ability to generate biomass after a short 8–14 week cropping cycle. Whilst brassica emergence and establishment were generally successful, the resultant plants had a lower biomass compared to similar brassica varieties recorded in previous studies (Ngala *et al*., 2015; Doheny-Adams *et al*., 2018).

The agronomic potential of brassica biofumigants is dependent on soil nutrients nitrogen and sulphur; and in the absence of these required nutrients such plants struggle and remain

stunted. Both sulphur and nitrogen are essential ingredients in the biosynthesis of GSL (Booth *et al*., 1991) and important for GSL accumulation in tissues. Furthermore, nitrogen is necessary for the biosynthesis of proteins, a process that influences the amount of biofumigant biomass produced. In this study, however, brassica plants were unfertilized in FE1 during their growing season. This could have had consequence on the brassica plants. Besides, since winter wheat (*Triticum aestivum*) was the previous crop on the field prior to establishing field experiment 1. Consequently, there could have been a potential lack of nutrient in the soil because wheat, a nutrient demanding crop requiring up to 243 kg N/ha (Barraclough, 2014), could have depleted the soil nutrients. Although the soil nutrient levels were not determined in this study, the lack of soil nutrition is presumed to be the most likely cause for the general low biomass obtained in field experiment 1 which reduces their potential biofumigation effect of the brassicas.

Soil nutrition during the brassica growing season is critical for their overall performance. Increasing the supply of sulphur and nitrogen fertilization rates improves plant height and the agronomic characteristics of *Brassicas* including their biomass (Taylor *et al*., 1991;; Öztürk, 2010, Björkman *et al*., 2011). The application of sulphur and nitrogen fertilizers was equally significant for the concentration of glucosinolate in brassica plants (Björkman *et al.*, 2011). According to Back *et al.* (2019), *Brassica juncea* treated with nitrogen of 100-150 kg/ha and sulphur of 25 kg/ha provided the best control of *Globodera pallida*. Glucosinolates are sulphur-rich plant metabolites and glucosinolates may represent up to 30% of the total sulphur content of a brassica plants. Since the field experiment 1 generally had low biomass and will constituently produce lower glucosinolate content for incorporation, the possible ITC release from the brassicas in field experiment one could have been insufficient to suppress stem nematode populations as our results confirmed.

In addition, a deficiency in soil nutrients could have limited the effect of partial biofumigation and the antagonistic effects of soil microbes which act synergistically to suppress plant-parasitic nematodes in a biofumigation system (Ngala *et al*., 2015). Soil microbes are known to play a role in partial biofumigation as they decompose glucosinolates released by brassica roots into the soils and convert the secondary metabolites to isothiocyanates (Ngala *et al*., 2015). However, the availability of soil nutrients such as nitrogen also determines their abundance. Bhattarai (2019) reported that the use of nitrogen fertilisers resulted in increased soil microbe activity. Hence, for biofumigation to be achieved, soil nutrients have an important influence on the growth of brassicas, their biomass and glucosinolate levels.

Moreover, a late sowing date may also have affected brassica production in field experiment 1. This field experiment was sown on 23/08/17. The consequence of late sowing can greatly affect brassicas' efficacy owing to poor biomass production, reduced glucosinolate accumulation and low soil temperatures at the time of incorporation (Back *et al.*, 2019). It is known that the highest total glucosinolate content is found in Brassica species grown at intermediate temperatures, high light intensity and dry conditions (Bjorkman *et al*., 2011). Though Ngala *et al*. (2015) recommended that sowing dates for brassicas should fall within June-August, an earlier date than the 23rd of August may be suitable.

6.5.4 The effect of brassica- legume mixtures on the growth and development brassicas for suppressing stem nematodes

Crop mixtures have many benefits in agricultural systems and that includes brassica-legume cover crop mixtures, which contribute to crop pest management and improving soil health. In comparison to single cover crops, brassica-legume mixtures have better multi-ecosystem services. The biofumigation efficiency of selected brassica species are preserved in the brassica-legume mixture, while the legumes enhance nitrogen availability (Couëdel *et al.*, 2019). Leguminous plants can fix nitrogen (N) from the atmosphere. This nitrogen fixing activity benefits not only the legumes, but also any intercropped or subsequent crops, therefore minimising or eliminating the need for N fertiliser use (Liu *et al.*, 2011). In field experiment 1, *B. juncea* and *E. sativa* grown in mixtures with the legume *Vicia villosa* (hairy vetch) showed a similar weight in biomass to when grown alone. Moreover, *R. sativus* while in combination with *V. villosa* had more biomass than *R. sativus* sown alone. This is noteworthy, because the seed rate for these brassicas in the brassica-legume mixture was half that of the brassicas sown alone. In such brassica-legume mixtures, the supplemental N fertilizer requirement are low for any accompanying non-leguminous plants to achieve optimum plant performance. Such was the case when vetch cover crop (*Vicia villosa*) provided nitrogen of 35 - 75 kg/ha to sweet corn (*Zea* mays) therefore reducing the need for supplemental N by up to 75% (Zotarelli *et al.*, 2009). Therefore, the increases in the biomass of brassica species in the presence of vetch may be related to the N fixation ability of the legume. This is in accordance with the benefit of cover crop mixtures which has been previously reported to produce greater biomass than cover crops sown alone (Khan and McVay, 2019). Brassica-legume mixtures may also achieve improved resource usage efficiency due to niche complementarity in utilising abiotic materials such as light, water, and nutrients, (Jensen, 1996).

6.5.5 The co-occurrence of *Ditylenchus dipsaci* and *D. gigas*

The two stem nematodes, *Ditylenchus gigas* and *D. dipsaci*, infecting field beans, are economically important plant parasites (Sikora and Greco, 1990). For single nematode infection, *Ditylenchus gigas* is generally more damaging because it causes more acute symptoms on field beans than *D. dipsaci* (Goodey, 1941; Hooper, 1971; Sikora and Greco, 1990; Sturhan and Brzeski, 1991). In addition, Hooper (1984), found *Vicia faba* plants severely infected by *D. dipsaci* and *D. dipsaci* "giant race" (syn *D. gigas*) showed that 3 and 67% of the seeds sample were infected, respectively. When crossing experiments were conducted with *D. dipsaci* and the "giant race" now described as *D. gigas,* F1 hybrids were formed, but they were infertile (Sturhan,1983).

There is limited information on efficient management strategies that focus on mixed *Ditylenchus* spp., particularly in field beans production. Mixed plant-parasitic nematode infections (within a nematode Genus and across several Genera) causing crop losses are common in agricultural fields. Such cases of mix infection have been reported for species within *Ditylenchus*. For example, Yu *et al*., (2012) reported the presence of both *D. destructor* and *D. dipsaci* using traditional morphology and PCR analysis, in garlic fields in Canada. The garlic plants were found to be stunted, and the basal bulbs became dark and decayed. In Iran, mix infection of *Ditylenchus gigas* and *D. dipsaci* were recorded as damaging on field beans (Azimi, 2017; Maafi *et al.*, 2013). In the UK too, the species *Ditylenchus dipsaci* "giant race" (syn *D. gigas*) and *D. dipsaci* were found together infecting field beans in field sites at Rothamsted (Hooper 1971). Additionally, this study further confirms the co-occurrence of both species infecting field beans in Malvern (Worcestershire) and Newport (Shropshire) sites in the UK.

Given the combined presence of *Ditylenchus* spp. in field beans, the choice of management measures should be effective against both species. This is important as effective management of one species may lead to the population development of the other. A concern over using cover-crops is that they serve as host to one of the species. For instance, in Chapter 5, it was found that while some brassica plants such as *Eruca sativa* and *Sinapis alba* may be non-host to *D. gigas* they however supported the reproduction of *D. dipsaci*.

6.5.6 Glucosinolate profiles of field grown Brassicaceae cultivars

The brassica cultivars used in the field experiments were chosen because they have been used as biofumigants against plant parasitic nematodes and are known to produce high concentrations of glucosinolates (GSL) associated with biocidal isothiocyanates (Ngala *et al*., 2015). In field experiment 2, above and below ground plant tissue was analysed for their GSL

content. A profile of 10 GSLs across the three brassica varieties was identified; the aliphatic GSLs progoitrin, sinigrin and glucoberin were the most common. Other aliphatic GSL, were less common and specific to certain brassica species including gluconapin, glucoraphanin and glucosinalbin. Indolic and aromatic GSLs present included glucotropeolin, glucobrassicin, gluconasturtin and neoglucobrassicin.

The primary role of GSL in brassica plants is to assist plants in defending themselves from herbivorous pests (Halkier and Gershenzon, 2006; Redovnikovic *et al*.,2008; Del Carmen Martnez-Ballesta, Moreno, and Carvajal, 2013). This function has been exploited and applied in crop production to suppress soil-borne plant pathogens, insect and nematode pests and weeds.

The efficacy of the brassica species in biofumigation is largely dependent on the specific type of GSLs generated than the overall GSL concentration. For example, Ngala *et al.* (2015) showed that allyl and 2-phenethyl ITCs resulting from *Brassica juncea* were effective against potato cyst nematode. The low concentration of glucobrassicin, progoitrin neoglucobrassicin and gluconapin found in the profiles of brassica samples indicated that they were unlikely to significantly change the overall GSL profile or affect the success of biofumigation. The types of GSL from *Brassica juncea* and *Brassica carinata* were identical, with *B. juncea* producing slightly higher quantities of sinigrin in comparison to *B. carinata*. This is similar to the findings of Lazzeri *et al.* (1993) who showed the presence of sinigrin in *B. carinata.* At the time of incorporation, the GSLs, sinigrin and gluconasturtiin were found to be significantly greater in *B. juncea* than in *Sinapis alba*. It may be argued that *B. juncea* and *B. carinata* have similar overall GSL profiles and that their usage as biofumigants should provide comparable outcomes. While this may be valid, other aspects must be considered, such as the amount of myrosinase activity (Dosz *et al*., 2014) and the relative quantity of ITC generated in each species in comparison to other degradation products mention in Section1.5.4 (Chapter 1). The determination of GSL content as a valuable determinant of ITC generation are not always indicative of the released ITC concentrations because of soil factors such as ITC sorption (Morra and Kirkegaard, 2002). However, the existence of a dominant GSL that creates an ITC toxic to other soil-borne pests may be a good indicator of suitable biofumigant against stem nematodes. Sinigrin dominated the sample of *B. juncea* and *B. carinata* which is in line with earlier research (Morra and Kirkegaard,2002; Gimsing and Kirkegaard,2006; Bellostas, Sørensen and Sørensen,2007). Sinigrin has been extensively studied in Brassicaceae for pest control and biofumigation (Lord *et al*., 2011; Brolsma *et al*., 2014; Vervoort *et al*., 2014; Ngala *et al*, 2015), and cultivars with high concentrations are of particular interest. Sinigrin is the parent GSL of allyl ITC, which has been demonstrated to be

effective against stem nematodes *Ditylenchus gigas* and *D. dipsaci* in Chapter 3; hence *B. juncea* and *B. carinata* might be useful biofumigant cultivars for stem nematode management. The third brassica assessed, *S. alba*, contained the aromatic glucosinolates sinalbin and glucotropaeolin at significantly higher concentrations than in the other brassica species tested. This is consistent with earlier research that found glucosinalbin and glucotropaeolin dominated in *S. alba* cultivars (Sarwar *et al.*, 1998; Doheny-Adams *et al.*, 2018). It has been recorded that the breakdown products of glucosinalbin increase mortality of plant-parasitic nematodes *in vitro* (Zasada *et al.*, 2009, Avato *et al*., 2013). The mobility of the potato cyst nematode, *G. pallida*, was also decreased with the presence of *S. alba* green manures (Lord *et al*., 2011). On the contrary, *G. rostochiensis* mortality *in-vitro* was unaffected by glucosinalbin breakdown products (Buskov *et al*., 2002) while macerated *S. alba* tissue had no influence on the hatching of *G. rostochiensis* juveniles (Valdes *et al*., 2011). Glucotropaeolin, which hydrolyses to benzyl ITC, was the second most dominant GSL found in *Sinapis alba*. In Chapter 3, benzyl ITC caused high mortality of *Ditylenchus gigas* and *D. dipsaci* at concentrations of 50 µg/ml. There are discrepancies between authors on what is the primary GSL associated with *Sinapis alba*. However, it appears that the cultivar used in this study may be more efficient as a biofumigant. This implies that GSL diversity within species is possible and adds to the significance of screening prospective biofumigants for GSLs of interest. In this study, total mean glucosinolate concentration per area of field is lower in *S. alba* than *B. juncea* and field toxicity of the corresponding ITCs post incorporation is also likely to be lower for *S. alba*.

The GSL concentrations found in the foliage and stems of *B*. *juncea*, *B. carinata* and *S. alba* are highest at the 50% flowering stage, which indicates the correct time to generate maximum ITC release following crop maceration and incorporation (Doheny-Adams *et al.*, 2018). In this study, however, brassica plants were incorporated at flower budding stage with less than 10% plants with flowers. In addition, the time brassica plants began flowering coincided with shorter daylength, low atmospheric and soil temperatures (average of 7ºC) recorded which in turn can result to lower GSLs compared to warmer temperatures in spring (Matthiessen and Kirkegaard, 2006).

6.6 Conclusion

Incorporation of brassica cover crops into agricultural soils as part of an integrated pest management system is sustainable and beneficial to the environment (Fourie *et al.*, 2016). Soil nutrition is critical for the performance of brassica plants as a biofumigant affecting the amount of GSL generated (Sarwar and Kirkegaard, 1998). Late sowing of brassica plants will negatively affect the development of brassicas as plants are exposed to shorter daylength. Despite having low GSL concentrations in brassicas, the suppressive impacts of brassica plants observed in this study may have been due to nematode-trapping by mustards and volunteer field beans. In addition, green manuring (the turning into the soil of undecomposed plant materials) could further lead to the additional reduction in nematode numbers.

7.0 Utilizing molecular techniques to identify and quantify *Ditylenchus* spp.

7.1 Introduction

The stem and bulb nematodes *Ditylenchus dipsaci* and *D. gigas* are economically important pests present in field beans growing regions across the UK (Stawniak, 2011). Telling such nematode species apart is difficult and time consuming because they are morphologically similar, while differentiating them from other species in the *Ditylenchus* genus is even more complex (Subbotin *et al.*, 2005). Moreover, *Ditylenchus* spp. are frequently present in mixed species populations, which makes their identification and quantification of individual nematodes even more challenging (Webster, 1967; Eisenback, 1993). Conventional methods used to separate species of *Ditylenchus* include morphological characters together with morphometrics and differential host tests. The latter approach is extremely difficult and varies across testing conditions and nematode populations (Perera *et al.*, 2009). Prior to the use of molecular analysis that distinguished the two species as *D. dipsaci* and *D. gigas*, field beans *Vicia faba* were attacked by what was considered a "normal" race and a "giant" race (Vovlas *et al.*, 2011).

It is often challenging and time-consuming to attribute characteristics to *Ditylenchus* species using phenotypic data alone, and accurate diagnostic can be limiting, hence the application of biochemical and molecular techniques can be reliable alternatives. Tenente and Evans (1997) studied the differences in protein composition of six races (giant/bean, oat, garlic, lucerne, red clover, teasel) of *Ditylenchus* spp. using PAGE (Polyacrylamide Gel Electrophoresis) and isoelectric focussing. This study could differentiate the “giant” race from the other races. In another study, the use of MALDI-TOF, a method of species identification based on the molecular mass of proteins such as ribosomes, was able to discriminate the oat and lucerne races of *Ditylenchus dipsaci* (Perera *et al*., 2009). The use of biochemical approaches to separate *Ditylenchus* spp. was rapidly advancing and promising; however, possible limitations could occur including different spectra elicited by different development stages, environmental conditions, and occasional cross-reactivity (Ahmed *et al.*, 2016) .

The use of molecular tools has become important in recent times. DNA-based tools such as amplified fragment length polymorphism (AFLP) markers (Esquibet *et al.*, 2003) and PCR-ITS-Restriction Fragment Length Polymorphism (RFLP) (Wendt *et al*., 1993; Kerkoud *et al*., 2007) were used to differentiate the ‘normal’ and ‘giant’ races. From these studies, the species *D. gigas* and *D. dipsaci* could be differentiated for all nematode life stages and environmental conditions. Advances in the use of molecular tools for

assessment of nematode abundance by quantitative PCR are being developed for application in agriculture (Ahmed *et al.*, 2016; Braun-Kiewnick and Kiewnick, 2018). One of such is a multiplex quantitative/real-time PCR assay which has been developed for the molecular identification and quantification of *D*. *dipsaci* and *D*. *gigas* (Jeszke *et al*. 2015). However, such assays were developed and tested with nematode specimens isolated from their environment. Their application using DNA extracted directly from infected plant material and soil, where a range of PCR inhibitors are present, has not been investigated.

7.2 Aim of study

The aim of this study was to compare between the traditional (using morphological features) and molecular methods of assessing stem nematodes from plant samples.

7.3 Null hypothesis

There is no correlation between the traditional and molecular methods of assessing stem nematodes.

7.4 Materials and methods

7.4.1 Genomic nematode DNA extraction

The genomic DNA was extracted from an individual or groups of nematodes in 20 µl of PCR water using the Purelink genomic DNA extraction kit (Invitrogen, United Kingdom). All steps were performed according to the instructions listed by the manufacturer, with DNA eluted in 30 µl of elution buffer. The extracted DNA suspension was stored at −20°C or used immediately for DNA amplification.

7.4.2 Universal and specie-specific DNA primers

Published universal (nematode) and species-specific real-time PCR assays were chosen from the literature (Table 7.1) to generate bacteria clones and detect individual *Ditylenchus* species. All primers were synthesized by Eurofins Scientific, UK

Table 7.1. A list of primers used in this study and their respective target genomic with product size

Names	Sense	Primer target	Sequence 5'-3'	Size (bp)	Reference
Vrain2F	Forward	Nematode	TTGATTACGTCCCTGCCCTT	966	Vrain *et al*. (1992)
Vrain2R	Reverse		TTTCACTCGCCGTTACTAAG		
DITuniF	Forward	*Ditylenchus*	CTGTAGGTGAACCTGC		
DITgigR	Reverse	*D. gigas*	GACCACCTGTCGATTC	271	Jeszke *et al*. (2015)
DITdipR	Reverse	*D. dipsaci*	GACATCACCAGTGAGCATCG	148	
TW81	Forward	Nematode	GTTTCCGTAGGTGAACCTGC	875	Volvas *et al*., (2008)
5.8MS	Reverse		GGCGCAATGTGCATTCGA		
ITS4	Forward	Plants*	TCTCCGCTTATTGATATGC	c.600	(White *et al.*, 1990)
ITS5	Reverse		GGAAGTAAAAGTCGTAACAAGG		

*These primers were designed as universal primers for fungi, but with an anneal of 50°C, they amplify plant and fungal DNA (Edwards *et al*., 2012).

7.4.3 Confirmation of *Ditylenchus* spp. identification by DNA sequencing

ITS rRNA was the molecular target of DNA amplification using nematode universal primers VRAIN2F (TTG ATT ACG TCC CTG CCC TTT) and VRAIN2R (TTT CAC TCG CCG TTA CTA AGG) (Vrain *et al*., 1992). The PCR reaction consisted of 5 µl of 5X PCR MyTaq™ Red Reaction Buffer (Bioline), 1 µl of 0.4 mM of each primer, 0.5 µl of MyTaq™ Red DNA Polymerase (Bioline), 5 µl of DNA sample and ddH_20 for a total volume of 25 µl. PCR conditions were: denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. PCR products were separated on a 1 % agarose gel and visualized and photographed under UV-light. The remaining PCR product was stored at 4°C or purified. The purification process was done as described by the manufacturer's instructions (Wizard® SV Gel and PCR Clean-Up System Kit, Promega). Purified DNA for each nematode specie was sequenced (Eurofins Genomics, UK) in both directions to obtain overlapping sequences of both DNA strands. The sequences were blasted in NCBI GenBank (http://www.ncbi.nlm.nih.gov/) to obtain 21 *Ditylenchus* sequences and one *Anguina* spp. sequence. These sequences were aligned using software packages MAFFT version 7 (Katoh *et al.*, 2019) and analysed using IQ-TREE version 2.1.3.

7.4.4 Species-specific PCR and quantitative (q)PCR

The species-specific primers set DITuniF/DITdipR and DITuniF/DITgigR were used to confirm the specificity of the primers under a gradient of PCR annealing temperatures. The PCR conditions were same as detailed above (Section 7.4.3) while the thermal cycler programme consisted of denaturation for 3 min at 95ºC; 40 cycles at 95ºC for 30 s, between 55°C to 68°C (gradient PCR) for 30 s and 72ºC for 30 s; a final extension at 72ºC for 5 min. PCR products were separated and visualised on a 1% agarose gel and were photographed under UV light.

Real-time PCR was carried out in 96-well plates using a Bio-Rad CFX96 (Biorad, Hemel Hempstead, UK) in a total reaction volume consisting of 5 µl DNA extract (Section 7.4.1), 13 µl of ddH_2O, 5 µl of 5× EVA Green PCR Master Mix and 1 µl of 0.4 mM of each of the primers (Jeszke *et al.*, 2015). Cycling conditions for *D. gigas* and *D. dipsaci* consisted of 95ºC for 5 min; 40 cycles at 95ºC for 20 s, 62ºC for 20 s, 72ºC for 30 s and at 82ºC for 15 s for the DITuniF/DITdipR and DITuniF/DITgigR primer sets. The melting phase began at

72ºC and ended at 95ºC, with an increase of 1ºC at each step. The cycle threshold (C_t) value for each reaction was assessed using the manufacturer's software.

Using the real-time PCR procedure above, ten individual juvenile stage four (J4) of *Ditylenchus dipsaci* and *D. gigas* were tested and their Ct values compared.

7.4.5 *Escherichia coli* cloning and plasmid extraction

Cloning bacterial cells with DNA of interest is a useful method of obtaining highly pure DNA of known sequence. This method was used to obtain DNA of the target site of the species- specific primers to be used as standards in qPCR experiments. To achieve this, sequences of 18s-ITS-5.8s-ITS-26s genomic region for *Ditylenchus* ssp. available on NCBI GenBank database were collated. These sequences were used to generate an alignment of 296 sequences using Bioedit (Hall *et al.*, 2011) for detecting sequence matches of universal (nematode) and species-specific primers (Table 7.1). The primer pair Vrain2F and 5.8MS were taken forward to generate PCR products of ≈635 bp for *D. gigas* and *D. dipsaci*. The resulting amplicons contained the target sequences for the respective species-specific primers.

The PCR products were purified using a Wizard PCR Prep Kit (Promega, United Kingdom). Purified PCR products were quantified on a gel and then ligated into a pGEM-T Vector (Promega, United Kingdom) and transformed into *Escherichia coli* JM109. White colonies were screened by amplification with the species-specific primers using the conditions described above for diagnostic PCR. Selection of positive clones were grown overnight in LB broth (Merck, United Kingdom) before plasmid DNA was extracted using a Wizard Plus SV Miniprep Kit (Promega, United Kingdom) while remaining *E. coli* clones were stored at −80ºC. All methods for purification, cloning and transformation were according to the manufacturer's instructions.

7.4.5.1 Standard curves

For each *Ditylenchus* species a standard curve was constructed using a ten-fold dilution across nine orders 10^{-1} to 10^{-9} with plasmid DNA obtained from *E. coli* clones. The starting concentration for each respective *Ditylenchus* spp. was 20 ng/µl. Efficiency of each standard curve was calculated following the equation:

$$E\% = \left[\left(10\left(\frac{-1}{slope}\right)\right) - 1\right] X100$$

7.4.6 Preliminary experiment

The development of a molecular method for stem nematode assessment from plant tissue samples was designed. Field beans plants (*V. faba* cv. Fuego) were grown in the glasshouse without prior inoculation of stem nematodes. After three months, the aerial portion of the plants were harvested (≈800 g) and freeze dried using a Girovac Freeze dryer (Girovac Ltd., Norfolk, UK) (≈100 g) and milled to powder (<2 mm). Three grams of *V. faba* powder was combined with 100 individuals of crushed (*D. gigas* or *D. dipsaci*) prior to DNA extraction. Total DNA of the mixture (stem nematodes and *V. faba*) was extracted using CTAB (Section 7.4.7.2). The resulting DNA was diluted ten-fold in two successive steps before species quantification was carried out as described in Section 7.4.4.

7.4.7 Plant samples from controlled experiments

Plant samples containing *D. gigas* and *D. dipsaci* plants from experiments 2 and 3 (Chapter 5) were analysed to determine the relationship between nematode counts and their associating cycle threshold (C_t) value. The samples were processed using the methods described in Section 7.4.7.1 and 7.4.7.2, and genetic material from *D. gigas* or *D. dipsaci* detected using the protocol in Section 7.4.4. Standard curves (Section 7.4.5.1) were included in each Q-PCR reaction.

7.4.7.1 Sample processing

Plant samples were washed with tap water to remove excess soil and debris before being sealed in plastic bags and frozen at -20°C. Frozen samples were freeze-dried in a Girovac Freeze dryer (Girovac Ltd., Norfolk, UK), sealed within individual plastic bags, and stored at room temperature until DNA extraction.

7.4.7.2 Genomic DNA extraction using CTAB

Total nucleic acids were extracted separately from fine (<2 mm) freeze dried plant tissue per individual subsample using the 2x CTAB (cetyl trimethylammonium bromide) procedure as described by Edwards *et al.* (2012) containing: 2% CTAB (w/v), 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM, EDTA pH 8.0 modified by adding 1% β-mercaptoethanol. The resulting DNA was eluted in 200 µl of TE buffer and assessed for quantity and quality using a NanoDrop 2000c™ (ThermoFisher Scientific, Loughborough, UK) spectrophotometer, before being diluted to a final working concentration of 40 ng/µl. The DNA procedure with or without the addition of 1% β-mercaptoethanol were compared for preliminary plant samples.

7.5 Data analysis

In order to examine the relationship between the cycle threshold and the log count of *Ditylenchus* spp. a Spearman correlation was performed. A Student t-test was done to compare the cycle threshold values of ten individuals of *Ditylenchus gigas* to that of *Ditylenchus dipsaci*. Analysis of variance (ANOVA) was performed to compare the cycle threshold values associated to factors (*Ditylenchus dipsaci* or *D. gigas*) and uninoculated nematode control at two concentrations (10 ng/µl and 100 ng/µl). Significant differences ($P<0.05$) were identified using emmeans and compact letter display was generated using multcomp package in R programming language (R Studio 4.0.2).

7.6 Results

7.6.1 *Ditylenchus* species sequence analyses

An ITS-rDNA sequence alignment of *Ditylenchus gigas* and *D. dipsaci* populations with sequences of *Ditylenchus* spp. available in GenBank was completed using a Maximum likelihood tree (Figure 7.1). The figure shows that the sequences of *Ditylenchus* spp. used in this study (*D.* dipsaci: DDNar1_VRAIN2F, DDNar2_VRAIN2F, DDNar3_VRAIN2F and *D. gigas* (DDCul2_VRAIN2F) match the identity of the species recorded in elsewhere (Figure 7.1).

Figure 7.1. The phylogenetic tree analysed based on Maximum Likelihood for all populations studied with addition of *Ditylenchus* populations obtained from GenBank based on the sequence alignment of the ITS rDNA under the GTR + I + G model. *Anguina tritici* served as an outgroup

7.6.2 Specificity of *Ditylenchus* spp. primers

The gradient PCR run with both primers sets and template DNA from *D. dipsaci* and *D. gigas*, respectively, showed four bright bands for a range of annealing temperatures between 55°C and 68°C (Figures 7.2). The highest annealing temperature (Ann T) 62°C yielding the brightest specific band was retained for each species and selected for further PCRs. Furthermore, no cross reactions were recorded at Ann T 62°C for DITuniF/DITgigR tested on *D. dipsaci* DNA but faint bands were seen when DITuniF/DITdipR tested on *D. gigas* DNA. The *D. gigas* and *D. dipsaci* specific primers amplified single bands of ≈270 and ≈148 bp for nematode samples respectively and repeatedly. Equally single nematode

detection using real-time PCR provided cycle threshold values of 35.8 ± 0.86 and 29.3 ± 0.26 for *D. dipsaci* and *D. gigas* respectively (Table 7.2).

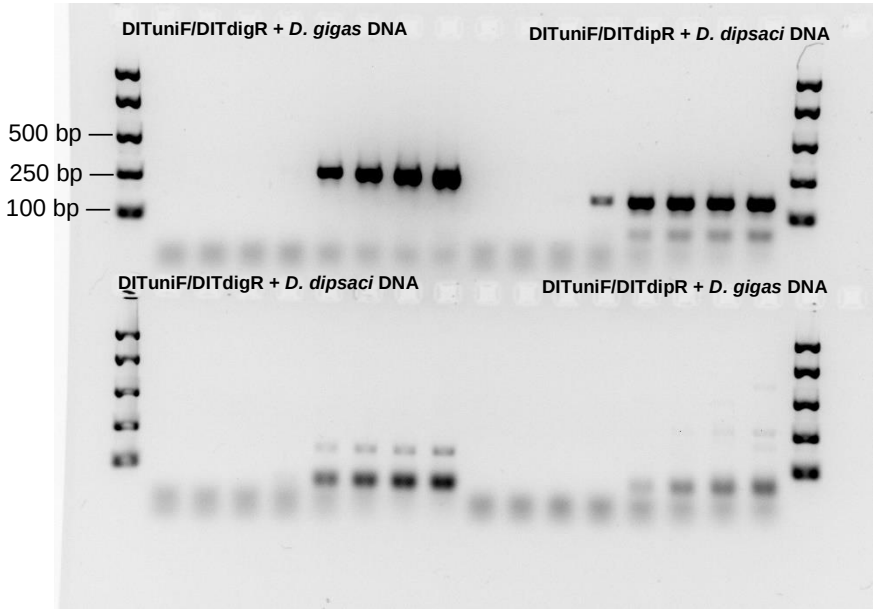

Figure 7.2. Gradient PCR with the species-specific primers set using *Ditylenchus gigas* and *D. dipsaci* DNA templates. Descending temperature ranged: 68.0ºC, 66.9ºC, 65.2ºC, 63.5ºC, 61.8ºC, 58.4ºC, 56.7ºC, 55.0ºC. Ladder: DNA ladder (Bioline EasyLadder I). Top-left DITuniF/DITdigR with *D. gigas* DNA; top-right DITuniF/DITdipR with *D. dipsaci* DNA; bottom-left DITuniF/DITdigR with *D. dipsaci* DNA; bottom-right DITuniF/DITdipR with *D. gigas* DNA

Table 7.2. The mean cycle threshold and standard error for ten individuals of juvenile stage four (J4) of *Ditylenchus dipsaci* and *D. gigas* nematodes

	Ditylenchus dipsaci	***Ditylenchus gigas***	***P-value***
Mean cycle threshold	35.8 ± 0.86	29.3 ± 0.26	<0.001

7.6.3 *Escherichia coli* clones

From the process of bacterial cloning, white colonies were obtained (Figure 7.3), indicating successful insertion of DNA into the plasmid. Using species-specific PCR, plasmid DNA were confirmed to have the respective *Ditylenchus* spp. inserts and afterwards their sequences confirmed 100% similarity to the individual *Ditylenchus* species. The dilution series was amplified with an efficiency of 107.7% and 112.5% for *D. dipsaci* and *D. gigas* and showed an R^2 value of 0.99 and 0.98 respectively (Figure 7.4).

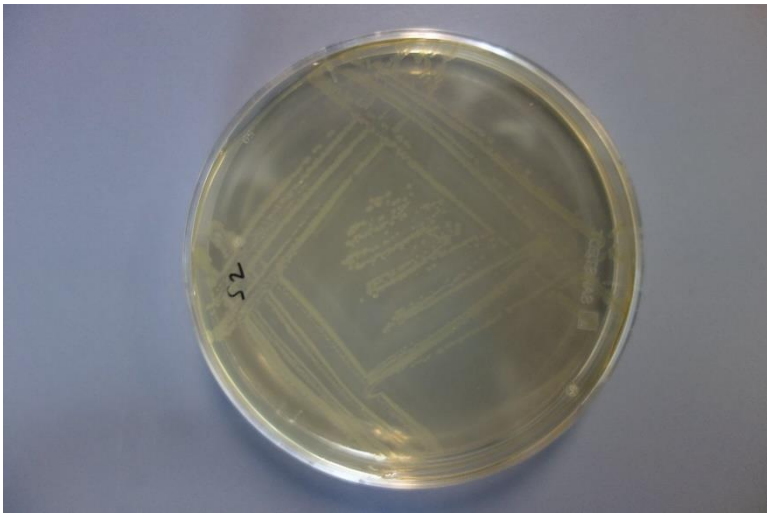

Figure 7.3. Plate showing white Escherichia coli colonies from cloning *Ditylenchus gigas* Vrain2F and 5.8MS.

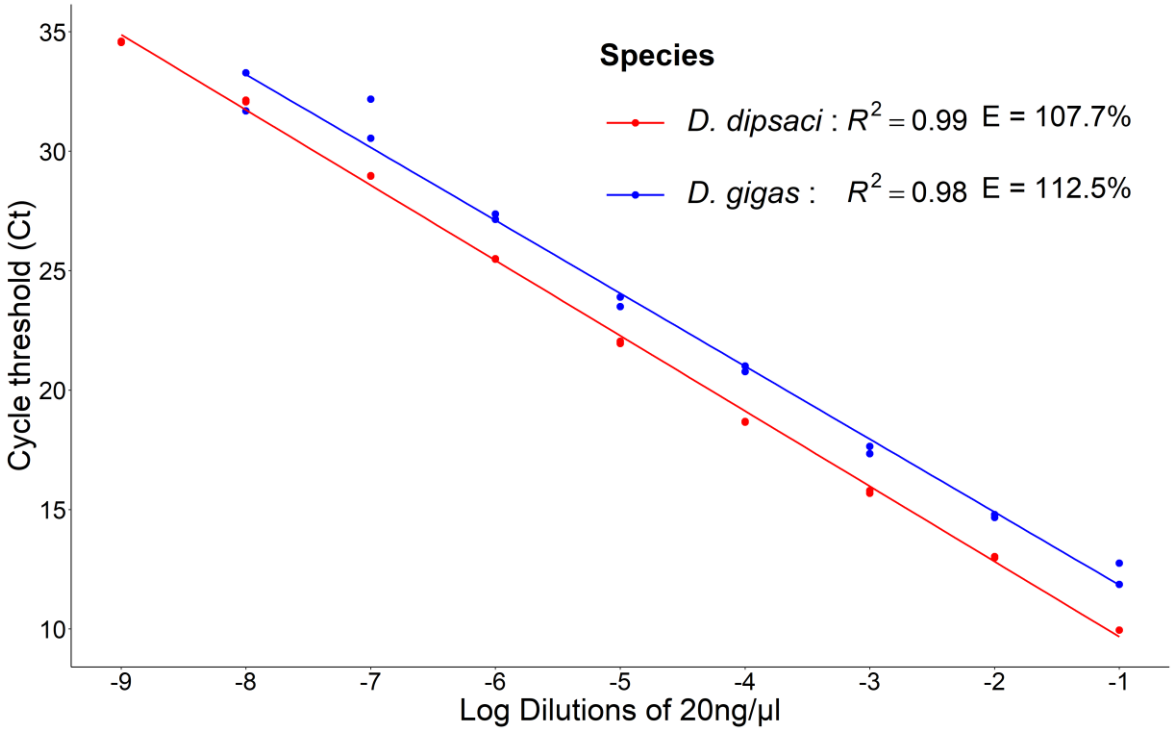

Figure 7.4. Standard curve for the real-time amplification showing a 10-fold dilution series of Ditylenchus dipsaci and D. gigas ranging from 20 x10-1 ng/µl to 20 x10-9 ng/µl.

7.6.4 Preliminary experiment

The DNA templates which were extracted from the mixture of field beans *Vicia faba* tissues and 100 individuals of *D. gigas* or *D. dipsaci* was used for qPCR at concentrations 10 ng/µl and 100 ng/µl total DNA. Their respective cycle threshold values derived from both species tested were obtained. At 10 ng/ul both species had significantly lower Ct values (≤35) than the controls (Uninoculated and NTC) whereas at 100 ng/µl only *D. dipsaci* had a Ct value significantly lower than the controls ($P<0.05$) (Figure 7.5). There should be no Ct values for the controls (uninoculated and NTC).

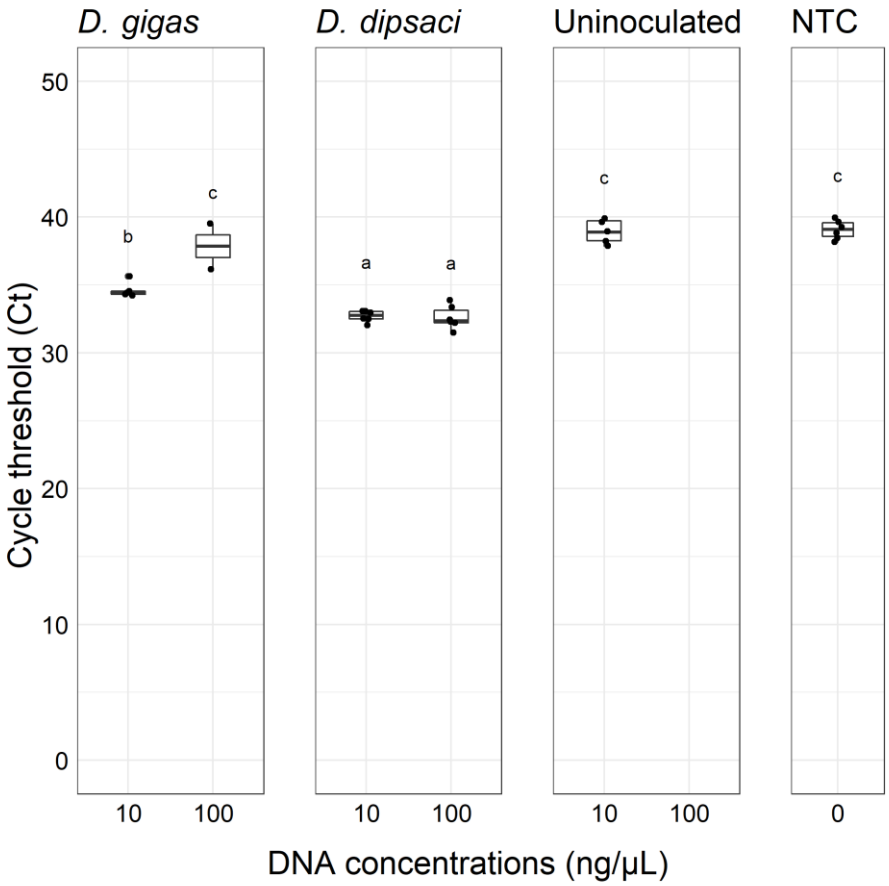

Figure 7.5. Cycle thresholds for stem nematodes (*Ditylenchus gigas* and *D. dipsaci*) detected in nematode inoculated and uninoculated *Vicia faba* plant tissues at 10 and 100 ng/µl DNA concentration. Means with the same letter are not significantly different from each other (P>0.05) according to Tukey's Honest Significant Difference test

7.6.5 Genomic DNA extraction using CTAB

The result of comparing the DNA extraction method with or without β-mercaptoethanol is indicated in Figure 7.6. The addition of 1% β mercaptoethanol improved the purity of the DNA obtained cause a cleaner sample of extracts.

Figure 7.6. DNA precipitation from a CTAB DNA procedure with (right-lighter pellet) and without (left-darker pellet) the addition of 1% β-mercaptoethanol. DNA obtained from the shoot of *Vicia faba* plant samples

7.6.6 Glasshouse

7.6.6.1 Glasshouse Experiment 2

Using spectrophotometry (NanoDrop 2000c™, ThermoFisher Scientific, Loughborough, UK) DNA concentration and purity appeared satisfactory (Table 7.3). The yield of isolated DNA using CTAB ranged from 104.60 ± 43.52 ng/µl in case of *Eruca sativa* to 1153.03 ± 591.89 ng/µl in the case of *Brassica juncea* (Table 7.3). Most of DNA samples extracted had A260/A280 ratios ranged from 1.68 ± 0.11 to 2.09 ± 0.04. Moreover, later amplification with the universal (plant) primers ITS 4 and 5 (White *et al*., 1990) at an annealing temperature of 50°C produce amplicons for most (90%) of the plant samples (Figure 7.7). The nematode molecular assessment with the pure or diluted gDNA (40 ng/µl) extracted from plant samples (second glasshouse experiment) containing *Ditylenchus gigas* or *D. dipsaci* or no nematode template failed to produce consistent cycle threshold for each sample replicates (data not shown). No significant negative correlation between the cycle threshold values and the log counts of nematodes was recorded (Spearman rank correlation r = -0.021, P= 0.8796). In addition, the cycle threshold values obtained from samples containing *D. gigas* or *D. dipsaci* were indistinguishable from the uninoculated controls.

Table 7.3. Genomic DNA concentration (ng/µl) and purity (A260/280) from legumes and brassica plants used in qPCR from Glasshouse Experiment 2, measurements conducted using spectrophotometry.

Plant Species	**Treatment**	**Nucleic Acid (ng/µl)**	**A260/280**
V. faba	*D. gigas*	488.23 ±334.25	1.85±0.04
	D. dipsaci	647.47 ±190.56	1.93±0.06
	Control	614.67 ±307.76	1.86±0.07
V. villoni	*D. gigas*	442.00 ±68.23	1.93±0.01
	D. dipsaci	536.60 ±217.13	1.95±0.08
	Control	287.93±221.81	1.99±0.06
B. juncea	*D. gigas*	1124.63±341.29	1.95±0.13
	D. dipsaci	1153.03±591.89	2.09±0.04
	Control	567.97±372.65	2.05±0.02
B. carinata	*D. gigas*	507.77±414.50	1.98±0.09
	D. dipsaci	588.60±411.59	1.93±0.21
	Control	478.93±193.48	1.99±0.06
S. alba	*D. gigas*	111.43±77.00	1.68±0.11
	D. dipsaci	323.13±153.60	1.90±0.03
	Control	587.80±701.41	1.88±0.17
E. sativa	*D. gigas*	104.60±43.52	1.75±0.15
	D. dipsaci	330.07±257.30	1.79±0.05
	Control	243.60±100.75	1.84±0.16
R. sativus	*D. gigas*	341.47±98.45	1.94±0.04
	D. dipsaci	456.57±241.21	1.88±0.14
	Control	197.67±71.69	1.75±0.10

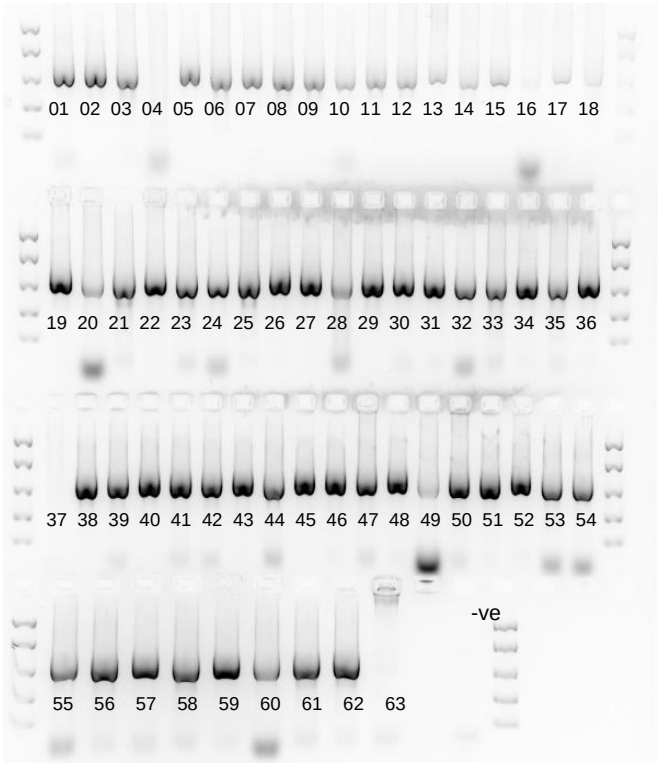

Figure 7.7. PCR with the universal primers ITS 4 and 5 (White *et al*., 1990) using the 63 DNA templates (40 ng/ml) obtained from Experiment 2 (Chapter 5). The bands are amplicons of plants DNA in order of plot numbers in experiment design and a negative control (-ve). Ladder: DNA ladder (Bioline EasyLadder I)

7.6.6.2 Glasshouse Experiment 3

A significant negative and moderate correlation between the cycle threshold values and the log counts number of nematodes was observed for *Ditylenchus dipsaci* (Spearman rank correlation r = -0.43, P= 0.003). There was a cluster of *V. faba* at the bottom-right of the scatter plot demonstrating this correlation (Figure 7.8).

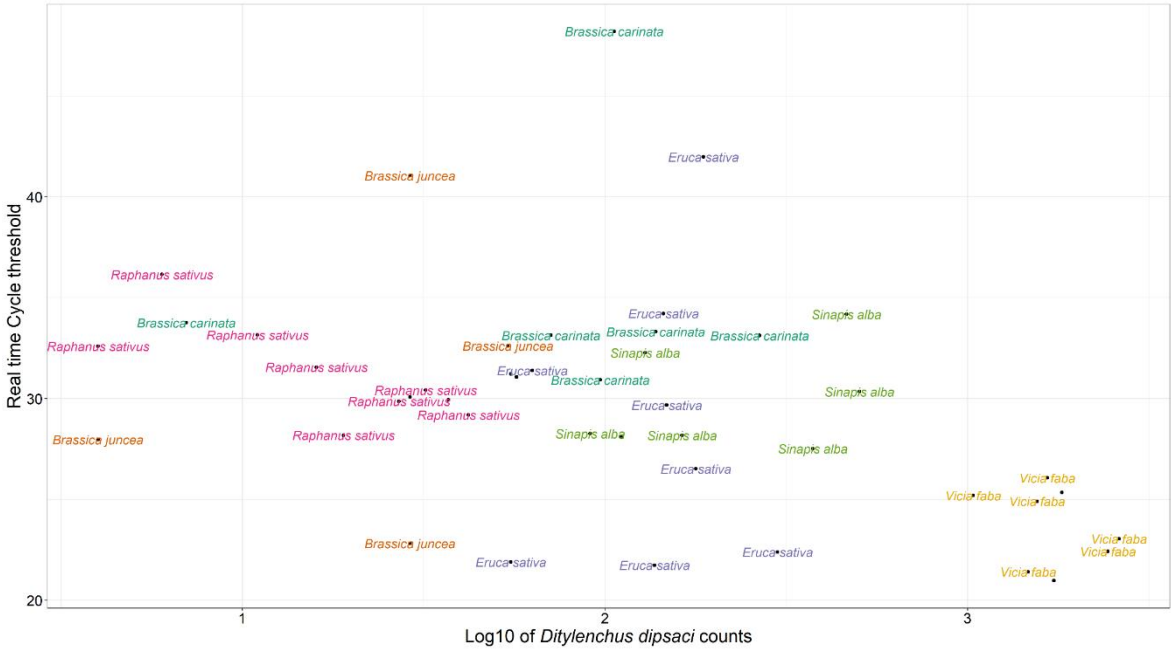

Figure 7.8. A scatter plot of the cycle threshold values of a real-time PCR with the ITS species-specific primers DITuniF/DITdipR and the log10 counts of *Ditylenchus dipsaci* obtained from legumes and brassica plants

7.7 Discussion

The two species, *Ditylenchus gigas* and *D. dipsaci*, have a substantial effect on the UK's field bean *Vicia faba* production systems, causing considerable losses. Rapid and reliable quantification of these nematodes in samples is an essential step in their management. Both species are distinguishable based on their morphology and morphometrics as previous studies have differentiated them as “giant” race and “normal” race. However, this traditional means of separately identifying and quantifying these two nematodes is time consuming and labour intensive. Species-specific primers for PCR have been developed to complement the traditional species identification for nematodes; and using sequences of the ITS ribosomal gene such plant parasitic nematodes can be identified (Subbotin and Moens, 2006). By using species-specific primers (Jeszke *et al.*, 2015), the characteristic bands of 270 bp and 147 bp for both *D. gigas* and *D. dipsaci* were obtained, which confirm their morphological identification and the specificity of the primers sets. The sequence comparison of the ITS region clearly separates the *Ditylenchus dipsaci* from *D. gigas*. The results reported here also showed up to 100% similarity between UK populations of both *D. dipsaci* and *D. gigas* and those submitted to the Genbank database on the ITS sequences. These results agree with that of Vovlas *et al*. (2011) who found *Ditylenchus gigas* and *D. dipsaci* populations originating from different regions clustered in two separate groups and had high similarities with individuals within their respective groups.

The qPCR protocol by Jeszke *et al.* (2015) with annealing temperature 63.5ºC failed to detect *Ditylenchus gigas* in this study and only detected *D. dipsaci* with a faint band. Annealing temperatures higher than the published 63.5ºC also failed to detect any of the *Ditylenchus* spp. However, decreasing annealing temperatures resulted in stronger bands indicating positive detection of the respective *Ditylenchus* spp. The optimum annealing temperature under the PCR conditions for this study was 62ºC, at such temperature non-specific detection was also minimised. Although there was faint cross-reaction between DITuniF/DITdigR primers and DNA of *D. dipsaci*, this occurred when using high concentrations of target DNA and is highly unlikely to occur with the concentration of target DNA expected within environmental samples. Jeszke *et al.* (2015) used a Rotor Gene 6000 cycler (Corbett Research) for their analysis while a Bio-Rad CFX96 (Biorad, Hemel Hempstead, UK) was used in this study. Having a different thermocycler and conditions might contribute to the different annealing temperatures, and this further emphasises the need to validate PCR protocols within each laboratory.

The traditional system of diagnosis and quantification of stem nematodes, which is based on extracting plant-parasitic nematodes, requires too much time to process large number of samples. Furthermore, the subsampling methods associated with the traditional system reduces the chance of detecting and correctly estimating the plant-parasitic nematodes. In this study, however, the process of milling homogenises the infested plant materials providing high repeatability between subsamples.

The validity of applying molecular techniques in environmental and controlled studies depends on obtaining representative extracts of nucleic acids from samples which must be of high quality and concentration (Aboul-Maaty and Oraby, 2019). In the current study, CTAB methods were modified with β-mercaptoethanol and yielded high-quality DNA from *Ditylenchus* infested legumes and brassicas. The main complications of plant DNA isolation are associated with the presence of plant derived inhibitors including pectin, polyphenols, polysaccharides and xylan (Schrader *et al.*, 2012). These compounds co-precipitate during the DNA extraction process and then affect DNA quality and quantity. The use of β-mercaptoethanol along with CTAB successfully removes these inhibitors especially polyphenols (Horne *et al*. 2004; Li *et al*. 2007) giving rise to a clear translucent DNA pellet. Hence, based on the quality of extracted DNA in this study, as determined by spectrophotometry, there were very low levels of these inhibitors.

From the preliminary experiment, there were greater variations and cycle threshold values recorded for gDNA samples at 100 ng/ml than there were for samples containing 10 ng/ml for both *D gigas* and *D dipsaci*. The possible reasons could be the presence of high/excess DNA concentration (of both plant and nematodes). DNA is capable of binding to magnesium ions present in PCR reagents to stabilize its own structure. This can effectively hinder the PCR reaction and particularly the function of Taq polymerase (Markoulatos *et al.*, 2002) and in some cases DNA can compete with the target template (Tamariz *et al.*, 2006). While the CTAB protocol in this study was improved to minimise these PCR inhibitors, trace amounts could be present, however their presence would be less of a problem with the lower gDNA concentration (10 ng/ml) used.

Although DNA products of high quality were obtained after applying CTAB to both legumes and brassica plants (second glasshouse samples), this was not sufficient to detect and estimate the quantity of *Ditylenchus* spp. present. There were low/no counts of these nematodes made using the traditional method of nematodes assessment. Such low nematode presence could have been overwhelmed by the high concentrations of plant DNA

in samples. Another possibility is that the qPCR assay has low sensitivity for reliably detecting the DNA of low numbers of individuals of *Ditylenchus* spp. when mixed with DNA from plant samples. This sensitivity fails to compare well with findings reported for other nematode species. Toyota *et al*. (2008) reported that qPCR sensitively detected a single second-stage of the cyst-forming nematodes *Globodera rostochiensis* from 1000 free-living nematodes. Mokrini *et al*. (2013) could detect a single individual of *Pratylenchus penetrans* when mixed with DNA from 80 individuals of *P. thornei*. The latter author used a primer set that include two primers and a TaqMan probe. The application of TaqMan technology in qPCR is more sensitive than the SYBR Green used in this study (Cao and Shockey, 2012; Tajadini *et al.*, 2014). For the subsequent sample set (glasshouse 3), there was a moderate correlation between the cycle threshold and the log count of *Ditylenchus dipsaci*. The results were consistent with what was recorded in the Chapter 5. This indicates a greater potential for molecular application for identifying *Ditylenchus gigas* and *D. dipsaci* in legumes and brassica plant tissues.

In recent years, CTAB, a method originally developed by Doyle and Doyle (1987) for plants, has been used to extract nematode gDNA. CTAB has the property of acting on nematode components as it is a cationic detergent which denatures proteins (enzymes) and solubilizes cell walls and lipid membranes (Heikrujam *et al.*, 2020). The successful application of CTAB method on nematodes have been recorded by de Oliveira *et al*. (2020) and Takeuchi *et al.* (2005) for plant-parasitic nematodes *Meloidogyne incognita* on tomato (*Solanum lycopersicum*) and *Bursaphelenchus xylophilus* on Japanese black pine (*Pinus thunbergia*) respectively. These authors have tested the application on a specific species of nematode and on a plant species. Moreover, the present study examined application of CTAB on detecting *Ditylenchus dipsaci* and *D gigas* across legume and brassica plant species. This is a first report on the application of such molecular procedures for *Ditylenchus* spp. diagnostics.

7.8 Conclusion

This study provides a simple and relatively affordable extraction method (CTAB combined with β-mercaptoethanol) for the extraction of DNA of *Ditylenchus* spp. from plant tissue. This DNA extraction method provided stable and high-quality DNA. The presence of *Ditylenchus dipsaci* and *D. gigas* in plant tissue samples can be quantified using a qPCR, however the assays may not be sufficiently sensitive enough to detect low nematode numbers.

8.0 General discussion

Field beans (*Vicia faba* L.) rank fourth in the world's cool-season legume crop hierarchy after chickpea (*Cicer arietinum* L.), field pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) in terms of total production. They are produced in many agro-ecological regions around the world, with the United Kingdom (UK) being the largest producer in Europe. (FAOSTAT, 2018). Most of UK's production is exported for human consumption or used as feed for animals.

Stem nematodes, such as *Ditylenchus dipsaci* and *D. gigas*, are among the most economically important pests degrading the quality and quantity of field bean global production (Hooper, 1984) and pose a serious threat during wet seasons. The use of seed that comes from infested stock can cause problems for several generations. Similarly, this nematode pest infests soils, complicating the establishment of new crops of field beans (Stawniak, 2011). In addition, the presence of stem nematodes in seeds is a primary reason for rejection of seeds for cultivation and processing for human consumption (Smith, 2010).

Field bean production has come under threat due to regulatory changes around pesticide usage. Following the implementation of EU pesticide regulation (EC)1107/2009, a number of major pesticides and pesticide classes were withdrawn from commercial use. Pesticides that pose a threat to biodiversity or are hazardous to humans have been prohibited. Alternative management options for *Ditylenchus* spp. in field beans are extremely limited. Farmers in the UK do not have the option of planting resistant cultivars, and crop rotation is less effective because *D. dipsaci* can reproduce on a wide variety of crops. There is, therefore, few recommended management measures available for field bean farmers at present. The demand for a safe, sustainable, and environmentally-friendly approach to managing these pests is highly sought after.

Biofumigation is a pest management method that uses plants, mainly those belonging to the Brassica family that produce glucosinolates. The glucosinolates in these plants are broken down enzymically when tissue damage occurs, therefore releasing various compounds, including isothiocyanates (ITC). Several pests and diseases are suppressed by ITC, including plant-parasitic nematodes (Ngala *et al*. 2015).

Hence, the aim of this thesis was to determine whether biofumigation is effective as a pest management method for *Ditylenchus* spp. in field beans. The experiments commenced with *in-vitro* experiments, which were designed to determine the effect of ITC on the mortality and

host finding behavior of *Ditylenchus* spp. Glasshouse experiments were conducted to find out if brassica biofumigants served as hosts for *Ditylenchus*. Afterwards, two field experiments conducted between 2017 and 2018 assessed the application of biofumigation in fields with a history of stem nematodes.

Natural ITC are toxic to a range of soil-borne pests and pathogens, including nematodes and they are the active ingredient in a biofumigation system. The ITC concentrations used in this study are thought to be comparable to levels achievable in the field as several studies have shown that high glucosinolate levels (resulting in high isothiocyanates) in various Brassica cultivars can suppress soil borne pests (Matthiessen *et al*., 2004; Gimsing and Kirkegaard, 2006, Wood *et al.* 2018). At 50 µg/ml concentration, the tested ITC, except for sulforaphane, caused over 70% mortality to *Ditylenchus gigas* and *D*. *dipsaci* within 72 h. The aromatic ITC particularly 2-phenethyl ITC had the most impact on the mortality for both species. Similarly, the concentration of ITC required to cause 50% mortality (LD_{50}) of the stem nematodes *D. gigas* and *D. dipsaci* were lower for aromatic ITC (2-phenethyl ITC and benzyl ITC) than aliphatic allyl ITC. This has implications for the choice of brassicas that will be suitable for biofumigation against *Ditylenchus* spp. The aromatic ITC 2-phenethyl ITC is derived from gluconasturtiin which is found mainly in the roots of brassicas such as *Brassica juncea* and *B. carinata*. On the other hand, sinigrin, a precursor to allyl ITC, is mainly found in the shoots of same brassicas (*B. juncea* and *B. carinata*). *Brassica carinata*, *B. nigra* and *B. juncea* generate a minimum of 50 µg/ml of allyl ITC (Bellostas *et al*., 2007) and would be suitable choice in the management of *Ditylenchus* spp. The parent glucosinolate for benzyl ITC is glucotropaeolin and is found in the roots of *Sinapis alba*. Glucosinolate profile and concentration was the basis for the selection of brassica plants used in field experiment (Chapter 6). Sulforaphane, on the other hand, failed to have nematotoxic activity on both *D. gigas* and *D. dipsaci* at the tested concentrations (Chapter 3). This suggests that structural differences among ITC relates to their ability to interact with the proteins of the nematodes investigated.

One limitation of the mortality assays described in Chapter 3, is their effectiveness in assessing stem nematodes that were dead or alive. Stem nematodes were assessed based on their response to stimuli from a probing needle (up to 7 times) and were considered alive following any movements, however slight. This approach could underestimate the efficacy of the ITC, and the concentrations tested on the activity of nematodes. For instance, a previous report observed decreased *Meloidogyne incognita* J2 activity at sub-lethal concentrations of benzyl ITC and that resulted in decreased egg production (Zasada *et al.*, 2009). This

highlights the need to conduct further investigation to fully understand the effects of ITC on the activities of nematodes. Following completion of the mortality assays, it was hypothesized that pre-exposure to ITC may inhibit the movement of stem nematodes towards their host.

Host attraction and movement towards hosts is vital for the survival of stem nematodes. In Chapter 4, a series of *in vitro* bioassays were conducted to test the effects of ITC on the movement and chemotaxis of *Ditylenchus* spp. At a sub-lethal concentration of 25 µg/ml, allyl ITC, benzyl ITC and 2-phenethyl ITC inhibited the ability of *D. dipsaci* and *D. gigas* to migrate towards the roots of field beans, while sulforaphane had no effect on them. Moreover, at a lower concentration of 12.5 µg/ml, allyl ITC had a greater effect in inhibiting migration compared to 2-phenethyl ITC and benzyl ITC at the same concentration, whereas nematodes exposed sulforaphane were unaffected. Nematodes possess amphids in their anterior region; these are important organs that function as chemoreceptors, which receive signals (such as through compounds in plant root leachates) from their host. Consequently, nematodes orientate towards plant root leachates and therefore propel themselves by undulating their body in response to the stimuli (Croll, 1970). The results of this attraction and mobility assays suggest that sub-lethal ITC concentrations such as 25 µg/ml allyl, 2-phenethyl and benzyl ITC are responsible for reducing movement and disrupting orientation of nematodes (Wu *et al*., 2014). Microscopic observations of treated stem nematodes revealed that sub-lethal-ITC caused distortions of their habits and normal movements. This finding has important implications for biofumigation as sub-lethal-ITC concentrations may be achieved under certain conditions in the field. For instance, partial-biofumigation, a term that describes biofumigant effects that occur during active plant growth and is associated with glucosinolates that are leached from the roots of brassica plants. The leached glucosinolates are thereby hydrolysed in the soil by myrosinase-producing organisms, releasing ITC as the brassica plants grow (Sakorn *et al*., 1999). In this study, the stem nematodes were exposed to sub-lethal-concentrations for 24 h, such exposure time was sufficient to inhibit stem nematode function and movement to its host root. This leads to the hypothesis that the period from which brassica plants are established to the time they are incorporated (≈ 12 weeks) may be sufficient for suppressing stem nematodes.

The choice of brassica cultivars selected for the two field experiments were informed by the sensitivity of *D. gigas* and *D. dipsaci* to ITC under *in-vitro* conditions and the ability of brassica species to produce high amounts of glucosinolates of the selected ITC. Two field experiments were performed to assess whether brassica biofumigants can suppress stem nematodes of field beans (Chapter 6). Positive results against stem nematodes *D. gigas* were observed with all cultivated brassicas (*Brassica juncea, B. carinata* and *Sinapis alba*)

whereas no effect was seen with *D. dipsaci* (Field experiment 2). Reductions in population densities of *D. gigas* may have been due to other factors since the glucosinolates levels generated were lower than expected. For example, sinigrin production pre-incorporation (plants at 50% flowering) was 13.12 and 6.19 mg/g for *B. juncea* and *B. carinata* respectively, while glucotropaeolin was measured at 2.95 mg/g for *S. alba*. Additionally, the amount of glucosinolates which are converted to ITC, 24 hours after incorporation in soil, is expected to be as low as 1% (Morra and Kirkegaard, 2002). The lowest glucosinolate concentration that was required to produce at least 50 µg/ml ITC (the effective concentration), assuming a 1% conversion, would be 5 mg/g. Using the 1% conversion, the glucosinolates concentrations recorded in this study would be expected to release allyl ITC at ≈130 and ≈62 µg/ml, from *B. juncea* and *B. carinata* respectively, and benzyl ITC at ≈29.5 µg/ml from *S. alba*. Results from Chapter 3 showed that 50 µg/ml allyl ITC and benzyl ITC was required to cause stem nematode mortality after 24 h. Under field conditions, the highest concentrations of ITC are typically produced immediately after macerating brassica tissue (2-5 h) and concentrations dissipates afterwards (Gimsing, Anne L. *et al.*, 2009). As such, the *in-vitro* mortality data is not directly comparable to the stem nematode suppression observed in the field. Additionally, reductions in stem nematode numbers may also be attributed to the release of other accompanied compounds, such as alkenals and alkenols (Potter *et al*., 1998) or sulphur-containing compounds such as dimethyl-disulphide (Bending and Lincoln, 1999). Similarly, stimulation of nematode antagonists by brassica residue incorporation may also contribute to the reduction observed (McLeod and Steel, 1999). In this study, the suppression of *D. gigas* is also attributed to the green manure effect. Nematode suppression was achieved in field experiment 2 with over nine-fold greater biomass (e.g., *B. juncea*) compared to the same brassica biomass obtained in field experiment 1, without stem nematode reduction. This concept has been reported previously for the suppression of plant-parasitic nematodes *Trichodorus* spp. and *Tylenchorhynchus* spp. after *B. juncea* incorporation (Vervoort *et al.*, 2014).

Even though the brassicas and their mixture with *Vicia villosa* tested in field experiment 1 failed to suppress stem nematodes because of low biomass generated, a few deductions may be drawn from it. The brassicas sown in a mixture with hairy vetch (*V. villosa*) appear to have produced better biomass, considering the seed rate was half that used where brassicas were sown alone. This indicates that there is a benefit to mixing brassica with legumes since the legumes provide a source of nitrogen through rhizobia mediated fixation. Moreover, the biofumigation efficiency of brassica species is preserved (i.e. biomass levels and glucosinolates levels) in a brassica- legume mixture (Couëdel *et al*., 2019). This implies that

the use of brassica-legume in agricultural systems is beneficial because they will manage pests well and improve soil health.

The use of brassica cover crops can result in the increase in plant parasitic nematode population densities. This has been reported for several nematodes, including *Meloidogyne* spp., but there is limited information in the literature on brassicas that host *Ditylenchus* spp. Host range evaluation studies were required to determine if brassica plants were suitable as hosts for *D. dipsaci* and *D. gigas* in order to devise appropriate brassica species in a biofumigation systems. In theory, biofumigation of land infested with stem nematodes will succeed if the selected brassicas do not support reproduction of plant parasitic nematodes (Ntalli and Caboni, 2017). Literature has reported that the *D. dipsaci* is able to develop on a wide host range from different plant families. Hence, it was hypothesized that, some brassicas may be hosts for *Ditylenchus* spp., and as such, would not be suitable for use as biofumigants. Three experiments were conducted to determine the host suitability of *D. gigas* and/or *D. dipsaci*. The initial methods used for this study were based upon the recommendations of Teklu *et al.* (2014), whereby soil is inoculated with the nematodes to mimic natural conditions in the field. This method, however, was found to be unsuitable due to the low recovery of nematodes from the soil and plants tested. Instead *Ditylenchus* spp. were introduced to brassica seedlings in a growth chamber with a high humidity. The modifications made in these experiments highlight the importance of optimising host status study protocols in order to limit the possibility of false negative results
Pot experiments using various brassica plants (Chapter 5) demonstrated that *Sinapis alba* and *Eruca sativa* were suitable host of *Ditylenchus dipsaci* but not *D. gigas*. However, *Brassica juncea, B. carinata* and *Raphanus sativus*, were poor hosts to the two *Ditylenchus* spp. making them preferable candidates for biofumigation against *Ditylenchus*. Clearly these results have important implications to growers of field beans who are considering biofumigation for the management of stem nematodes.

From Chapters 3 and 5, there appears to be a relationship between brassicas that are suitable hosts of *Ditylenchus* spp. and the sensitivity of stem nematodes to ITC associated with the same brassicas. Brassica species that were able to host *Ditylenchus* spp. were also found to produce ITC that were less toxic to them. For instance, glucoraphanin, the glucosinolate precursor, for the ITC sulforaphane is found in the brassica species *Eruca sativa* (Ntalli, 2016; Aissani *et al*., 2015, Lord *et al*., 2011), which is also known as hosts to *D. dipsaci* (Chapter 5). However, *Sinapis alba* is a suitable host for *D. dipsaci* (Chapter 5) but is a producer of benzyl ITC, which is toxic to stem nematodes (Chapter 3). This could be

possible because for host suitability study, stem nematodes were inoculated on the shoot of *S. alba* and reproduced successfully. The shoot of *S. alba* is known to generate glucosinolates that produce unstable ITC which are not toxic to nematodes. However, benzyl ITC, which is toxic to nematodes is associated with a glucosinolate produced in the roots of *S. alba*. Stem nematodes can use water films on their host to migrate up and infect the aerial region. This could be their strategy to escape toxic substances produced from their host roots. Therefore, it could be hypothesized that stem nematodes have developed tolerance to certain ITC and are able to avoid plant organs generating toxic volatile compounds.

It is essential that nematodes can be recognized without hesitation for management practices to be successful. In Chapter 7, molecular assays were utilized to study the populations of *Ditylenchus* spp. There was successful application of qPCR to determine relative quantity of *Ditylenchus* spp. on plant samples. Moreover, the modified genomic extraction (CTAB method) protocol used in this study can facilitate the process of diagnosing the samples quickly and efficiently.

In summary, this thesis shows ITC (allyl, 2-phenetheyl and benzyl) can cause the death of *Ditylenchus dipsaci* and *D. gigas* and at sub-lethal concentrations, they affect their orientation and host finding ability, but such was not the case for sulforaphane. In addition, soil incorporation of brassica plants is a feasible management measure against stem nematodes in field beans. However, some brassica plants such as *Sinapis alba* and *Eruca sativa* are host to *Ditylenchus dipsaci* and may not be suitable for such management program.

8.1 Further work

This study has demonstrated the potential of using brassica in a biofumigation system against *Ditylenchus gigas* in field beans. Farmers may benefit from the information provided in this thesis, although further research is recommended to answer practical questions.

During the field studies, the biofumigation succeeded in suppressing *D. gigas* but not *D. dipsaci*. First, it would be interesting to investigate different varieties of *Brassica* spp. other than the ones used in the study for their suppressive effect on both stem nematode species. This would avoid the situation where one pest species is suppressed while the other is not. There is also a risk that some biofumigant brassicas could act as a host for stem nematodes, particularly for *D. dipsaci* that has a wide host range. Screening more brassica plants to determine whether they are suitable host of stem nematodes is recommended as the search for suitable brassica plants for managing stem nematode pest are conducted.

Results from Field Experiment 1 (2017) indicated the benefit of brassica-legume mixtures. Further research should investigate which cover crop combinations provide the best suppression of stem nematodes. Furthermore, biofumigation needs to be considered in the framework of integrated pest management systems for stem nematodes. Even when ITC release is low, the green manure effect may have substantial effect on stem nematodes. Determining the synergistic role of different management measures could provide maximum benefit for stem nematode suppression.

Plants invest up to 40% of their photosynthetically obtained carbon in root diffusates (Badri and Vivanco, 2009). These can be exploited for use in nematode management. Part of the root diffusates of brassica plants are glucosinolates which are released into the soil (Elliot and Stowe, 1971). It has been demonstrated that these glucosinolates can be broken down by myrosinase-producing soil organisms to release concentrations of ITC, toxic to parasitic-nematodes in a process known as partial-biofumigation (Ngala *et al.,* 2015). However, the factors responsible for this has not been fully elucidated. Since root exudation occurs mainly at the root meristematic apex, comparing the root architecture of various brassicas and selecting those with more fibrous structure may provide efficient distribution of their glucosinolates. This will target regions where nematodes are and provide maximum nematode suppression.

Finally, this study has shown the effect of sub-lethal concentrations of ITC on nematode orientation and movement. Under the influence of ITC, the body form of stem nematodes changes from sigmoid to coil. It is known that stem nematodes coil when under adverse environmental conditions. However, the underlying physiological changes linked to ITC have not been well studied. One study report that ITC stimulate the expression of heat shock proteins, which play a role in stress response, therefore causing excessive energy loss and nematode inactivity (Brolsma *et al*., 2014). It would be interesting to identify the key physiological process behind such ITC bioactivity and understand how it inhibits the nematode nervous system in future research.

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Plant Science into Practice

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23 December 2020

Dear Nasamu

Report of analysis for glucosinolates testing

We have assigned names to the glucosinolates by comparison to oilseed rape and published literature and to the best of our knowledge we believe these to be correct.

Results are below:

Block	1	2	3	4	5	6
Sample type	Ethiopian mustard	Ethiopian mustard	Ethiopian mustard	Ethiopian mustard	Ethiopian mustard	Ethiopian mustard
Sample number	1	4	7	10	13	17
NIAB Labtest sample number	165778	165783	165787	165790	165793	165796
Individual Glucosinolate	micromoles/g	micromoles/g	micromoles/g	micromoles/g	micromoles/g	micromoles/g
Glucoberin	1.70	1.55	0.99	1.27	1.29	1.57
Progoitrin	0.20	0.18	0.14	0.18	0.06	0.02
Sinigrin	6.62	8.07	9.49	8.24	3.22	1.49
Glucobrassicin	0.55	0.48	0.43	0.32	0.11	0.05
Gluconasturtiin	0.66	0.82	1.20	0.91	2.08	0.31
Neoglucobrassicin	0.05	0.06	0.10	0.05	0.05	0.01
Total Glucosinolate	9.78	11.16	12.35	10.97	6.81	3.45

Block	1	2	3	4	5	6
Sample type	White mustard	White mustard	White mustard	White mustard	White mustard	White mustard
Sample number	2	5	8	11	14	18
NIAB Labtest sample number	165780	165784	165788	165791	165794	165797
Individual Glucosinolate	micromoles/g	micromoles/g	micromoles/g	micromoles/g	micromoles/g	micromoles/g
Glucoberin	1.33	1.35	1.46	0.99	1.66	1.31
Progoitrin	0.20	0.17	0.12	0.13	0.05	0.02
Sinigrin	0.00	1.25	0.00	0.00	0.00	0.00
Glucoraphanin	0.31	0.34	0.28	0.26	0.22	0.08
Glucosinalbin	2.24	2.28	3.10	4.30	1.93	0.42
Glucotropaeolin	3.18	3.49	3.38	4.70	2.60	0.33
Gluconasturtiin	0.55	0.46	0.41	0.38	0.47	0.22
Neoglucobrassicin	0.06	0.04	0.05	0.06	0.05	0.00
Total Glucosinolate	7.87	9.38	8.80	10.82	6.98	2.38

Block	1	2	3	4	5	6
Sample type	Indian mustard	Indian mustard	Indian mustard	Indian mustard	Indian mustard	Indian mustard
Sample number	3	6	9	12	15	16
NIAB Labtest sample number	165782	165786	165789	165792	165795	165798
Individual Glucosinolate	micromoles/g	micromoles/g	micromoles/g	micromoles/g	micromoles/g	micromoles/g
Glucoberin	1.77	1.68	1.57	1.28	1.37	1.58
Progoitrin	0.18	0.19	0.14	0.12	0.05	0.06
Sinigrin	11.22	19.65	23.28	14.40	3.64	6.54
Gluconapin	0.11	0.18	0.21	0.13	0.04	0.06
Glucobrassicin	0.08	0.09	0.14	0.09	0.01	0.03
Gluconasturtiin	1.11	3.22	3.27	1.96	0.21	0.66
Neoglucobrassicin	0.09	0.13	0.16	0.12	0.01	0.03
Total Glucosinolate	14.56	25.14	28.77	18.10	5.33	8.96