Utilisation of single and multiple species cover crops for the suppression of soil-borne nematodes of *Narcissus*

A thesis submitted for the award of the degree of Doctor of Philosophy

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By

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GENERAL ABSTRACT

Ditylenchus dipsaci, Pratylenchus penetrans, and *Aphelenchoides subtenuis* parasitise *Narcissus*. Cover crops reduce plant parasitic nematodes through several mechanisms, including non/poor host, allelopathy and trap cropping. This study assessed the impact of cover crops on plant parasitic nematodes associated with *Narcissus* and beneficial nematode communities. Several cover crops were tested under greenhouse conditions for their host suitability to *P. penetrans*, and ten were rated as poor hosts (Chapter 3). Four selected cover crops (French marigold, oilseed radish, alfalfa and forage chicory) were tested in three field experiments (Chapter 6). The abundance of *Pratylenchus* spp., fungivorous and bacterivorous nematodes, was monitored before planting, three months after planting and six weeks post-incorporation. All cover crops significantly reduced *Pratylenchus* spp. and increased the abundance of fungivorous and bacterivorous nematodes (Chapter 6). The impact of cover crops on soil nematode communities and soil food web indices was assessed using High-throughput Sequencing targeting the *18S rRNA* gene (Chapter 7). Cover crop treatments did not impact beta diversity; therefore, no adverse effects on nematode communities. To better understand how cover crops reduce *P. penetrans, in vitro* assays were conducted to assess the nematodes' behavioural responses after exposure to root exudates (Chapter 4). Root exudates from forage chicory and alfalfa did not affect the behaviour of *P. penetrans*. Finally, *in vitro* assays were conducted to test the nematicidal potential of three different brassica isothiocyanates against *P. penetrans* (Chapter 5). Benzyl was the most toxic (LD_{50}=3.2 µg ml^{-1}), 2-Phenylethyl (LD_{50}=5.2 µg ml^{-1}) was the second and lastly Allyl (LD_{50}=9.9 µg ml^{-1}). Collating the results of the experimental work in this PhD thesis strongly suggests that French marigold, oilseed radish, forage chicory, and alfalfa are potential options for managing *Pratylenchus* spp. in *Narcissus* fields without deleterious effects on non-target nematodes. Moreover, *P. penetrans* is associated with various cash crops grown in the UK; therefore, these cover crops could become part of integrated nematode management and in rotations to confer agroecosystem services, such as improved soil fertility.

DECLARATION

I, Vongai Chekanai, hereby declare that the work presented in this dissertation is my own. Any external assistance and relevant sources of information referred to in the report have been duly acknowledged or cited within the text and appropriately referenced.

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RESEARCH OUTPUTS

Scientific publications:

1. Chekanai, V., Neilson, R., Roberts, D., Edwards, S., & Back, M. (2024). *In vitro* nematicidal efficacy of brassica-derived isothiocyanates against the root lesion nematode, *Pratylenchus penetrans*. *Nematology*, *1*, 1-10. https://doi.org/10.1163/15685411-bja10347
2. Chekanai, V., Neilson, R., Clark, M., Edwards, S. G., Roberts, D., & Back, M. (2024). Management of and future perspectives on plant parasitic nematodes associated with Narcissus grown in the UK. *The Journal of Horticultural Science and Biotechnology*, 1-10. https://doi.org/10.1080/14620316.2024.2380398

Media/Blogs:

1. Bayer Crop Science UK blog feature: https://cropscience.bayer.co.uk/blog/articles/2023/03/cover-crops-for-pcn-and-other-plant-parasitic-nematodes-which-species-work
2. Farming UK blog feature: https://www.farminguk.com/news/project-to-tackle-plant-parasitic-nematodes-in-daffodils-and-potatoes_60260.html
3. Scottish Agronomy blog feature: https://scottishagronomy.co.uk/cover-crops-for-pcn-and-other-plant-parasitic-nematodes- which-species-work/

Oral presentations

1. Harper Adams Annual Research Conference: Utilization of single and multiple species cover crops for the suppression of soil-borne nematodes of *Narcissus,* (3 September 2024).
2. Hutchinsons Potato Day, A H Worths farms - “Responses of root lesion and free-living nematodes to cover cropping” (17 July 2024).
3. 35th ESN Conference, Cordoba Spain: Response of *Pratylenchus* spp., *Aphelenchus, Aphelenchoides* and bacterivore nematodes to cover cropping in *Narcissus* fields, (17 April 2024).
4. James Hutton Institute Annual PHD Event: Developing a cover crop scheme for managing plant parasitic nematodes in UK-grown *Narcissus* (Daffodils), (7 March 2024).
5. AAB Advances in Nematology, London - “Responses of plant parasitic and free-living nematodes to cover cropping in *Narcissus* fields” (7 December 2023).
6. Hutchinsons Potato Day, A.H Worths farms - “Responses of root lesion and free-living nematodes to cover cropping”. (9 July 2023).

7. James Hutton Institute PHD Annual event 2023, Dunkeld "Responses of plant parasitic and free-living nematodes to cover cropping in *Narcissus* fields". (7 March 2023).
8. Student Colloquium Conference, Harper Adams University - "Host plant status of selected cover crops to root-lesion nematodes (*Pratylenchus penetrans*)" (3 December 2022).
9. AAB Advances in Nematology, London - "Host plant status of selected cover crops to root-lesion nematodes (*Pratylenchus penetrans*)" (7 December 2022-online).

Poster presentations

1) James Hutton Institute Ecological Sciences Departmental Review, Dundee, (26-27 February 2024) "Responses of plant parasitic and free-living nematodes to cover cropping in *Narcissus* fields".
2) PhD Student Annual Colloquium 2022, Harper Adams University - "The potential of brassica isothiocyanates for controlling root-lesion nematodes (*Pratylenchus penetrans*) under in vitro conditions" (3 December 2021)

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LITERATURE REVIEW

Chapter modified from: Chekanai, V., Neilson, R., Clark, M., Edwards, S. G., Roberts, D., & Back, M. (2024). Management of and future perspectives on plant parasitic nematodes associated with *Narcissus* grown in the UK. *The Journal of Horticultural Science and Biotechnology*, 1-10. DOI: 10.1080/14620316.2024.2380398.

This Chapter reviews the current status of nematodes associated with *Narcissus* in the UK, their pathogenicity and management, and highlights areas of potential research towards sustainable management of nematodes in *Narcissus*.

1.1 INTRODUCTION

Narcissus is a monocotyledon native to Spain but has been naturalised in many regions of the world, with over 40 species grown as ornamental plants for flower and bulb production (Akram *et al.,* 2021). Above and beyond their ornamental/decorative use, *Narcissus* bulbs contain numerous alkaloids with pharmaceutical properties, such as galantamine, and other alkaloids with antimalarial, antifungal, (Hotchandani and Desgagne-Penix, 2017) antiviral and antitumor properties (He *et al.,* 2015). Over 70% of global *Narcissus* production is in the UK and the Netherlands (Hanks, 2013). In the UK, *Narcissus* is grown in the Isles of Scilly, Lincolnshire, Cornwall, Cambridgeshire, Wales, and Scotland, with a total area under cultivation of 4400 ha (DEFRA, 2023). The UK *Narcissus* sector is valued at over £110 million and includes two markets, one of cut flowers and another through exportation of bulbs to North America, Germany, Scandinavia, and France (DEFRA, 2023; Hanks, 2013). Figures for economic crop losses due to plant parasitic nematodes (PPNs) are a work in progress for most crops, yet estimates are essential for policy development, resource allocation and research prioritisation. The losses in the UK *Narcissus* sector due to PPNs are unquantified. Losses occur from direct nematode damage, nematode-disease complexes, loss of revenue from export due to contamination by quarantine nematodes, and loss of bulb viability due to hot water treatment.

In the UK, *Narcissus* is planted in September, and new bulblets are initiated from the mother bulbs. These grow rapidly over 3-4 months in spring and summer and then slowly in autumn and winter (Lillywhite *et al.,* 2017). Growers in different areas of the UK specialise in either flower or bulb production (Lillywhite *et al.,* 2017). The warm Gulf Stream influences Cornwall, giving rise to the highest mean annual temperature (MAT) of 11.5 °C recorded in the UK, where *Narcissus* is mainly grown for cut flowers. Growers in Lincolnshire and Cambridgeshire (9.5-10.5 °C MAT) focus on cut flower and bulb production at commercial scale. Scotland, which has a temperate maritime climate and a MAT ranging from 8.6-10.3 °C, mainly produces bulbs for export (Lillywhite *et al.,* 2017). In terms of production scale, Cornwall has a mixture of small- and commercial-scale growers. The two largest producers of *Narcissus* flowers in the UK are based in Cornwall.

Narcissus is impacted by several pests and pathogens, including insects and nematodes, which cause direct damage and mediate disease complexes or bulb contamination (Hanks and Chastagner, 2018). The most impactful nematodes are *Ditylenchus dipsaci* (commonly referred to as stem and bulb nematode)*, Pratylenchus penetrans* (root lesion nematode) and *Aphelenchoides subtenuis* (leaf and bud nematode). In addition, species of *Trichodorus, Paratrichodorus,* and *Longidorus* have anecdotally been reported to transmit viruses to *Narcissus*. At the same time, *Globodera pallida* and *G. rostochiensis* (potato cyst nematode, PCN), which can inhabit soils adhering to bulbs, are a concern because contamination can render the bulbs ineligible for export to several countries due to phytosanitary concerns associated with local potato production. Furthermore, *P. penetrans* and *A. subtenuis* are associated with fungi such as *Cylindrocarpon destructans* and *Fusarium* spp. leading to disease complexes (Hanks, 2013).

Management of nematode populations in *Narcissus* production is constrained by the lack of nematode-resistant varieties, bulk, and mechanised handling of bulbs with minimal inspection, sorting and sharing of equipment used for potatoes (a host for PCN) (Hanks, 2013). Moreover, UK growers contemporarily leave *Narcissus* bulbs in fields for two or more years instead of lifting them annually, which provides nematodes with a continuous host and thus, bulbs are not subjected to annual hot water treatment (Hanks, 2013; Lillywhite *et al.,* 2017). Further complexity is driven by nematode biology; for example, *D. dipsaci* undergoes anhydrobiosis and survives for up to 20 years without host plants (Perry and Moens, 2011). Thus, the management of PPNs is challenging once a field is infested. This problem is compounded by the lack of available land for *Narcissus* production, leading to sub-optimal short crop rotations. Moreover, changes in assessing plant protection products from risk- to hazard-based criteria (Regulation (EC)1107/2009) have resulted in no nematicides currently available in the UK with label permission for use on *Narcissus*. As such, it is imperative to identify alternative nematode management strategies to support a continued and sustainable *Narcissus* industry in the UK.

1.1.1 Pests and diseases of *Narcissus*

Major pests in narcissus fields include nematodes (*D. dipsaci*. *P. penetrans*), narcissus flies (*Merodon equestris, Eumurus strigatus, E. tuberculatus*), bulb scale mite (*Stenotasonemus laticeps*), bulb mites (*Rhizoglyphus* and *Histiostoma* spp.) and *Norellia spinipes*, the narcissus leaf miner (Hanks and Chastagner, 2018). Large and small narcissus flies are the most significant pests, while mites usually attack pre-damaged bulbs, with most found in stored bulbs. Although a minor problem, moths (*Hepialus* spp.), which cause holes in the outer scales of bulbs, and virus vector aphid species are common in *Narcissus* fields. Slugs and snails cause minor damage to foliage, except keel slugs, which attack bulbs and may transmit virus diseases between plants (Hanks, 2013).

Fusarium oxysporum f.sp. *narcissi* pathogen causes basal rot a major disease of *Narcissus*. Another disease is 'neck rot', similar to basal rot symptoms except that it starts at the top instead of the bottom of the bulb (Hanks and Carder, 2003). Several fungal species have been isolated from bulbs with neck rots, including *Botrytis narcissicola, Penicillium* spp., *and Stagonospora curtisii*. The foliage of narcissus plants is also attacked by other fungal species, predominantly *B. narcissicola*, which contributes 10% yield losses, *Ramularia vallisumbrosae*, found mainly in Cornwall, and *Sclerotinia polyblastis,* which is primarily found in the Netherlands (Hanks and Chastagner, 2018). Fungicide spray programmes have been developed to treat fungal foliar diseases, although basal rot is insensitive to the available treatments (Hanks, 2013).

Bacterial pathogens rarely attack *Narcissus*; however, *Pectobacterium carotovorum* causes basal rot in *Narcissus* varieties such as Paper White. This pathogen can render the crop of whole fields unsaleable, with symptoms including soft rot, blackened tissue, and foul-smelling plants (Hanks and Chastagner, 2018). Sanitation is the primary management option for this disease. *Pseudomonas* species cause bacterial streaks that result in vascular discolouration of bulbs and collapse of stem bases in the field. Dipping the bulbs in antibiotics and storing them at temperatures below 2 °C helps reduce the disease burden (Hanks, 2013).

1.2 NEMATODES OF *NARCISSUS*

1. 2.1 Stem and bulb nematode (*Ditylenchus dipsaci*)

Ditylenchus dipsaci is a polyphagous nematode species associated with c. 500 plant species belonging to approximately 50 angiosperm families. *Ditylenchus dipsaci* infects several crops including *Narcissus* spp., alfalfa (*Medicago sativa* L.), onion (*Allium sepa* L.), potato (*Solanum tuberosum* L.), broad bean (*Vicia faba* L.), carrots (*Daucus carota* L.), garlic (*Allium sativum* L.), sugar beet (*Beta vulgaris* L.), pea (*Pisum sativum* L.), sunflower

(*Helianthus annuus* L.), and oat (*Avena sativa* L.) (Bridge and Starr, 2007; Tenuta *et al.,* 2014). Extensive intraspecific variation exists in the nematode phenotype, with more than 20 recognised races of *D. dipsaci* (Subbotin *et al.,* 2005). The three races known to infect *Narcissus* in the UK are the *Narcissus*, tulip and hyacinth races, the latter being rare but occasionally reported in the southwest of England. *Ditylenchus dipsaci* is listed as an EPPO A2 quarantine pest.

Ditylenchus dipsaci has separate males and females, and the life cycle starts with an egg, followed by juvenile stages (1-4) and an adult. Reproduction occurs via amphimixis, and mating occurs in the host plant tissues rather than the soil (Hanks, 2013). The duration of the life cycle of *D. dipsaci* is 19-23 days at 15 °C with a life span ranging from 45-73 days (Gubina, 1982). Females are highly fecund, laying up to 200-500 eggs deposited in clutches within the plant host (Yuksel, 1960). The largest egg clutches are mainly found in the stem parenchyma tissue, the preferred feeding site for *D. dipsaci*. *Ditylenchus dipsaci* is spread by soil and seed, with the fourth-stage juveniles (J4) being the most infective stage. The J4 survives in both soil and infected plant tissues in an anhydrobiotic state; moves through the soil along moisture films, especially after irrigation or rainfall, and invades the host via leaf stomata, lenticels at the stem bases, buds, or wounds (Cook and Yeates, 1993). Movement within the plant can be intercellular or intracellular; nematodes feed on the stem and leaves and migrate up the plant passively as the plant grows (Seinhorst, 1950). Upon successful invasion, nematodes begin feeding on the cell contents and secrete pectinase, which dissolves cell walls, leading to cell separation, cell enlargement and tissue malformation (Riedel and Mai, 1971). Infected tissues with a collapsed middle lamella are typically soft and fleshy with a glistening, mealy texture (Duncan and Moens, 2013). When infected, *Narcissus* bulbs cut transversely exhibit brown concentric rings and, when cut longitudinally, have necrotic lesions originating from the neck of the bulb. Leaf symptoms are typically pale yellowish swellings that appear as blisters or spickels (Figure 1.1). Secondary infections can occur due to fungi, the small *Narcissus* fly, or bulb-scale mites (Hanks, 2013). Patches of weak and stunted plants can usually be observed from the second year onwards, and ‘eelworm wool’ can erupt from the bulb's basal plate or neck (Hanks, 2013). Once the heavily infested bulbs completely rot, some nematodes leave the host bulb and migrate to attack adjacent bulbs (Gratwick, 1992). Other nematodes do not develop past the preadult stage J4; they aggregate and coil to form ‘eelworm wool’ as a survival strategy where they lie dormant for over 20 years (Jones *et al.,* 2013; Sturhan *et al.,* 2015).

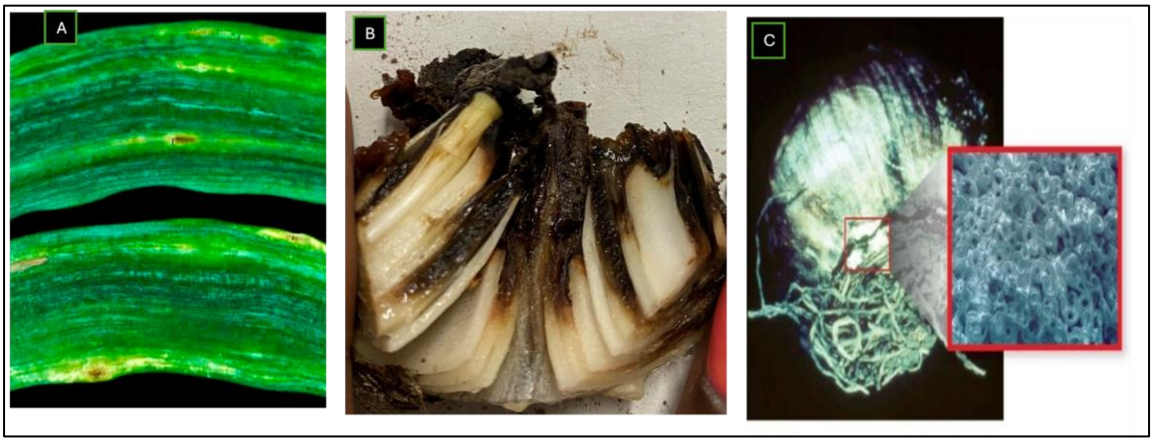


Figure 1.1. A) Symptoms on *Narcissus* leaves are shown on the left as pale yellowish swellings that appear as blisters or spickels (Byther, 2024). B) Brown concentric rings observed after a bulb was cut transversely and conversely, and C) ‘Coiled, clumped eelworm wool, *D. dipsaci* fourth-stage juveniles eelworm wool’ are seen erupting from the neck of the bulb, under a transmission electron microscope (courtesy of Roland N. Perry).

As with other nematode species, *D. dipsaci* can be transferred from one field to another by infected *Narcissus* bulbs or contaminated farm equipment (Boag, 1985). Nematodes can also be distributed via debris transferred from post-harvest bulb storage facilities. Bulbs infected with *D. dipsaci* deteriorate even in storage at 18-22°C (Winfield and Hesling, 1966), where nematode reproduction can continue (Webster, 1964); therefore, bulbs are lifted early and hot water treated before planting (Hastings and Newton, 1934; Horton, 1958; Slootweg, 1962). Furthermore, *D. dipsaci* can be introduced via either livestock faeces if a field is used for grazing or field application of compost, as desiccated J4 nematodes can remain viable in the intestines of pigs, cattle, and composting methods (Palmisano *et al.,* 1971).

Soil type and conditions influence the occurrence of *D. dipsaci* in fields. The movement of *D. dipsaci* was greater in loamy soils than sandy soils (Seinhorst, 1950) while *P. penetrans* is commonly found in sandy soils (Castillo and Vovlas, 2007). Temperature greatly influences the reproduction of this nematode; for instance, Griffith *et al*. (1997) observed an increased penetration rate of *D. dipsaci* females on red clover from 4% at 4 °C to 12% at 20°C. The maximum activity of *D. dipsaci* under UK conditions is between spring and autumn (Evans *et al.,* 1993), though a changing climate may alter this behaviour.

Ditylenchus dipsaci is frequently undetectable when present in fields in low abundance. Detection of *D. dipsaci* in the soil depends on environmental conditions, with the highest populations observed after rainfall events (Wallace, 1962). Sampling depth is an essential consideration as results have been variable, with *D. dipsaci* reported concentrated at 5 cm depth in a field survey of garlic (Blauel *et al.,* 2021) at 6 cm depth in fallowed soil (Lewis and Mai, 1960), and at 10 cm depth in both oat (Wallace, 1962) and alfalfa fields (Tseng *et al.,* 1968).

The damage threshold associated with *D. dipsaci* in *Narcissus* is poorly documented, but Seinhorst (1950) suggested that 2-5 *D. dipsaci* nematodes per kg of soil lead to significant yield losses. Furthermore, Hesling (1972) established that inoculating healthy bulbs with at least four *D. dipsaci* led to severe damage and destruction of *Narcissus* bulbs within a growing season.

1.2.2 Root-lesion nematodes (*Pratylenchus penetrans*)

Pratylenchus spp. are widely distributed in tropical to cool and temperate regions and are highly polyphagous, infecting up to 350 plant species, including cereals, pulse crops, coffee, potatoes, bananas, sugarcane, ornamentals, and *Narcissus* (Castillo and Vovlas, 2007). Of the 101 *Pratylenchus* spp. identified (Janssen *et al.,* 2017), only *P. penetrans* is known to be associated with *Narcissus*. In the UK, *P. penetrans* was recorded in carrots, potatoes, peas, beans, cereals, and permanent pasture (Boag, 1979, 1980; Boag and Lopez-Llorca, 1989; Boag *et al.,* 1990; Orlando *et al.,* 2020; Southey, 1959).

In the UK, *P. penetrans* occurs with other *Pratylenchus* species, including *P. thornei, P. crenatus*, and *P. neglectus* (Orlando *et al.,* 2020). However, these species have not been recorded in *Narcissus*. The evidence of *P. penetrans* feeding on *Narcissus* was first reported nearly 70 years ago (Slootweg, 1956); therefore, contemporary host suitability studies are required to evaluate the pathogenicity of this nematode species to modern varieties of *Narcissus*.

Pratylenchus penetrans reproduces through obligatory amphimixis. The life cycle is 54-65 days, with each female producing approximately 16-35 eggs (Turner and Chapman, 1972). The life cycle begins with eggs laid in clusters in plant roots or soil. Eggs hatch, and juveniles moult through four stages before developing into adult nematodes. First-stage juveniles moult within the egg into second-stage juveniles (J2), which remain unhatched and quiescent until stimulated by hatching diffusates from root exudates produced from young host plants (Castillo and Vovlas, 2007). This mechanism is an adaptation to plant parasitism, which prevents young juveniles from depleting energy reserves in search of host plants (Duncan and Moens, 2013). Hatching also depends on temperature, soil moisture and texture, plant species and age (Duncan and Moens, 2013). Juveniles (J2) hatch roughly one week after egg deposition by using their stylet to perforate the eggshell, thus creating a slit through which they exit and immediately migrate towards host roots to infect. All growth stages are vermiform, and except for J1, all other stages can penetrate and reside in the root cortex (Jones and Fosu-Nyarko, 2014). *Narcissus* bulbs form new roots in autumn, and those infected with *P. penetrans* form longitudinally striped lesions (Figure 1.2), which continue to enlarge until the roots are entirely decayed. Plants infected by root lesion nematodes typically show brown to reddish root lesions at the point of nematode entry, movement/migration, and exit. The nematodes are attracted to the apical meristems of the roots, and root penetration causes the attraction of more nematodes. Feeding occurs through repetitive stylet thrusting and the release of salivary enzymes, which degrade cell walls and cause cell death (Duncan and Moens, 2013). Infected *Narcissus* plants are stunted with tiny roots and bulbs, appear patchy and eventually die before bulb lifting (Courtney, 1961). Upon the death of the plant host, nematodes migrate into the soil and spread to nearby hosts (Duncan and Moens, 2013). Data from field and greenhouse experiments have shown that soil-borne populations of *P. penetrans* are lowest between the bulb lifting and formation of roots of the subsequent crop under heavy rainfall (Slootweg, 1956). Entry points of *P. penetrans* on the host plant also facilitate infection by pathogens, such as the *Cylindrocarpon destructans,* leading to the formation of a fungi-nematodes disease complex *called Narcissus* root rot. This is common, especially in the Isles of Scilly and Eastern England (Lane, 1984).

As few as ten *Pratylenchus* nematodes per kilogram of soil can cause severe root rot due to the small root biomass typically formed by *Narcissus* bulbs. These roots are often not replaced during the growing period, while their fleshy nature makes them susceptible to secondary infection by other pathogens (Slootweg, 1956). Treating *Narcissus* with fungicides such as metalaxyl for fungal rots increases *P. penetrans* abundance because the enhanced root growth improves nematode reproduction (Powell, 1971; Wood and Foot, 1982).

Figure 1.2. *Narcissus* roots showing typical symptoms caused by *P. penetrans* attack. Necrotic lesions (shown by red arrow) identify infected roots—picture courtesy of Johnny Visser, Field Crops, Wageningen University and Research (Visser, 2021).

1.2.3 Bulb and leaf nematode (*Aphelenchoides subtenuis*)

Nematodes of the genus *Aphelenchoides* (foliar nematodes) typically feed on aboveground plant parts. However, *Aphelenchoides* can also feed on fungi, making them facultative plant parasites (Hunt, 1993). *Aphelenchoides subtenuis* is atypical because it primarily feeds on the roots of *Narcissus* (Mor and Spiegel, 1993) and has also been found in pseudostems and corms of other ornamental hosts such as *Gladiolus* (Mohammad *et al.,* 2006). The host range of *A. subtenuis* includes onion, garlic, *Narcissus*, crocus, *Gladiolus*, clover, and tulips and was first reported from *Narcissus* bulbs and foliage in the 1930s from the Isles of Scilly (Goodeyi, 1935). Female nematodes lay their eggs in the root parenchymal cells, and juveniles hatch inside the roots, which subsequently collapse (Mor and Spiegel, 1993). While *A. subtenuis* does not cause much direct damage to *Narcissus,* it can form associations with other pathogens, such as *Fusarium* spp., leading to basal plate disease (Mor and Spiegel, 1993). When infected, leaves of *Narcissus* turn yellow, plants may be weak and stunted, bulbs display greyish discolouration, and when cut, brown rings with a waxy appearance can be observed (Goodeyi, 1935). The outer bulbs of infected *Narcissus* plants have blisters and crinkles (Mor and Spiegel, 1993), flowering is reduced, and the plant foliage is yellow and stunted, leading to eventual death. *Narcissus* plants infected by *A. subtenuis* associated with basal plate disease have 55% fewer flowers and a 45% reduction in height (Vigodsky-Haas and Lavi, 1986). To survive desiccation, some species, such as *A. besseyi,* suspend metabolism and ageing by entering an anhydrobiotic state (Huang and Chiang, 1975). Recent unpublished reports in the UK indicate the presence of *Aphelenchoides* spp. in *Narcissus* bulbs (Ailsa, Cargill, “Pers comm”). However, the species was not determined, and there is no clear evidence that the nematode feeds on the bulbs.

1.2.4 Virus vector nematodes and potato cyst nematodes

Trichodorus, *Paratrichodorus* and *Longidorus* spp. are unlikely to cause direct damage to the bulbs but are of economic importance due to being virus vectors (Brunt, 1980; Taylor and Brown, 1981). *Trichodorus* and *Paratrichodorus* species transmit Tobacco Rattle Virus (TRV) mediated by chemical cues (van Griethuysen *et al.,* 2024), while *Longidorus* spp. (needle nematodes) are vectors of Raspberry Ringspot Virus (RRSV) and Arabis Mosaic Virus (ARMV). Although these viruses have been recorded in *Narcissus*, it is unclear whether nematodes acted as vectors (Sturhan, 1999). There are no control options for viruses infecting *Narcissus*; the primary method is rogueing/removal of infected plants.

Globodera pallida and *G. rostochiensis* are parasites of potatoes that create a permanent feeding site called a syncytium inside the plant tissues. Female nematodes feed, grow spherical and eventually break through the roots. After mating and fertilisation, the globose female dies and dries, then tans into a cyst, enclosing next-generation eggs (Jones *et al.,* 2013). The cyst stays in the soil for up to 20 years until environmental conditions become favourable for hatching (Grainger, 1964); consequently, control and eradication are difficult. *Globodera pallida* and *G. rostochiensis* are included in the EU Plant Health Directive 2000/29/EC as A2 quarantine pests (Pickup *et al.,* 2018). Although *Narcissus* is not a known host to PCN, it is impacted by association. For example, in Scotland, *Narcissus* can only be grown in soil that has passed pre-planting soil tests or where potatoes or other hosts of PCN have not been cultivated for at least 12 years. However, bulbs are exempt from these restrictions if they are entirely soil-free and exhibit no identifiable risk of spreading PCN. Growing *Narcissus* for replanting or cut flowers is also exempt from these restrictions.

1.3 CURRENT MANAGEMENT STRATEGIES FOR NEMATODES ASSOCIATED WITH *NARCISSUS*

1.3.1 Hot water treatment

Hot water treatment is a traditional technique to eradicate pests and diseases. For *Narcissus* bulbs, hot water treatment is done in stages. The balance between the optimum temperature to kill the target pathogen and to preserve the bulb from damage is crucial (Hanks, 2013). Fully dormant and lightly infested bulbs are initially held at 22°C and low humidity for one week. The reduced humidity discourages the growth of pathogenic fungi, and the lower pre-warming temperature slows down shoot initiation, thereby preventing heat damage by subsequent higher sterilising temperatures (Hanks, 2013). Nevertheless, this temperature may allow stem and bulb nematodes to enter dormancy (Gratwick and Southey, 1986). The next step is pre-soaking the bulbs in water at 30°C for three hours and sterilising at 47°C for another three hours before immediately cooling for at least 12 hours. Bulbs are left to dry, conditioned and planted when the bulbs are considered sufficiently dry. This reduces the incidence of cross-contamination or re-infection (Gratwick and Southey, 1986). Before legislation to reduce chemical interventions, formalin would have been added to the pre-soak tanks to prevent fusarium rot and kill any active nematodes (Hanks, 2013). Heat is assumed to kill nematodes within bulbs more readily than active nematodes that have escaped into water tanks during hot water treatment (Gratwick, 1992). While this may appear counterintuitive, damaged bulb tissue produces weakly nematicidal compounds that are enhanced by high temperatures. *Ditylenchus dipsaci* can also enter the bulb core and evade treatment (Gratwick and Southey, 1986). Seed bulb fitness is hindered by hot water treatment. Previously, hot water treatment was frequently coupled with fungicide application (Hanks and Chastagner, 2018), but currently, no approved fungicides are available for *Narcissus*. Hot water treatment excludes control of *Pratylenchus penetrans* because the nematodes do not enter bulbs (Slootweg, 1956).

1.3.1 Quarantine and sanitation measures

Ditylenchus dipsaci survives and spreads quickly on bulbs and foliage (Hooper and Southey, 1978); therefore, control is centred on excluding infested materials from uninfested sites. Growers are encouraged to use nematode-free bulbs, preferably those certified with a phytosanitary certificate (DEFRA, 2023). Most *Narcissus* crops will be planted in fields typically independently sampled by regulatory seed inspectors for PCN. Thus, these *Narcissus* crops will be eligible for the high health status of certified stock.

Growers are recommended to conduct regular inspections of crops and remove any infected plants within a one-metre radius to mitigate nematode spread. Disinfecting farm equipment before use on new fields can prevent the spread of *D. dipsaci*. Bulb waste from grading areas should not be returned to the fields or composted (DEFRA, 2023). All *Narcissus* fields require consistent removal of weeds and groundkeeper bulbs, as weeds can increase nematode abundance (Seinhorst, 1950). Farmers burn off foliage to stimulate growth faster and reduce foliage pathogens (Figure 1.3). To reduce costs, sometimes burn-off is combined with ploughing in bulbs and buying new seeds rather than lifting bulbs from old flower fields. However, this practice will exacerbate any existing PPN problem. Incineration of infested plant stocks at the end of the season is also done in bean production to eliminate *Ditylenchus* spp. Burning has minimal effect on *P. penetrans*, as shown by cherry brush burning, where *P. penetrans* at soil depth <15 cm was unaffected (Mai and Parker, 1972).

Figure 1.3: A tractor-driven burner burning off *Narcissus* foliage in early July 2022 in St Mary's, Isles of Scilly, Cornwall (Photograph is the Author’s own).

1.3.4 Crop rotations

Crop rotations should aim to keep the fields free of *D. dipsaci* or other PPN associated with *Narcissus* for at least four years (DEFRA, 2023). In Scotland, bulbs are grown in 1:7 rotations, and onion is not part of the rotation because it hosts both *D. dipsaci* and *P. penetrans*. However, crop rotations typically fail to manage nematodes adequately due to the polyphagous nature of *P. penetrans* and *D. dipsaci* (Jones *et al.,* 2013). Secondly, *Narcissus* is often under monocropping in the Isles of Scilly due to a lack of available land.

1.3.5 Chemical control

The UK *Narcissus* industry previously relied heavily on nematicides such as formaldehyde, aldicarb, thionazin, pirimiphos-methyl, carbofuran, phoxim, methyl bromide, chloropicrin, which, due to Regulation (EC) 1107/2009, are now banned from use. Velum Prime® (fluopyram) and Nemathorin® (fosthiazate) are currently nematicides available in the UK, but neither has label permission for use on *Narcissus*.

1.4 COVER CROPS FOR THE MANAGEMENT OF PLANT PARASITIC NEMATODES

Historically, growing cover crops was implemented to protect or improve the soil. Cover crops reduce soil erosion, enhance nutrient and moisture retention, suppress pests and diseases, and improve soil aggregation (Couëdel *et al*., 2019). Other benefits to the soil, which might encourage nematode suppression, include soil structural effects, plant cover effects and organic matter addition, which create microenvironments favourable to antagonistic flora and fauna (Wang *et al*., 2001).

1.4.1 Modes of action

Cover crops suppress nematodes through allelopathy, have poor/non-host effects, and act as trap crops. Poor or non-host cover crops reduce nematode abundance by starvation. Trap crops allow nematodes to feed but cannot reproduce, and allelopathic crops which produce nematicidal/nematostatic compounds (Ntalli and Caboni, 2017). Some cover crops may suppress nematode populations by multiple mechanisms, independently or simultaneously (Hooks *et al.,* 2010; Grabau *et al.,* 2017). For example, marigolds reduce populations by being poor or non-host plants, through allelochemical production, by trapping nematodes (Pudasaini *et al*., 2008; Wang *et al*., 2001). Susceptible trap crops have been successfully used in managing PCN populations by using susceptible potato varieties (Whitehead, 1992); however, the cost of production makes it not economically viable. Resistant trap crops are more economically viable, and so far, non-tuber-bearing *Solanum* species such as *Solanum sisymbriifolium, Solanum nigrum, Solanum villosum, Solanum linnaeanum* and *Solanum scabrum* have proved to be excellent candidates to manage root-knot nematodes and PCN (Scholte, 2000; Chitambo *et al*., 2019; Perpétuo *et al*., 2021). *S. sisymbriifolium* (sticky nightshade) has the potential to reduce PCN densities by 75-80% in Portugal (Timmermans *et al*., 2005). Similar levels of suppression (85%) were observed if sown around May/June in rotation with potatoes in ongoing field trials in the UK.

1.4.2 Allelopathic plants

Plants from different families produce biocidal secondary metabolites, which directly kill PPNs. Five prominent plant families are important in nematode management. The family Asteraceae produces polythienyls, alkaloids and polyacetylenes; the Brassicaceae produces glucosinolates; Fabaceae and Boraginaceae exude alkaloids; and Poaceae releases glucosides (Thoden *et al*., 2009). *Sorghum bicolor* and Sudangrass (*Sorghum bicolor* subsp Sudanense) produce a toxic secondary metabolite called dhurrin, a type of cyanogenic glucoside, which represents 30% of the dry weight of seedling shoots (Vetter, 2000). Dhurrin is hydrolysed by the enzyme dhurrinase to glucose and p-hydroxy-(S)-mandelonitrile and converted to hydrogen cyanide (HCN). The whole process is called cyanogenesis, and the released HCN is highly nematicidal to *Meloidogyne hapla* (Widmer and Abawi, 2002), *Meloidogyne chitwoodi* (Mojtahedi *et al*., 1993)*,* and *Meloidogyne incognita* (Stapleton *et al*., 2010; Curto *et al*., 2012). *In vitro* assays showed that dhurrin inhibits hatching of *M. incognita* eggs, but no nematistatic effects were observed on J2s (Curto *et al*., 2012). The same study identified hybrids (*S. bicolor* × *S. sudanensis*) as poor hosts to *M. incognita* (reproduction factor <1) in glasshouse experiments. Greenhouse studies showed reduced galling and penetration of *M. hapla* in lettuce plants after sudangrass was incorporated in potted soil before nematode inoculation (Widmer and Abawi, 2002).

Tagetes species (marigolds) possess fungicidal, insecticidal and nematicidal properties (Jankowska *et al.,* 2009; Silveira *et al.,* 2009) and can be used in intercropping or green manure or applied as plant extracts (Buena *et al.,* 2008; Hooks *et al.,* 2010; D'Addabbo *et al.,* 2014; Bhattacharyya, 2017). α-terthienyl produced in roots of *Tagetes* spp. is photoactivated by near UV light (325-400 nm), producing toxic reactive oxygen species. Exposure of dauer juveniles of *Caenorhabditis elegans* and *M. incognita* J2 nematodes to α-terthienyl under dark conditions showed that α-terthienyl penetrates the hypodermis, causing oxidative stress (Hamaguchi *et al*., 2019). Besides these allelopathic effects, *Tagetes* spp. are trap crops for *P. penetrans*, whereby egg hatching occurs, but the juvenile development fails (Pudasaini *et al*., 2008). An aqueous extract of *Tagetes patula* applied to tomato plants inhibited galling by 62.2% and reduced the number of J2 of *M. incognita* by 61.5% (Franzener, 2007). Around 20 years ago, experimental work was conducted in the Isles of Scilly, Cornwall, to evaluate *Tagetes patula* for suppressing nematode populations in

Narcissus. Cultivation of *T. patula* improved *Narcissus* crop vigour for at least five years and reduced *P. penetrans* (Tompsett, 2004). However, the results of this study were inconclusive due to the absence of *P. penetrans* in the untreated control. As such, further research is needed before *Tagetes* can be recommended to growers as a cover crop for *P. penetrans* suppression.

1.4.4 Brassica biofumigants

Biofumigation suppresses pests and pathogens through hydrolysis of biocidal metabolites called glucosinolates (GSLs) produced by Brassicaceous plants after they are chopped and incorporated into the soil (Kirkegaard and Sarwa, 1998). Upon tissue damage of *Brassica* spp., glucosinolates are hydrolysed by myrosinase, and the end products are sulphur-containing biocidal compounds called isothiocyanates (ITCs), nitriles and epithionitriles (Matthiessen and Kirkegaard, 2006). Several other plant families produce glucosinolates, which could potentially be used as biofumigants, but Brassicas are more popular due to their contribution to numerous soil ecosystem services, which include water conservation and soil fertility improvement (Chen and Weil, 2011; Kristensen and Thorup-Kristensen, 2004). The use of Brassicaceae crops effectively manages root-knot (*Meloidogyne*), cyst (*Heterodera* and *Globodera*) and root lesion (*Pratylenchus* spp.) nematodes (Fourie *et al*., 2016). The most common biofumigation crops are *Brassica juncea* (Indian mustard)*, Raphanus sativus* (oilseed radish), *Eruca sativa* (rocket) and *Sinapis alba* (white mustard), and these differ in their ITC production capacities, with *B. juncea* being the highest producer of allyl glucosinolate. Residual GSLs are either weakly absorbed, degraded by microorganisms, leached or mineralised in the soil. ITCs rapidly decline in soils because they have a short duration; their half-life is mostly a few hours. The short duration is linked to chemical nature and physical properties. Unlike GSLs, ITCs strongly adsorb to soil organic matter and react with nucleophilic ions in the soil (Gimsing and Kirkegaard, 2009). The shortest recorded half-life of ITCs in field soil was <10 min (Aissani *et al*., 2013).

1.5 NON-TARGET EFFECTS OF COVER CROPS ON SOIL NEMATODE COMMUNITIES

Cover crops do not selectively affect PPNs in the soil; they act on all soil-living organisms, leading to a shift in soil and aquatic microfauna and microflora. Numerous studies have explored the effects of cover crops on non-plant parasitic nematode communities (Forge *et al*., 2003; Wang *et al*., 2004; Georgieva *et al*., 2005). The abundance and diversity of different nematode trophic groups change after cover crop use (Masson *et al*., 2022). The magnitude of the influence of cover crops on nematode communities is dictated by the functional type and cover crop species (Grabau *et al*., 2017; Gao *et al*., 2020). Cover crops that produce high shoot biomass are associated with increased bacterivores, carnivores and omnivores (Dietrich *et al*., 2021). Green manuring with Sunn hemp (*Crotalaria juncea) increased* predatory and omnivorous nematodes (Wang *et al*., 2001); these nematodes may reduce populations of other nematode groups in soil (Neilson *et al*., 2020). In other studies, bacterivore and fungivore nematodes were stimulated by Sunn hemp (McSorley *et al*., 2008; Wang *et al*., 2008). Soil amended with seed meal of Indian mustard (*Brassica juncea*) did not negatively impact C. *elegans* compared to PPNs (Yu *et al*., 2007). Biofumigation with *Brassica hirta* and *Sorghum sudanense* significantly lowered PPN levels and increased fungivore and bacterivore nematodes. The observed decline in fungivore nematodes after biofumigation is attributed to actual/direct soil disinfestation or an increase in antagonists (Valdes *et al*., 2012).

Oilseed radish improves bacterial decomposition processes, while rye and rapeseed enhance fungal decomposition (Gruver *et al*., 2010). The lower nitrogen content in rye and rapeseed biomass might explain the more fungal-mediated decomposition and fewer dauer larvae or bacterial decomposition. Bacterivorous nematode taxa such as Heterorhabditidae, Diplogasteriana and *Panagrolaimus* are increased by growing cover crops with a low C: N ratio; conversely, the reverse holds for cover crops with a high C: N ratio (Dupont *et al*., 2009). Incorporating vetch root biomass (C: N=8) increased dauer juveniles compared to rye (C: N=22) in greenhouse and field conditions (Georgieva *et al*. 2005). Growing cover crops changes the rhizosphere's soil function and microbial community structures. The consequences of these probable side effects include increasing the abundance and competitiveness of PPNs and other detrimental pathogens (Jin *et al*., 2019; Valdes *et al*., 2012). Assessing the potential non-target effects on soil nematode and microbial communities is crucial before recommending cover crops to growers to maintain the trade-off balance between PPNs and non-plant parasitic nematodes.

1.6 RESEARCH OUTLOOK

This chapter has highlighted the current management options for PPNs associated with *Narcissus* and their limitations. To successfully manage PPNs, extensive investment in breeding resistant varieties remains the most practical and effective strategy. Some varieties of potatoes, clover, alfalfa, fava bean, oats and garlic are partially resistant to stem and bulb nematodes (McDaniel and Barr, 1994; Peng and Moens, 2003; Starr *et al.,* 2013; Mwaura *et al.,* 2015). Thomas (1958) observed differences in susceptibility to *D. dipsaci* penetration among two *Narcissus* cultivars — the foliage of cv. Soleil D’or was more infested than bulbs, whereas, for cv. Carlton, the opposite was true. Nonetheless, this study did not investigate the basis of these observed differences. *Narcissus* has a moderately wide genetic diversity with over 40 species and 26,000 cultivated varieties (Akram *et al.,* 2021), which could provide a primary gene pool for developing elite *Narcissus* varieties with nematode resistance. A current project, "The Scottish Daffodil Project", aims to collect various *Narcissus* samples, sequence extracted foliage DNA, and assemble the chloroplast genome, which will help to understand the diversity of plant traits (Mackintosh *et al.,* 2021). Engagement and collaborations with such projects in the context of nematode-resistant characteristics will provide a starting point for nematode resistance breeding programmes.

Controlled environment pest control practised in the Netherlands, mainly for tulips and lilies, could also be implemented for *Narcissus*. Bulbs are held at 1% oxygen and 4% carbon dioxide, killing nematodes within the bulbs. This is a potential low-cost alternative to hot water treatment (Lillywhite *et al.,* 2017). Determining damage thresholds for nematodes affecting *Narcissus* is arguably necessary to reduce production costs by removing default management interventions. The damage threshold previously reported for *P. penetrans* is based on root rot disease from their interaction with the fungal pathogen *C. destructans* rather than direct nematode damage. Damage thresholds differ with nematode species, cultivar, soil texture, moisture and temperature (Duncan and Moens, 2013). While *A. subtenuis* is associated with *Narcissus* in the UK*,* information on its pathogenicity and impact on bulb yields of commonly grown *Narcissus* cultivars in the UK is undocumented. Furthermore, additional work is required to inform growers of the key symptoms caused by nematodes on *Narcissus*.

Cover crops can potentially suppress PPNs via several mechanisms, including being a non/poor host, trap crops, allelopathic crops, and biofumigants (Osipitan *et al.,* 2019; Van Eerd *et al.,* 2023). In the UK, the fallow period between June and September between *Narcissus* cropping cycles is crucial to breaking PPN cycles, thus depriving a susceptible host and reducing nematode abundance. Growers typically grow Indian mustard (*Brassica juncea*) and ryegrass as summer cover crops, although the benefits concerning nematode suppression in *Narcissus* are unknown. Nevertheless, both cover crops are hosts for *P. penetrans* (Neupane and Yan, 2023; Taning *et al.,* 2024).

1.7 RESEARCH AIM AND OBJECTIVES

This research aimed to explore the potential of different cover crops for managing plant parasitic nematodes of economic importance in *Narcissus* in the UK without harmful non-target effects on beneficial nematode communities.
The specific objectives were:

1. To evaluate the host plant status of different cover crops for *Pratylenchus penetrans* under greenhouse conditions (Chapter 2)
2. To select cover crops that are poor hosts as observed in Objective 1 and assess their effects on important nematodes of *Narcissus*, non-target beneficial fungivore and bacterivore nematodes under field conditions using morphological identification (microscopy). *Pratylenchus* and *Ditylenchus,* and *Aphelenchoides* spp. are difficult to distinguish using morphological features therefore, molecular methods were used for species identification. Additionally, only *P. penetrans* is known to be associated with *Narcissus,* but *Pratylenchus* spp. typically occur as a species mixture in the field. Therefore, real-time quantitative PCR was used to identify and quantify four common *Pratylenchus* spp. (*P. penetrans, P. crenatus, P. neglectus,* and *P. thornei*). *D. dipsaci* and *A. subtenuis* are the reported pathogens of *Narcissus;* therefore, conventional PCR was used to test for their presence. (Chapter 3)
3. To assess the impact of cover crop treatments (selected from Objective 2 for their ability to reduce *Pratylenchus* spp.) on soil nematode communities under the same field conditions using Next-Generation Sequencing (Chapter 4)
4. To investigate the mechanisms by which certain cover crops (forage chicory and alfalfa, identified in Objectives 1 and 2) reduce the abundance of *Pratylenchus* spp. by examining the motility, migration, and chemotaxis of *P. penetrans* in response to root exudates *in vitro.* (Chapter 5)
5. To understand the mechanisms through which oilseed radish reduces the abundance of *P. penetrans*. Oilseed radish is a known brassica biofumigant and reduced *P. penetrans* numbers in greenhouse experiments (Objective 1) and the abundance of *Pratylenchus* spp. in field experiments (Objective 2); therefore, the effects of pure brassica isothiocyanates on motility and mortality of *P. penetrans in vitro* were evaluated (Chapter 6)

CHAPTER 2

HOST SUITABILITY OF SELECTED COVER CROP SPECIES TO A ROOT-LESION NEMATODE, *PRATYLENCHUS PENETRANS,* UNDER GREENHOUSE CONDITIONS

2.1 INTRODUCTION

Pratylenchus penetrans is an important pathogen of *Narcissus*, but its management is constrained by the limited availability of nematicides, and crop rotations are not feasible due to limited land availability (Upcott *et al*., 2023). Cover crops can potentially manage plant parasitic nematodes as poor or non-hosts, which starve the nematodes, thereby providing a non-chemical nematode management option. There have been inconsistent reports regarding the management of *P. penetrans* with a broad range of cover crop species. Cover crops species such as perennial ryegrass (*Lolium perenne*), alfalfa (*Medicago sativa*), pearl millet (*Pennisetum glaucum*), French marigold (*Tagetes patula),* forage sorghum (*Sorghum bicolor*), crown vetch (*Coronilla varia*) and sweet clover (*Melilotus alba*) have been reported as either poor or non-hosts for *P. penetrans* (Miller, 1978; Thies *et al*., 1995; Bélair *et al*., 2002; Neupane and Yan, 2023; Siddiqui and Alam, 1988; Pudasaini *et al*., 2006). However, certain crops such as oilseed radish (*Raphanus sativus*), oat (*Avena sativa*), Phacelia (*Phacelia tanacetifolia*), and rye (*Secale cereale*) have been reported to support multiplication of *P. penetrans* (Thies *et al*.,1995; Bélair *et al*., 2002). Nonetheless, host suitability is dependent mainly on cover crop cultivars, soil and environmental conditions, geographic area, and pathogenicity of nematode species/populations (Viaene *et al*., 2013; Rudolph *et al*., 2017). For example, seven populations of *P. penetrans* isolated from across Europe exhibited different pathogenicity on alfalfa, carrot, and French marigold (Estifanos, 2019). Duration of experiments, initial nematode abundance, and cover crop seeding rate are other important factors known to influence the response of nematodes to cover crops (Forge *et al*., 2000; Schomaker and Been, 2013; Taning *et al*., 2023). To maintain the future sustainability and resilience of the UK *Narcissus* sector, it is vital to identify and evaluate different cover crop species/cultivars available on the market to potentially manage *P. penetrans*.

2.1.1 Objectives

This study aimed to explore the potential of different cover crops for managing plant parasitic nematodes of economic importance in *Narcissus*, specifically:

1. To evaluate the host plant status of different cover crops for *Pratylenchus penetrans* under greenhouse experimental conditions.

2.1.2 Hypothesis

H_1: There is a significant difference in the host plant status of different cover crops for *Pratylenchus penetrans* under greenhouse conditions.

H_0: There is no significant difference in the host plant status of different cover crops for *Pratylenchus penetrans* under greenhouse conditions.

2.2 MATERIALS AND METHODS

2.2.1 Nematode cultures

Two independent experiments were carried out using pure cultures of *P. penetrans* maintained at Harper Adams University Nematology Laboratory. The laboratory maintains pure cultures of different *Pratylenchus* species therefore, culture purity confirmation was conducted before use. Forty nematodes were randomly selected, and DNA was extracted from each nematode using a Purelink Genomic DNA kit according to the manufacturer's instructions. DNA amplification was done by combining three forward primers (PP-F2a: ATGGGTTCGAATTGGTGTGG, PP-AltF2b: TGAGTTCGAGTTGGTGTGG, PP-AltF2c: ATGGGTTCGCGTTGGTGTGG) and one reverse primer (PP-R2: AGGACCGAATTGGCAGAAGG) in conjunction with the probe (Pen-Probe2: CACATGTTGCATGCAACTGCCACC). The DNA was amplified using Taqman qPCR at the following conditions: 95˚C for 3 minutes, 35 cycles for 10 seconds at 95°C, and 69°C for 60 seconds, as described previously (Orlando *et al*., 2024).

Mass culturing of *P. penetrans* was done on carrot discs made from fresh carrots in a laminar flow cabinet. Forceps, knife, and surfaces were disinfected using absolute ethanol (>99%). The carrots were dipped in absolute ethanol, flamed over a gas burner, and peeled using a sterile knife aided by sterile forceps. Sterilization of the knife and forceps was done between each carrot by flaming. The carrots were chopped into 1 cm-thick discs of approximately 3-4 cm diameter and instantly placed into sterile Petri dishes (one disc per Petri dish). The Petri dishes were sealed using parafilm and incubated at 23 ±1°C for three weeks. Non-contaminated discs were selected and then inoculated with nematodes sterilized in 4000 mg L-1 Streptomycin sulfate. Approximately 20 mixed-stage nematodes were transferred to each carrot disc in a drop of water, sealed with parafilm, and incubated at 21°C for eight weeks. The nematodes used for the greenhouse experiments were extracted from the carrot discs using the modified Baermann funnel technique (Hooper, 1986).

2.2.2 Cover crop treatments and experiment establishment

The cover crops were selected based on previous reports of being a poor host to *P. penetrans* (The cover crop treatments are listed in Table 2.1). Cover crops from eight plant families were selected with *Phacelia* as a positive control due to its known good host status to *P. penetrans*. Fallow soil was used as a negative control. Greenhouse experiments were conducted to determine the host status of cover crops to *P. penetrans*. Sterilised/autoclaved loam soil (John Innes, UK) was mixed with sand at a ratio of 1:1 to avoid slumping and to increase the sand content as *P. penetrans* is frequent in sandy fields (Florini *et al*, 1987). Approximately 1000 cm^3 of the mixture was filled into individual 1-litre plastic pots (13 cm internal diameter). The soil was watered to field capacity before planting, and cover crop seeds were directly sown at variable depths according to the suppliers' recommendations. The seeding rates and number of plants per pot are listed in Table 2.1. Cover crop treatments (see Table 2.1) were replicated six times in a randomised complete block design. Seedling thinning was done according to the supplier's recommended seed rate, seven days after planting. Nematode inoculation was done seven days after the day of thinning. An aqueous suspension containing an initial population (P_i) of approximately 1000 mixed life-stage *P. penetrans* was pipetted and buried into three holes, 0.7 cm in diameter and 4 cm deep, near the plant roots. The experiments were maintained in a glasshouse with controlled conditions of 24 ± 2 °C mean day and 16 °C mean night temperature for eight weeks. Supplementary light was provided to achieve an 18:6 light: dark photoperiod. Watering was done every other day (approx. 100 ml per pot), and no fertilisers were added to the cover crops.

2.2.3 Nematode extraction, enumeration, and staining

The experiments were terminated eight weeks after nematode inoculation, and plant roots were carefully separated from the soil before nematode extraction. All the soil in each pot was mixed by hand, and a subsample of 200 cm^3 soil (Wiesel *et al*., 2015) was measured using a volumetric cylinder and used for nematode extraction. Plant roots were gently washed with tap water and dabbed with an absorbent paper towel to dry, and chopped with scissors into 1 cm pieces. The roots were weighed, and the fresh weight was recorded before a subsample of 5 g was macerated in 50 ml of tap water for 60 seconds in a blender at maximum speed. For treatments that yielded less than 5g, all the roots were used for nematode extraction. Nematode extraction from soil and root subsamples was done using a modified Baermann method. Soil and root samples were separately spread on standard facial tissue (20 x 20 cm, Kleenex, UK) placed over a 1 mm plastic wire mesh supported by a plastic-coated wire letter tray (40 x 30 x 8 cm), suspended on a rectangular plastic tray (56 X 40 X 12 cm). containing a shallow pool of tap water. The samples were left for 48 hours, and the suspension (with nematodes) was sieved through a 38 µm nylon sieve. The captured nematode suspension was poured into 45 ml Falcon tubes and left to settle for three hours before excess water (35 ml) was pipetted out until the nematodes were left in a 10 ml suspension. Nematodes were then poured into gridded nematode counting dishes (7.5 x 3.5 cm, Wageningen University, Netherlands) and counted under a stereomicroscope (M80, Leica Microsystems, Milton Keynes, UK) at 40x magnification. Nematodes obtained from the 5 g subsample were used to estimate the total number of nematodes in the root system. The mean final population (P_f) was calculated as: P_f = total number of nematodes extracted from the soil + total number of nematodes extracted from the whole root system. Nematode reproduction factor (Rf) was calculated as follows: Rf = P_f / P_i, where P_i is the initial population inoculated into the pots at the start of the experiment. Based on the Rf, the host status of the cover crops was obtained by classifying the cover crops into five categories: excellent host (Rf > 4.0), good host (2.0 ≤ Rf ≤ 4.0), maintenance host (1.0 ≤ Rf ≤ 2.0), poor host (0.15 ≤ Rf < 1.0) and non-host (Rf < 0.15) (Ferris *et al*., 1993).

Acid fuchsin staining of selected cover crop roots (those that produced more than 5g of roots and one replicate of Japanese oats) was used to determine whether nematodes had successfully penetrated the roots (Byrd *et al*., 1983). The roots were gently washed with water to remove soil, cut into 2 cm pieces using sterilised scissors, and soaked in a 1% sodium hypochlorite solution for five minutes. The root pieces were rinsed three times in tap water, transferred to a beaker of boiling acid fuchsin stain (3.5 g of fuchsin, 250 ml glacial acetic acid, and 750 ml of water), and left to boil for two minutes. The roots were briefly rinsed in tap water, placed in a 9 cm diameter Petri dish containing acidified glycerol (one drop of glacial acetic acid per 100 ml of glycerol), and visualised under a stereomicroscope (M80, Leica Microsystems, Milton Keynes, UK) at 40x magnification.

2.2.4 Data analysis

Mean final nematode abundances and reproduction factors associated with the different cover crop treatments were analysed using one-way analysis of variance (ANOVA) after the assumptions of ANOVA (homogeneity of variances, normality, independence) were met. Pairwise mean comparison was done using Tukey Honest Significant Difference (HSD) ($p \leq 0.05$). A comparison of soil and root nematode abundances was done using a non-parametric Wilcoxon signed-rank test ($p \leq 0.05$) for each cover crop treatment, as the assumptions of ANOVA were not met. An F-test (to compare variances of the two datasets) was conducted to decide whether a combined analysis could be done, but the variances of the experiments were different ($p \leq 0.05$); therefore, the results were analysed separately. All data analysis was done using R 2023.06.1, Build 524 (R core team, 2022).

2.3 RESULTS

2.3.1 Final nematode abundances, reproduction factors and host status

Final *P. penetrans* abundance recovered from soil significantly differed between cover crop treatments (Table 2.2) for experiments 1 and 2 ($p<0.001$). Nematode abundance was, as expected, lowest (94 and 110 in Experiment 1 and 2, respectively) in the fallow negative control and significantly different from all cover crop treatments ($p\leq 0.05$). Except for Japanese oat, mean nematode abundance was greatest (1305 nematodes) in the *Phacelia* positive control compared to the other cover crops in both experiments (Table 2.2). In both experiments, final nematode abundances in all cover crop treatments were significantly higher ($p\leq 0.05$) than the fallow control (Table 2.2). The lowest (277 and 440) nematode abundance was associated with Alfalfa in both experiments. Reproduction factors significantly differed (ranging from 0.27 to 1.3) between cover crop treatments in the two experiments ($p<0.001$). All crop cover treatments, except for *Phacelia*, Japanese oat, and Viterra Mix (Experiment 2 only), were categorized as poor hosts for *P. penetrans* in both experiments (Table 2.2).

Table 2.1. Cover crop treatment lists used in the two greenhouse experiments

Common name/Cultivar	Scientific name	Family	Source	Seed rate kg/ha	Number of plants/pot
1. French marigold (MP04371)	*Tateges patula* L.	Asteraceae	Elsoms Seeds, UK	8	2
2. Chicory (Wild succory 334N)	*Cichorium intybus* L.	Asteraceae	Chiltern Seeds, UK	6	3
3. Japanese oat (Pratex)	*Avena sativus* L.	Poaceae	P.H. Petersen Germany	100	4
4. Spider flower (Sparkler mix)	*Cleome hassleriana* L.	Capparaceae	Syngenta Seeds, UK	6	6
5. Indian mustard (Brons)	*Brassica juncea* L.	Brassicaceae	Joordens, Netherlands	10	2
6. Oilseed radish 1 (Angus)	*Raphanus sativus* L.	Brassicaceae	P.H. Petersen Germany	30	2
7. Oilseed radish 2 (Contra)	*Raphanus sativus* L.	Brassicaceae	P.H. Petersen Germany	30	2
8. Oilseed rape (Tennyson)	*Brassica napus* L.	Brassicaceae	Elsoms Seeds, UK	25	2
9. Viterra Mix	*R. sativus* + *B. juncea*	Brassicaceae	Elsoms Seeds, UK	15	2
10. Lupin (ILDIGO)	*Lupin angustifolius* L.	Fabaceae	Elsoms Seeds, UK	120	2
11. Phacelia (Angelia)	*Phacelia tanacetifolia Benth*.	Boraginaceae	Elsoms Seeds, UK	10	5
12. African nightshade (DeCyst Broadleaf)	*Solanum scabrum* L.	Solanaceae	AHDB, UK	6	2
13. Alfalfa (Artemis)	*Medicago sativa* L.	Fabaceae	Joordens, Netherlands	25	4
14. Fallow control	-	-	-	-	-

Table 2.2 Mean final nematode abundance of *P. penetrans (*n=6, ± standard errors), p value and CV%, reproduction factors, and host status of selected cover crop treatments eight weeks after inoculation with an initial population of approx. 1000 mixed life stages. *Host status was classified under five different categories: excellent host (E) (Rf > 4.0), good host (G) (2.0 ≤ Rf ≤ 4.0), maintenance host (M) (1.0 ≤ Rf ≤ 2.0), poor host (P) (0.15 ≤ Rf < 1.0) and non-host (N) (Rf < 0.15) (R_f <0.15). Oilseed radish 1=Angus, Oilseed radish 2=Contra. Mean final abundance is the total amount of nematodes in 1kg soil + those in the root system. Different letters after the means indicate significant differences according to the Tukey HSD test (p≤0.05).*

Cover crop	Mean final abundance		Reproduction factor		Host status	
	Exp 1	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 1*	*Exp 2*
Phacelia	1152±73.1 h	1305±51.3 h	1.15 a	1.30 a	M	M
Japanese oat	1041±42.7 gh	1157±32.0 gh	1.04 ab	1.16 ab	M	M
Viterra mix	832±46.3 fg	1038±19.9 fg	0.83 bc	1.04 bc	P	M
Oilseed rape	695±55.7 ef	914±54.4 ef	0.69 cd	0.91 cd	P	P
Blue lupin	697±144.0 cd	723±27.9 cd	0.70 cde	0.72 de	P	P
Indian mustard	623±50.6 de	768±74.8 de	0.62 def	0.77 de	P	P
Spider flower	553±24.8 de	735±50.0 de	0.55 ef	0.73 de	P	P
Oilseed radish 1	525±51.3 de	754±36.2 de	0.53 ef	0.75 de	P	P
African nightshade	523±37.7 de	684±70.9 de	0.52 ef	0.68 de	P	P
French marigold	498±31.8 cd	584±36.4 cd	0.50 ef	0.58 ef	P	P
Forage chicory	490±20.7 cd	636±43.3 cd	0.49 ef	0.63 ef	P	P
Oilseed radish 2	462±15.5 cd	570±25.8 cd	0.46 fg	0.57 ef	P	P
Alfalfa	277±13.5 b	440±80.4 b	0.27 gh	0.44 f	P	P
Fallow control	94±14.7 a	110±18.6 a	0.09 h	0.11 g	-	-
P value	<0.001	<0.001	<0.001	<0.001		
CV%	22.5	15.9	22.5	15.9		

2.4.2 Nematode distribution between soil and roots

A greater number of nematodes were recovered from the soil than from the plant roots in all tested cover crop treatments in both experiments (p≤0.05) (Table 2.3). The greatest (117 nematodes) abundance of *P. penetrans* was recovered from the roots of Japanese oat (Experiment 2, Figure 2.1). No nematodes were recovered from or observed in the roots of French marigold in either experiment. In Experiment 1, no nematodes were recovered from either *Phacelia* or Indian mustard (Table 2.3).

Figure 2.1. Individual *Pratylenchus penetrans* observed inside Japanese oat roots eight weeks after planting.

Table 2.3 *P. penetrans* abundance from soil and roots (n=6, ± standard errors) and total fresh root weight associated with cover crop treatments eight weeks after inoculation with an initial population of approx. 1000 mixed life stages. Asterisk (*) represents significant differences (p≤0.05) between soil and root *P. penetrans* abundance for each cover crop treatment after Wilcoxon signed rank test.

Cover crop	*Pratylenchus penetrans* abundance in soil and roots				Total fresh root weight (g)	
	Exp 1		*Exp 2*			
	soil	*roots*	*soil*	*roots*	Exp1	Exp 2
Phacelia	1152±73.1	0±0*	1304±51.3	1±0.8*	0.1	0.4
Japanese oat	1018±47.3	23±5.4*	1039±32.0	117±47.6*	2.9	3.7
Viterra mix	831±46.3	1±0.5*	1037±19.9	1±0.4*	1.4	1.4
Oilseed rape	694±144	1±0.5*	913±54.4	1±0.3*	2.3	3.1
Blue lupin	678±57.7	19±6.7*	703±27.9	20±7.6*	1.7	2.3
Indian mustard	623±50.6	0±0*	767±74.8	1±0.7*	2.2	2.3
Spider flower	550±24.8	3±1.2*	729±50.0	6±2.0*	2.5	3.6
Oilseed radish 1	524±51.3	1±0.5*	754±36.2	3±1.9*	3.8	4,2
African nightshade	520±37.7	3±0.4*	678±70.9	6±2.1*	8.8	10.4
French marigold	498±31.8	0±0*	584±36.4	0±0*	5	5.2
Forage chicory	485±20.7	5±0.7*	625±43.3	11±4.7*	7.3	9.1
Oilseed radish 2	461±15.5	1±0.5*	568±25.8	2±0.6*	3.1	3.6
Alfalfa	274±13.5	3±1.3*	429±80.4	11±3.2*	2.5	3.1
Fallow control	94±14.7	-	110±18.6	-	-	-

2.5 DISCUSSION

This chapter reports the host status of selected cover crops for *P. penetrans* based on reproduction factors obtained from eight-week greenhouse experiments. Out of all cover crops investigated in this study, only *Phacelia* the positive control and Japanese oat were considered maintenance hosts. In contrast, all other tested cover crop treatments reduced *P. penetrans* abundance and were considered poor hosts.

Although *Phacelia* was expected to be a good host (Kimpinski *et al*., 2000), the current findings agree with Taning *et al*. (2024), who rated *Phacelia* as a maintenance host.

Japanese oat was identified as a maintenance host, consistent with past reports (Estifanos, 2019; Forge *et al*., 2000; Taning *et al*., 2024). French marigold was consistently a poor host in both experiments, similar to previous field and greenhouse experiments, where *P. penetrans* abundance was suppressed (Miller, 1969; Estifanos *et al*., 2019; Kimpinski *et al*., 2000; Evenhuis *et al*., 2004; Pudasaini *et al*., 2006). French marigold can be grown as green manure (Siddiqui and Alam, 1987), in rotation (Miller and Reporter, 1969; Ploeg, 1999; Estifanos *et al*., 2019; Kimpinski *et al*., 2000; Evenhuis *et al*., 2004; Pudasaini *et al*., 2006), intercropped with cash crops (Abid and Maqbool, 1990) or applied as plant extracts (Miller and Reporter, 1969). *Tagetes patula* roots release alpha-terthienyl, a biocide and hatch inhibitor, leading to an active decline of *P. penetrans* populations in soils after three months of field growth (Siddiqui and Alam, 1987; Pudasaini *et al*., 2006). In this study, alfalfa was rated as a poor host to *P. penetrans* in both experiments. Previous studies have published similar results (Hafez *et al*., 1990; Barnes *et al*., 1995; Thies *et al*., 1995). Similarly, blue lupin is considered a poor host in both experiments, which agrees with earlier greenhouse observations (Lundborg, 2022). In both experiments, spider flower, African nightshade, and forage chicory were consistently poor hosts to *P. penetrans*.

In both experiments, Indian mustard, oilseed rape, and oilseed radish were rated as poor hosts to *P. penetrans*. Previous studies have reported similar observations (Mbiro and Wim Wesemael, 2016; Knoetze *et al*., 2023; Taning *et al*., 2024). Brassica cover crops are biofumigants against several PPNs, including *G. pallida* (Ngala *et al., 2015)* and *Pratylenchus neglectus* (Potter *et al.,* 1999). At cover crop termination, glucosinolates in the chopped Brassica tissues are converted into biocidal isothiocyanates by the enzyme myrosinase. The biofumigation effects of Brassica cover crops may be masked if the selected species support the reproduction and multiplication of the target PPN (Vervoot *et al*., 2014; Grabau *et al*., 2017). Most cover crops in the UK are grown as mixes to capitalise on the benefits of both crops. In this study, Viterra mix (oilseed radish + Indian mustard) was rated as poor and a maintenance host in the two experiments, but the difference between the reproduction factors (0.83 and 1.04) is marginal. Work to understand the interactions of cover crop mixtures with PPNs is in progress.

In contrast with the current findings, several studies have reported that Indian mustard (Rudolph *et al*., 2017; LaMondia, 2021; Neupane and Yan, 2023), oilseed radish (Visser and Molendijk, 2015; Grabau *et al*., 2017; Estifanos *et al*., 2019; Neupane and Yan 2023), oilseed rape (MacDonald and Mai 1963; Forge *et al*., 2000; Bélair *et al*., 2002; Estifanos, 2019; LaMondia, 2021), alfalfa (Miller, 1978; Nelson *et al*., 1985), blue lupin (Colbran, 1979) are good hosts to *P. penetrans*. Earlier studies also contradict current observations as they identified Japanese oat as a poor host to *P. penetrans (*Colbran, 1979; Vrain *et al*., 1996; LaMondia *et al*., 2002*)*. However, cultivars of these cover crops evaluated in previous studies differed from our study, thus raising the potential that intra-species variations in host responses to PPNs exist, which have also been suggested for Japanese oat and oilseed rape previously (Townshend, 1989; Webb, 1996). As such, caution should be taken when making cover crop recommendations to avoid generalising host responses. Similar to our study, more *P. penetrans* were recovered from the soil compared to cover crop plant roots (Neupane and Yan 2023). Root lesion nematodes move freely between soil and roots. Therefore, nematodes residing in both habitats must be quantified to ensure the accuracy of the final population in host suitability tests (Grabau *et al*., 2017; MacGuidwin and Bender, 2018). In this study, the poor root growth observed on most of the cover crops could have influenced nematode distribution between roots and soil. However, in cover crops that had good root growth, such as forage chicory and African nightshade, no nematodes were observed in the roots after staining, compared to Japanese oats, in which a few nematodes were observed.

2.6 CONCLUSIONS

Rhizosphere soil associated with *Narcissus* typically comprises more than one genus/species of economically important nematodes (Hanks, 2013). Host suitability studies are therefore necessary to select cover crops that are poor hosts to more than one *Pratylenchus* spp. or, better yet, more than one genus of parasitic nematodes. Although greenhouse host suitability trials do not always indicate what will happen under field conditions, they enable accurate calculation of P_f / P_i data, unachievable in the field (Manuel *et al*., 1980). Testing these cover crops under field conditions in different geographical areas to validate these results was done in the next Chapter. Farmers in the UK have increased awareness of sustainable soil management partly due to incentivised cover crop government-funded programs such as “Greening Measures” under Ecological Focus Areas (EFA) (Storr *et al*., 2019). The selection of cover crops mainly depends on the agronomic needs of the production system, including improving soil fertility and structure, weed, and pest management (White *et al*., 2015). All the tested cover crops except African nightshade grow well in England and Scotland (White *et al*., 2015; Storr *et al*., 2019); therefore, growers can easily adopt them into the *Narcissus* cropping cycle as summer cover crops before *Narcissus* is grown. Additionally, *P. penetrans* is present in all *Narcissus* production areas in the UK and is associated with a broad range of cash crops that form a typical UK rotation. Thus, dependent upon the results of field trials, candidate cover crops such as oilseed radish, French marigold, forage chicory, alfalfa have the potential for broader use in managing *P. penetrans*.

CHAPTER 3

RESPONSE OF *PRATYLENCHUS, APHELENCHUS, APHELENCHOIDES* SPP., AND BACTERIVORE NEMATODES TO COVER CROPPING IN *NARCISSUS* FIELDS.

3.1 INTRODUCTION

Narcissus is associated with pathogenic nematodes *Ditylenchus dipsaci, Pratylenchus penetrans,* and *Aphelenchoides subtenuis,* which directly feed on plants and interact with fungal pathogens, causing disease complexes (Hanks & Chastagner, 2018). As discussed in depth in Chapter 1, there are no commercially available nematode-resistant varieties or nematicides available on the market, the latter due to regulatory legislation. Increasing the length of crop rotations is not feasible due to limited land (Upcott *et al*., 2023). Both *P. penetrans* and *D. dipsaci* are polyphagous, with most, if not all, of the typical rotational crops grown in the UK known as hosts to both nematode species (Castillo and Vovlas, 2007; Tenuta *et al*., 2014). There is, therefore, an urgent need to explore alternative chemical-free plant parasitic nematode (PPNs) management strategies. Cover crops have the potential to reduce plant parasitic nematode abundance via several modes of action, including acting as a trap crop that prevents nematode reproduction, allelopathy, (partial or full) biofumigation, and being a non/poor host (Ntalli and Caboni, 2017). *Narcissus* bulb lifting typically occurs in June, creating a fallow period until September, when bulbs are planted again (Lillywhite *et al*., 2017). This fallow period is an ideal window for growing nematode-suppressive cover crops.

Cover crops may change or influence the diversity and abundance of nematode communities within the rhizosphere, affecting soil processes such as decomposition, carbon and nutrient cycling (Jin *et al*., 2019). The soil rhizosphere has a taxonomically and functionally diverse community of nematodes, with their composition and structure indicative of the health status of the soil (Gao *et al*., 2020; Lu *et al*., 2020). Yeates *et al*. (1993) grouped nematodes into a minimum of five functional feeding groups: plant, fungal and bacterial feeders, predators, and omnivores. In soil, bacterivore nematodes are the most abundant, followed by plant parasites> fungivores> omnivores> and predators (van den Hoogen *et al*., 2019). These nematodes have different trophic roles in the soil, are sensitive to environmental change, and respond differently to cover cropping. Microbial-feeding nematodes regulate the diversity, activity and functioning of fungi and bacteria in the soil (Thakur and Geisen, 2019). Bacterial feeders and predatory nematodes directly and indirectly contribute 8-19% of nitrogen mineralisation under field conditions (Beare, 1997). They contribute by feeding on decomposer microbes and releasing ammonium through excreta and nitrogen immobilisation (Ferris *et al*., 1998). Predaceous nematodes feed on fungivore and bacterivore nematodes, channelling resources from low to high trophic levels, thereby regulating nitrogen mineralisation (Wardle and Yeates, 1993). Ideally, cover crops should suppress plant parasitic nematodes without reducing non-target beneficial nematodes. Thus, monitoring the abundance of beneficial nematodes, such as fungivores and bacterivores nematodes is essential to develop cover crop schemes for sustainable nematode management. Nematodes associated with *Narcissus* also affect a broad range of important crops grown in the UK; therefore, the identification of suppressive cover crops has the potential for broader use. Moreover, cover crops that support the multiplication of important PPNs can be avoided as they can potentially elevate nematode abundance (Neupane and Yan, 2023).

Greenhouse experiments in Chapter 2 identified oilseed radish 2 (Contra), French marigold, forage chicory, and alfalfa as poor hosts with the least reproduction factors (Rf<0.6) therefore, these were selected for field testing. Japanese oats, *Phacelia* (maintenance hosts), and Indian mustard (poor host) were included in the treatment list because they are commonly grown and suitable for local growing conditions. Additionally, Japanese oats (Pratex) is marketed as a poor host to *P. penetrans,* which disagrees with greenhouse findings; therefore, additional testing under field conditions was necessary. All cover crops could not be tested in the first year due to limited labour, therefore, forage chicory and alfalfa were tested only in the second year.

3.1.1 Objectives

The Chapter aimed to assess the effects of selected cover crops tested in greenhouse experiments (Chapter 2) on plant parasitic nematodes of *Narcissus* while monitoring beneficial fungivore and bacterivore nematodes under field conditions. *Pratylenchus* and *Ditylenchus* and *Aphelenchoides* spp. are difficult to distinguish using morphological features; therefore, molecular identification methods were employed. Additionally, only *P. penetrans* is known to be associated with *Narcissus,* but *Pratylenchus* spp. typically occur as a species mixture in the field. Therefore, real-time quantitative PCR was used to identify and quantify four common *Pratylenchus* spp. (*P. penetrans, P. crenatus, P. neglectus,* and *P. thornei*). *D. dipsaci* and *A. subtenuis* are the reported pathogens of *Narcissus;* therefore, conventional PCR was used to test for their presence.

The specific objectives were:

1. To evaluate the impact of selected cover crops on the abundance of *Pratylenchus* spp., *Ditylenchus* spp., fungivore (*Aphelenchoides, Aphelenchus* spp.) and bacterivore nematodes using morphological identification (microscopy).

2. To test for the presence of *P. penetrans, P. neglectus, P. crenatus* and *P. thornei* and assess whether individual species respond to cover crop treatments using qPCR.
3. To test for the presence of *A. subtenuis* and *D. dipsaci* (in the three field sites using conventional PCR.

3.1.2 Hypotheses

H_1: Cover crops have an impact on the abundance of *Pratylenchu*s spp., *Ditylenchus* spp., fungivore (*Aphelenchoides, Aphelenchus* spp.) and bacterivore nematodes.

H_1: *P. penetrans*, *P. neglectus, P. crenatus*, and *P. thornei* are present in the tested field sites, and individual species do not respond to cover crop treatments.

H_1: *A. subtenuis* and *D. dipsaci* are present in the tested field sites.

H_0: Cover crops have no impact on the abundance of *Pratylenchu*s spp., *Ditylenchus* spp., fungivore (*Aphelenchoides, Aphelenchus* spp.) and bacterivore nematodes.

H_0: *P. penetrans*, *P. neglectus, P. crenatus*, and *P. thornei* are absent in the tested field sites, and individual species do not respond to cover crop treatments.

H_0: *A. subtenuis* and *D. dipsaci* are absent in the tested field sites.

3.2 MATERIALS AND METHODS

Six *Narcissus* fields in which *P. penetrans* and *D. dipsaci* were previously recorded were selected in each region (Scotland or the Isles of Scilly). None of the sites tested were positive for *D. dipsaci*; therefore, sites with a high abundance of *Pratylenchus* spp. were selected for use. In the first year (2022), two sites were established (Montrose and the Isles of Scilly), and in the second year (2023), only one site in Perth was established as a repeat of the first-year experiments. Before the experiments commenced at each site, *Narcissus* bulbs were lifted, and the field was ploughed and harrowed to a fine tilth before planting. Cover crop species and varieties were drilled at the seeding rates detailed in Table 3.1. Brassica cover crop treatments received 100 kg ha^{-1} N and 25 kg ha^{-1} S fertiliser as Sulfan (24% N, 15% SO_3) at planting because they need supplementary nutrients for maximum production of glucosinolates.

3.2.1 Field Site 1

The site was located near Montrose, Scotland (56.85672, -2.12874), on a sandy loam soil (69% sand, 21% silt, 10% clay) with a pH of 5.4-6.0 ($CaCl_2$). The cover crop treatments were as follows: oilseed radish, French marigold, *Phacelia*, Indian mustard, and two fallow controls: one disturbed at the incorporation stage and one left undisturbed. The treatments were arranged in a randomised complete block design (RCBD) with four replications in 12 x 3.66 m plots separated by 3 m paths between blocks. A small plot drill with Amazon Suffolk coulters was used for seed drilling at the supplier's recommended seeding rates. The trial plots were rolled after drilling using a tractor-mounted roller to improve soil-seed contact and protect the seed from predators. Planting was done on the 6th of June 2022, and soil sampling was done to assess the initial nematode abundances (Pi). Brassica cover crops produce the highest levels of glucosinolates at peak flowering (Kierkgaard and Sarwar, 1998). Therefore, for maximum biofumigation potential, at peak flowering of oilseed radish and Indian mustard (7 September 2022), soil sampling was done to determine nematode abundance at the pre-incorporation stage. Cover crop plant tissues were chopped and incorporated into the soil using a tractor-mounted 4 m Standen Powavator (Standen UK, PV400140) rotavator. Afterwards, the plots were rolled to seal the soil surface using a tractor-mounted roller (Figure 6.1). Approximately six weeks after cover crop incorporation (2 November 2022), sampling was done to assess final nematode abundance (Pf) at the post-incorporation stage.

3.2.2 Field Site 2

The field was situated on the Isles of Scilly (49.92706, 6.28622), also on a sandy loam soil (72% sand, 24% silt, 4% clay) with a pH of 5.3-5.9 ($CaCl_2$). The cover crop treatments were oilseed radish, French marigold, *Phacelia*, Indian mustard and a fallow control, which was disturbed at the pre-incorporation stage. An undisturbed fallow control was not feasible due to limited available land. Land was prepared by lifting bulbs, ploughing and power-harrowing to a fine tilth. Planting was done on 8th July 2022. The field was small and could not accommodate a drill; therefore, the cover crops were broadcast using an Earthway 2750 11kg Nylon Bag Seeder and Spreader calibrated for the different seed types. Seeding was done at the recommended seeding rate. Plots were thereafter compressed manually with feet, as no roller was available. The plots measured 3 x 1 m, and the treatments were arranged in RCBD with five replicates. After planting, the plots were watered using a watering can, and the cover crops reached peak flowering on 26th November 2022. Soil sampling was conducted to determine nematode abundance at the pre-incorporation stage (Figure 6.2A). The cover crops were chopped and incorporated using a rotavator. Afterwards, the plots were rolled with a tractor-mounted roller. Final nematode abundance was assessed on 4th February 2023 (8 weeks post-incorporation) due to delays with the availability of machinery and labour.

3.2.3 Field Site 3

This experiment was an iteration of the experiments conducted in 2022, using cover crops that reduced *Pratylenchus* spp.: oilseed radish, French marigold, forage chicory, alfalfa and disturbed fallow control. The experiment was conducted on sandy loam soil near Perth, Scotland (56.62405, -3.17936) (73% sand, 23% silt, 4% clay) with a pH of 5.3-5.5 ($CaCl_2$). Bulbs were lifted from the site, and the field ploughed and harrowed to a fine tilth. Planting was done on 29th June 2023. All seeds were broadcast using an Earthway 2750 11kg Nylon Bag Seeder and Spreader at the recommended seed rate due to the unavailability of drilling equipment. The treatments were arranged in RCBD with five replicates on 3 x 4 m plots. Approximately three months (29th September 2023) after planting, at the peak flowering stage, soil sampling was done to assess nematode abundance at the pre-incorporation stage (Figure 3.2). Due to the prolonged heavy rainfall, the soil was too wet to incorporate cover crops. Therefore, this site does not have post-incorporation stage data.

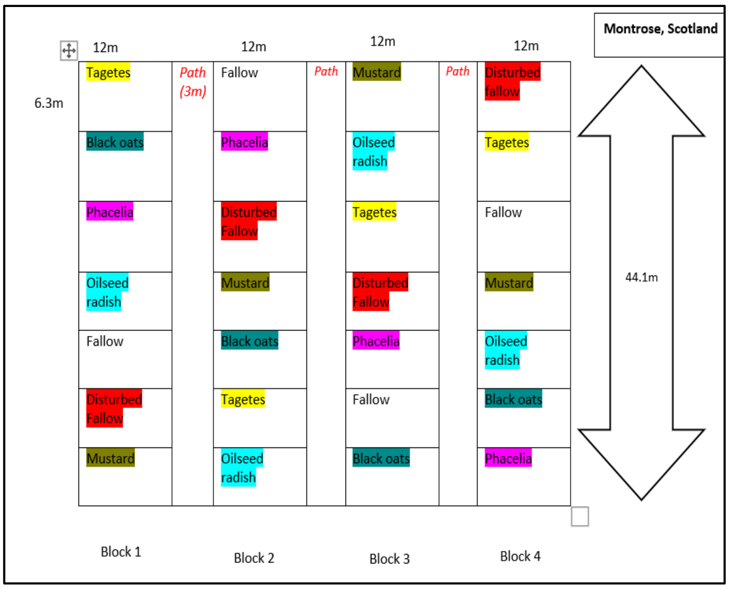
Table 3.1. Cover crops sown across the three experimental sites, their respective recommended seed rates and sampling dates. Different cultivars of French marigold, Indian mustard and forage chicory were used because the seed of cultivars used in greenhouse experiments was unavailable at the suppliers. The treatments were replicated five times in the Isles of Scilly and Perth and four times in Montrose. Due to limited labour, reduced replicates were used in Montrose, as there were more treatments compared to the other sites

Site	Cover crop (Cultivar)	Scientific name	Family	Source	Seed rate kg ha^{-1}	Soil sampling intervals and dates
Field Site 1: Montrose	French marigold (French marigold)	*Tateges patula* L.	Asteraceae	Joordens, Netherlands	8	Planting (06/06/2022), Pre-incorporation (7/09/2022), Post-incorporation (2/11/2022)
	Oilseed radish 2 (Contra)	*Raphanus sativus* L.	Brassicaceae	P.H. Petersen, Germany	30	
	Indian mustard (Caliente Rojo)	*Brassica juncea* L.	Brassicaceae	Tozer Seeds, UK	10	
	Phacelia (Angelia)	*Phacelia tanacetifolia* Benth.	Boraginaceae	Elsoms Seeds, UK	10	
	Japanese oat (Pratex)	*Avena sativus* L.	Poaceae	P.H. Petersen, Germany	100	
	Disturbed fallow	-	-	-	-	
	Undisturbed fallow	-	-	-	-	
Field site 2: Isles of Scilly	French marigold (French marigold)	*Tateges patula* L.	Asteraceae	Joordens, Netherlands	8	Planting (08/07/2022), Pre-incorporation (26/11/2022), Post-incorporation (04/02/2023)
	Oilseed radish 2 (Contra)	*Raphanus sativus* L.	Brassicaceae	P.H. Petersen, Germany	30	

	Indian mustard (Caliente Rojo)	*Brassica juncea* L.	Brassicaceae	Tozer Seeds, UK	10	
	Phacelia (Angelia)	*Phacelia tanacetifolia* Benth.	Boraginaceae	Elsoms Seeds, UK	10	
	Disturbed fallow	-	-	-	-	
Field site 3: Perth	French marigold (French marigold)	*Tateges patula* L.	Asteraceae	Joordens, Netherlands	8	Planting (29/06/2023), Pre-incorporation (29/09/2023), (cover crops not incorporated)
	Oilseed radish 2 (Contra)	*Raphanus sativus* L.	Brassicaceae	P.H. Petersen, Germany	30	
	Alfalfa (Artemis)	*Medicago sativa* L.	Fabaceae	Joordens, Netherlands	25	
	Forage chicory (Commander)	*Cichorium intybus* L.	Asteraceae	Barenbrug, UK	8	
	Fallow control	-	-	-	-	

Figure 3.1. Field Site 1, Montrose, Scotland. Photograph A: Cover crops three months after planting (at the incorporation stage). Photographs B and C: Experimental plots after chopping and incorporation using a rotavator.

A. Field map for field site 1, Montrose Scotland 2022

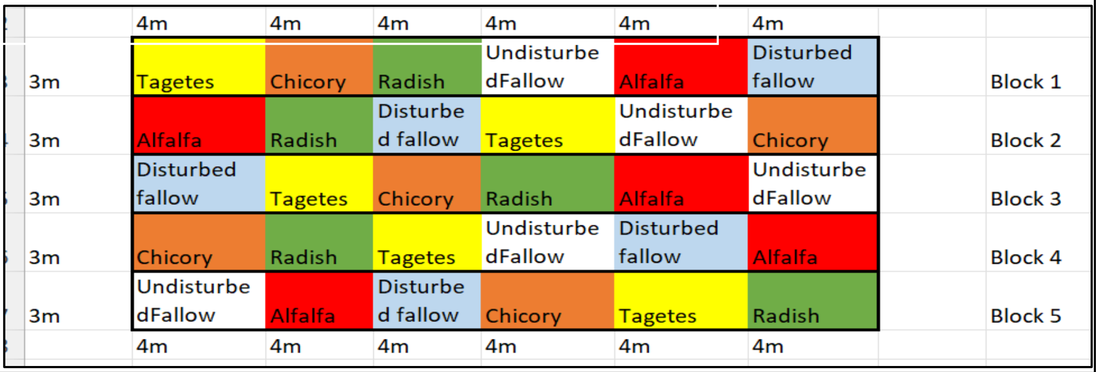


12m	Path (3m)	12m	Path	12m	Path	12m
Tagetes		Fallow		Mustard		Disturbed fallow
Black oats		Phacelia		Oilseed radish		Tagetes
Phacelia		Disturbed Fallow		Tagetes		Fallow
Oilseed radish		Mustard		Disturbed Fallow		Mustard
Fallow		Black oats		Phacelia		Oilseed radish
Disturbed Fallow		Tagetes		Fallow		Black oats
Mustard		Oilseed radish		Black oats		Phacelia
Block 1		Block 2		Block 3		Block 4

B. Field map for field site 2, Isles of Scilly, Cornwall 2022

		Phacelia
Tagetes	Fallow	Contra
Mustard	Contra	Tagetes
Fallow	Phacelia	Mustard
Phacelia	Fallow	Contra
Mustard	Tagetes	Fallow
Contra	Mustard	Phacelia
Tagetes	Fallow	Contra
Phacelia	Mustard	Tagetes

C. Field map for field site 3, Perth Scotland 2023



	4m	4m	4m	4m	4m	4m	
3m	Tagetes	Chicory	Radish	UndisturbedFallow	Alfalfa	Disturbed fallow	Block 1
3m	Alfalfa	Radish	Disturbed fallow	Tagetes	UndisturbedFallow	Chicory	Block 2
3m	Disturbed fallow	Tagetes	Chicory	Radish	Alfalfa	UndisturbedFallow	Block 3
3m	Chicory	Radish	Tagetes	UndisturbedFallow	Disturbed fallow	Alfalfa	Block 4
3m	UndisturbedFallow	Alfalfa	Disturbed fallow	Chicory	Tagetes	Radish	Block 5
	4m	4m	4m	4m	4m	4m	

Figure 3.2 Field maps for the three trial sites

Figure 3.3. Photograph A: Field Site 2, Isles of Scilly, England, cover crops (French marigold and *Phacelia* shown) four months after planting (at the incorporation stage). Photograph B: Field Site 3, Perth, Scotland, cover crops (French marigold, oilseed radish and forage chicory shown) three months after planting (at the incorporation stage).

3.2.4 Soil sampling and nematode extraction

Soil sampling was performed at planting, three months after planting (pre-incorporation stage) and post-incorporation of the cover crops (Table 2.1). Twenty soil cores were collected from each plot at 20 cm depth using a grass plot sampler (2.3 cm diameter soil core) (Van Walt Ltd, UK). The sampling pattern was W-shaped with equidistant sampling points along the pattern. The soil cores were gently hand-mixed to make a composite soil sample. Nematodes were extracted from a 200 g subsample for microscopy counts using a modified Baermann tray (Whitehead and Hemming, 1965) for 48 h. The nematodes were collected in a 10 ml suspension from which three 1 ml subsamples were pipetted onto counting slides, and the nematodes were identified to either genus or trophic group level (Yeates *et al*., 1993) under a compound microscope (Leica, Germany) at 40x magnification. The mean of the three subsamples was used to calculate the total number of nematodes in 200 g using the formula: *[total number of nematodes per 200 g soil sample = average number per subsample x 10 (total suspension volume in mL)].*

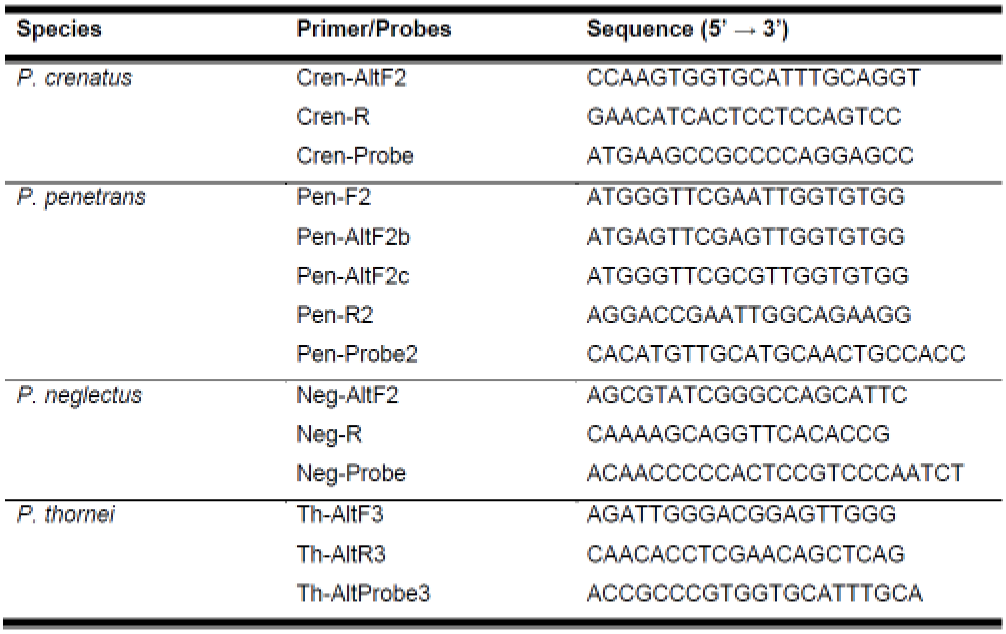
3.2.5 Quantitative detection of *Pratylenchus* species using qPCR

Pratylenchus spp. typically occur as a species mixture in the UK (Orlando et al., 2020), with only *P. penetrans* known to be associated with *Narcissus* (Courtney, 1961). Real-time quantitative PCR diagnostics was used to detect and quantify four common *Pratylenchus* species: *P. crenatus, P. penetrans, P. neglectus,* and *P. thornei* from the three sites. Due to resource limitations, the responses of individual *Pratylenchus* spp. to cover crop treatments were tested in treatments that showed a reduction of nematodes by microscopy (Table 2.2). Nematodes were extracted from 200 g soil using a modified Baermann funnel method (Brown and Boag, 1988). The collected nematode suspension was reduced to *ca* 1.5 ml and freeze-dried before DNA extraction. Genomic DNA was extracted using a Purelink Genomic DNA extraction kit (Invitrogen, UK) according to the manufacturer’s instructions. DNA was eluted in 100 µl of DNA elution buffer, and a 2 µl aliquot was used in qPCR reactions testing for each of the four target species. Details of the primers and probes for the four species are shown in Table 3.3. Taqman Real-Time PCR was used to amplify the DNA at the following conditions: 95°C for 3 minutes, 35 cycles for 10 seconds at 95°C, and 69°C for 60 seconds (Orlando *et al*., 2024). The PCR reaction mix was made of 10 µl of Taqman^{TM} Fast Universal PCR Mastermix 2X (ThermoFisher, Scientific, UK), 0.6 µM of primers, 0.25 µM of probe, and PCR grade distilled water to a final volume of 20 µl and 2 µl of template DNA. Each of the four species was estimated by dividing the total DNA copy number per sample by the mean DNA copy number per individual. The mean DNA copy numbers for the four *Pratylenchus* species (Orlando *et al*., 2024) are listed in Table 3.4. The total DNA copy number was obtained after cloning the D2-D3 region of the 28S rDNA into a plasmid, measuring the DNA concentration in the plasmid and calculating the gene copy number using the formula: number of copies = (amount (ng) × 6.022 × 10^{23}) / (length (bp) × 1 × 10^{9} × 650) (Daniell *et al*., 2012). The standard curves for qPCR were then obtained by a serial dilution ranging from 10^{8} to 10^{1} copies μl^{-1} in 10 mM Tris (pH 8.0) (Orlando *et al*., 2024).

Table 3. 2. Cover crop treatments in which qPCR was performed to evaluate the effects of cover crops on individual *Pratylenchus* species.

Year	Site	Treatments used
Year 1: 2022	Montrose, Scotland	Disturbed fallow French marigold
	Isles of Scilly, England	Disturbed fallow, French marigold, Oilseed radish
Year 2: 2023	Perth, Scotland	Disturbed fallow, French marigold, Oilseed radish, Forage chicory, Alfalfa

Table 3.3. Primers and probes used for diagnostic PCR of *P. penetrans, P. crenatus*, *P. neglectus,* and *P. thornei* (Orlando *et al.,* 2024*)*.



Species	**Primer/Probes**	**Sequence (5' → 3')**
P. crenatus	Cren-AltF2	CCAAGTGGTGCATTTGCAGGT
	Cren-R	GAACATCACTCCTCCAGTCC
	Cren-Probe	ATGAAGCCGCCCCAGGAGCC
P. penetrans	Pen-F2	ATGGGTTCGAATTGGTGTGG
	Pen-AltF2b	ATGAGTTCGAGTTGGTGTGG
	Pen-AltF2c	ATGGGTTCGCGTTGGTGTGG
	Pen-R2	AGGACCGAATTGGCAGAAGG
	Pen-Probe2	CACATGTTGCATGCAACTGCCACC
P. neglectus	Neg-AltF2	AGCGTATCGGGCCAGCATTC
	Neg-R	CAAAAGCAGGTTCACACCG
	Neg-Probe	ACAACCCCCACTCCGTCCCAATCT
P. thornei	Th-AltF3	AGATTGGGACGGAGTTGGG
	Th-AltR3	CAACACCTCGAACAGCTCAG
	Th-AltProbe3	ACCGCCCGTGGTGCATTTGCA

Table 3. 4. Average DNA copy numbers per individual for the four target *Pratylenchus* species (Orlando *et al*., 2024)

	P. neglectus	*P. penetrans*	*P. crenatus*	*P. thornei*
Average copy number ± se	5292 ± 266	9555 ± 297	7775 ± 199	3624 ± 109

3.2.6 Molecular identification of *Aphelenchoides subtenuis*

Microscopy identification revealed the presence of *Aphelenchoides* spp. at all experimental sites. The presence of *A. subtenuis* (previously reported to be associated with *Narcissus*) was tested using conventional PCR. Soil sampling was conducted as described Chapter

3.2.4, while nematode extraction was carried out as described in Chapter 3.2.5. Ten *Aphelenchoides* nematodes were randomly selected, handpicked from nematode suspension and placed in 18 µl PCR water. Nematodes were disrupted mechanically by adding three 1 mm glass beads to the tubes and homogenising for 30 s at 30 Hz in a tissue disruptor (Retsch M300, Germany) (Jesus *et al*., 2016). Afterwards, the tubes were centrifuged at *16000 g*; 4 µl of proteinase K (100 µg ml^{-1}) and 2 µl of 10X PCR buffer (Gotaq, Promega, UK) were added to each tube to facilitate enzymatic digestion of nematode tissue. Finally, the PCR tubes containing the digested nematodes were incubated at 60 °C for 1hr, at 95 °C for 15 min, and at 10 °C for 10 minutes. The primers used to amplify the mtCOI DNA region were forward: mtCOI-F (CCTACTATGATTGGTGGTTTTGGTAATTG) and mtCOI-R (GTAGCAGCAGTAAAATAAGCACG) (Kanzaki and Futai, 2002). The reaction mixture was made up of 8.5 µl PCR grade water, 3 µl of 5X GoTaq buffer, 0.06 µl TaqPolymerase, 0.6 µl of 0.01 mM forward and reverse primer, 0.6 µl of 94.75 µM dNTPs and 2 µl DNA template. The PCR reaction was as follows: 94 °C for 5 min, 42 cycles at (94 °C for 30 s, 48, 51 and 54 °C (gradient PCR) for 30 s, 72 °C for 2 min), and 72 °C for 10 min (Sánchez-Monge *et al*., 2017). *Pratylenchus penetrans* was used as a non-target control, while a known *A. subtenuis* positive control was unavailable. The PCR products were separated by gel electrophoresis (1% agarose gel using 6X GelRed loading buffer (Biotium)) and visualised under UV light.

3.2.7 Molecular identification of *Ditylenchus dipsaci*

Narcissus is associated with *D. dipsaci*, but microscopy does not allow straightforward differentiation between the different *Ditylenchus* species. Therefore, the presence of *D. dipsaci* in the three field sites was tested using conventional PCR. Nematodes were extracted from 200 g of soil using a modified Baermann funnel method (Brown and Boag, 1988). The collected nematode suspension was reduced to *ca* 1.5 ml and freeze-dried before DNA extraction. Genomic DNA was extracted using a Purelink Genomic DNA extraction kit (Invitrogen, UK) according to the manufacturer’s instructions. DNA was amplified using ITS rRNA primers specific for *D. dipsaci*; forward: CTGTAGGTGAACCTGC and reverse: GACATCACCAGTGAGCATCG (Jeszke *et al*., 2015). The PCR reaction mixture was made up of 8.5 µl PCR grade water, 3 µl of 5X GoTaq PCR buffer, 0.6 µl of 0.01 mM forward and reverse primers, 1.82 µl of 94.75 µM dNTPs, 0.06 µl Taq polymerase and 2 µl of template DNA. The PCR conditions were as follows: 3 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 61, 62, 63.5 and 65 °C (gradient PCR), 30 s at 72°C and then 5 min at 72°C. *Pratylenchus penetrans* was used as a non-target control, while a known *D. dipsaci* positive control was unavailable. Gel electrophoresis and visualisation were done as described in the previous section (6.2.6).

3.2.8 Data analysis

To analyse the effects of cover crop treatments on nematode abundance, a generalised linear mixed effects model (LME) was used. Cover crop treatment and time of sampling were considered fixed effects predictors. A nested time: block was added to account for correlation in repeated measures within the same block. The 'lme4' package in R was used (Bates *et al*., 2015; R Core Team, 2022). The output of the model (*model <- lmer (abundance~ Covercrop + Time: Covercrop + (1|Block) + (1|Time: Block), data = data*) shows the effects of cover crops and time on nematode abundance.

Overall, time effects are not of interest since they are not meaningful, but rather the effects of time by species. Therefore, instead of using *Covercrop * Time* as the fixed effects, *Covercrop + Time: Covercrop* is more suitable. This model estimates the species effect and the effects of time in each species (without assuming an overall time effect). Whenever there was significance, the emmeans multcomp package in R (Hothorn *et al*., 2022) was used for pairwise comparison (Tukey HSD post hoc tests).

The qPCR data were compared to microscopy counts by fitting a linear regression model. To test the effects of cover crop treatments on individual *Pratylenchus* spp. from qPCR, a generalised linear mixed effects model was fitted with cover crop treatment, time of sampling as fixed effects and block as a random effect to test the impact of cover crop treatments on the individual target species at different time points. All analyses and graphs were performed using R Studio.

3.3 RESULTS

3.3.1 Montrose – Field Site 1 (Table 3.5)

A significant, 58%, reduction of *Pratylenchus* spp. was observed after growing and incorporating French marigold (p=0.01). Growing Indian mustard significantly increased *Pratylenchus* spp. by 319% at the pre-incorporation stage (p=0.02), suggesting that Indian mustard is a good host for *Pratylenchus* spp. during the growing cycle. Oilseed radish, *Phacelia* and Japanese oats did not affect the abundance of *Pratylenchus* spp. (p>0.05). Incorporating oilseed radish and Indian mustard significantly increased the abundance of bacterivorous nematodes, with the highest increase observed in oilseed radish (335%). Japanese oats, Phacelia and oilseed radish significantly affected the abundance of *Aphelenchoides* spp. after incorporation (p<0.05). Indian mustard significantly increased *Aphelenchus* spp. at the pre-incorporation stage (p=0.04). Except for French marigold and Japanese oats, all cover crops, including disturbed fallow, significantly increased the abundance of *Aphelenchus* spp. at the post-incorporation stage.

3.3.2 Isles of Scilly – Field Site 2 (Table 3.6)

Significant reductions ($p<0.05$) in *Pratylenchus* populations were observed after growing and incorporating oilseed radish (75%) and French marigold (70%). In contrast, Indian mustard significantly increased *Pratylenchus* spp. abundance by 113% ($p=0.01$). The abundance of *Pratylenchus* spp. was unaffected by disturbed fallow and *Phacelia* treatments ($p>0.05$). After incorporation, all cover crops significantly increased bacterivores ($p<0.05$). Oilseed radish and Indian mustard significantly increased *Aphelenchoides* spp. at the post-incorporation stage ($p<0.05$). Except for the disturbed fallow control, cover crops significantly increased the abundance of *Aphelenchus* spp. after incorporation ($p\leq0.05$).

3.3.3 Perth – Field Site 3 (Table 3.7)

Except for the disturbed fallow treatments, all the cover crop treatments significantly reduced the abundance of *Pratylenchus* spp. ($p<0.05$). Growing French marigold, oilseed radish, forage chicory, and alfalfa for three months without incorporation reduced *Pratylenchus* nematodes by 60%, 53%, 54%, and 67%, respectively. Growing cover crops did not affect the abundance of bacterivores, *Aphelenchus* and *Aphelenchoides* spp. ($p>0.05$). No data was recorded for the incorporation stage, as flooding prevented the incorporation of the cover crops.

Table 3.5. Mean abundances (n=4) ± standard error of mean per 200 g soil of *Pratylenchus, Aphelenchus*, *Aphelenchoides* spp. and bacterivore nematodes at planting (Pi), three months after planting (pre- incorporation = Pre) and six weeks post-incorporation (Pf) of oilseed radish (*Raphanus sativus*), Indian mustard (*Brassica juncea*), French marigold (*Tagetes patula*), Japanese oats (*Avena sativa*) and *Phacelia (Phacelia tanacetifolia)* at the field site 1 near Montrose, Scotland. Significant changes in nematode abundances at different times of sampling for each treatment compared to the undisturbed fallow control are indicated with asterisks after Tukey HSD post hoc test (p≤.05).

		Pratylenchus spp.		Bacterivores		
Cover crop treatments	Pi	Pre	Pf	Pi	Pre	Pf
Undisturbed fallow	41 ± 4	44 ± 3	32 ± 3	546 ± 66	2014 ± 1016	2646 ± 1791
Disturbed fallow	22 ± 9	26 ± 15	29 ± 16	812 ± 227	1416 ± 401	2360 ± 748
French marigold	50 ± 7	31 ± 5	21 ± 7*	502 ± 47	1150 ± 424	2666 ± 953
Oilseed radish	61 ± 16	46 ± 12	48 ± 12	1376 ± 352	2781 ± 735	5990 ± 1233*
Indian mustard	26 ± 9	109 ± 38*	26 ± 13	873 ± 128	1895 ± 672	3558 ± 778*
Phacelia	59 ± 20	100 ± 35	41 ± 13	788 ± 149	2550 ± 1045	2742 ± 606
Japanese oats	40 ± 4	96 ± 45	38 ± 10	621 ± 141	1568 ± 395	1778 ± 315
		Aphelenchus spp.		*Aphelenchoides* spp.		
Undisturbed fallow	11 ± 5	23 ± 9	22 ± 9	5 ± 2	9 ± 4	7 ± 1
Disturbed fallow	7 ± 2	30 ± 13	78 ± 45*	4 ± 1	7 ± 3	35 ± 8
French marigold	8 ± 1	16 ± 2	35 ± 11	14 ± 6	19 ± 6	16 ± 3
Oilseed radish	7 ± 3	44 ±28	146 ± 28*	7 ± 3	43 ± 20	222 ± 50*
Indian mustard	4 ± 1	54 ± 21*	72 ± 18*	2 ± 0	23 ± 5	34 ± 13
Phacelia	19 ± 5	50 ± 14	76 ± 28*	8 ± 2	16 ± 7	51 ± 15*
Japanese oats	11 ± 4	34 ± 10	51 ± 8	9 ± 4	11 ± 4	49 ± 24*

Table 3.6. Mean abundances (n=5) ± standard error of mean per 200 g soil of *Pratylenchus, Aphelenchus*, *Aphelenchoides* spp. and bacterivore nematodes at planting (Pi), three months after planting (pre-incorporation = Pre) and eight weeks post-incorporation (Pf) of oilseed radish (*Raphanus sativus*), Indian mustard (*Brassica juncea*), French marigold (*Tagetes patula*) and *Phacelia (Phacelia tanacetifolia)* at the field site 2 on the Isles of Scilly, England. Significant differences in nematode abundances at different times of sampling for each treatment compared to the disturbed fallow control are indicated with asterisks after Tukey HSD post hoc test (p≤0.05).

	Pratylenchus spp.			Bacterivores		
Cover crop treatments	Pi	Pre	Pf	Pi	Pre	Pf
Disturbed fallow	31 ± 5	30 ± 5	24 ± 5	581 ± 186	684± 192	1227 ± 154
French marigold	73 ± 10	14 ± 3*	22 ± 7*	710 ± 214	885 ± 242	1380 ± 107*
Oilseed radish	63 ± 14	15 ± 1*	16 ± 1*	504 ± 70	976 ± 231	1657 ± 259*
Indian mustard	77 ± 29	164 ± 40*	78 ± 22	655 ± 235	963 ± 216	1310 ± 64*
Phacelia	50 ± 4	51 ± 10	48 ± 3.0	356 ± 72	531 ± 242	1447 ± 233*
	Aphelenchus spp.			*Aphelenchoides* spp.		
Disturbed fallow	208 ± 30	258 ± 104	318 ± 104	2 ± 1	8 ± 3	35 ± 10
French marigold	108 ± 45	211 ± 91	462 ± 79*	1 ± 1	3 ± 3	40 ± 8
Oilseed radish	53 ± 13.7	143 ± 44	471 ± 90*	5 ± 2	8 ± 2	83 ± 58*
Indian mustard	67 ± 30	183 ± 50	441 ± 160*	3 ± 1	6 ± 2	70 ± 15*
Phacelia	79 ± 19	124 ± 38.3	358 ± 109*	4 ± 1	9 ± 2	24 ± 7

Table 3.7. Mean abundances (n=5) ± standard error of mean per 200 g soil of *Pratylenchus, Aphelenchus*, *Aphelenchoides* spp. and bacterivore nematodes at planting (Pi) three months after planting (pre-incorporation) in oilseed radish (*Raphanus sativus*), French marigold (*Tagetes patula*), forage chicory (*Cichorium intybus*), alfalfa (*Medicago sativa*) and disturbed fallow control under field conditions at the field site near Perth, Scotland. Significant changes in nematode abundances relative to the disturbed fallow control at different sampling times are indicated with asterisks after the Tukey HSD post hoc test (p≤0.05).

	Pratylenchus spp.		Bacterivores	
Cover crop treatments	Pi	Pre-inc	Pi	Pre-inc
Disturbed fallow	13 ± 1	10 ± 2	1038 ± 137	978 ± 229
French marigold	10 ± 1	4 ± 1*	558 ± 81	648 ± 95
Oilseed radish	15 ± 2	7 ± 1*	929 ± 171	815 ± 238
Forage chicory	13 ± 2	6 ± 1*	599 ± 106	710 ± 94
Alfalfa	15 ± 2	5 ± 1*	1007 ± 137	985 ± 135
	Aphelenchus spp.		*Aphelenchoides* spp.	
Disturbed fallow	45 ± 1	41 ± 4	9 ± 1	8 ± 1
French marigold	39 ± 2	39 ± 2	7 ± 1	5 ± 1
Oilseed radish	39± 3	41 ± 5	10 ± 1	9 ± 2
Forage chicory	41 ± 4	39 ± 2	8 ± 1	9 ± 1
Alfalfa	27 ± 5	23 ± 4	12 ± 5	10 ± 2

3.3.4 Detection of four *Pratylenchus* species at experimental sites

qPCR was conducted to test for four commonly found *Pratylenchus* species, namely *P. penetrans, P. crenatus, P. neglectus* and *P. thornei,* at all field sites. Montrose site tested positive for *P. crenatus* and *P. thornei*. The Isles of Scilly site tested positive for *P. penetrans* and *P. crenatus,* while in Perth site tested positive for *P. crenatus* only.

3.3.5 Montrose – Field Site 1

While qPCR detected *P. crenatus* and *P. thornei* from this site, a comparison of microscopy nematode counts and qPCR data showed a weak positive relationship (R^2=0.052), which was non-significant (p=0.18). Counts from microscopy were higher than the estimated abundance provided by real-time PCR, with qPCR detecting only 29% of the *Pratylenchus* spp. observed by microscopy counts. No differences in the abundance of individual species were observed between cover crop treatments (p>0.05).

3.3.6. Isles of Scilly – Field Site 2

qPCR detected *P. penetrans* and *P. crenatus* in samples from this site, accounting for 15% of the *Pratylenchus* spp. observed by microscopy counts. A comparison of microscope counts and qPCR data showed no relationship (R^2=-0.03; p=0.73). Cover crop treatments had no significant effects on the four individual *Pratylenchus* spp. species (p>0.05).

3.3.7 Perth – Field Site 3

Only *P. crenatus* was detected in samples from this site. No relationship (R^2=0.02; p=0.13) was found between total qPCR estimated abundance and nematode counts performed with microscopy. Compared to microscopy counts, qPCR overestimated the total number of *P. crenatus*. French marigold and forage chicory significantly reduced the abundance of *P. crenatus* compared to the fallow control (Table 3.8).

Table 3.8. *Pratylenchus crenatus* abundance as detected by qPCR for each cover crop in Perth. Asterisks indicate a significant difference relative to the disturbed fallow control after pairwise comparisons (Tukey HSD p≤0.05) of the means.

	Pratylenchus crenatus abundance/200g soil	
Cover crop treatments	Pi	Pre-inc
Disturbed fallow	74 ± 1	45 ± 2
French marigold	83 ± 1	19 ± 1*
Oilseed radish	47 ± 2	30 ± 1
Forage chicory	100 ± 2	28 ± 1*
Alfalfa	63 ± 2	40± 1

3.3.8 Molecular identification of *Aphelenchoides subtenuis* and *Ditylenchus dipsaci*

Separation of the PCR products obtained from the samples on agarose gel yielded no visible band under UV light. Gradient PCRs were done to ensure nematode detection was not hindered by unsuitable annealing temperatures; however, no amplification was observed at any of the tested temperatures for both nematode species. Without a positive control, these results are not adequate to confirm the absence of the target nematodes.

3.4 DISCUSSION

The results of this chapter reveal that certain cover crops effectively reduce *Pratylenchus* spp. abundance without exerting adverse effects on beneficial fungivorous and bacterivorous nematodes. Previous research similarly showed that incorporating cover crops increases the abundance of beneficial nematodes whilst reducing PPNs (Gruver *et al*., 2010; Valdes *et al*., 2012; Lu *et al*., 2016; Waisen *et al*., 2022). A recent meta-analysis revealed that cover crops stimulate plant parasites and bacterivores (Puissant *et al*., 2021). This may seem contradictory to current findings. However, an increase in total PPN abundance does not mean that all the plant parasitic nematodes present will increase, as the response depends on the cover crop's host status. It is plausible that the cover crop treatments reduced some plant parasites that were not assessed in this study.

In the current study, the bacterivore and fungivore nematodes increased six weeks after incorporating some cover crops, with oilseed radish having the most substantial effect. Japanese oats, oilseed radish, and Phacelia consistently stimulated bacterivorous nematodes (Van Himbeeck *et al*., 2024). Incorporated plant material acts as a substrate for fungi and bacteria, which proliferate and are fed on by bacterivorous and fungivorous nematodes (Bonkowski, 2004). Cover crops that produce high shoot biomass are associated with a higher abundance of bacterivorous, omnivorous, and predator/carnivore nematodes (Dietrich *et al*., 2021). The shoot biomass of the cover crops was not assessed at the pre-incorporation stage; therefore, a conclusion cannot be made to explain the influence of oilseed radish on bacterivores after incorporation. The abundance of fungivore and bacterivore nematodes remained unchanged at the third site, Perth, where cover crops were not incorporated. An increased abundance of fungivore (*Aphelenchus* spp.) nematodes observed before incorporation in Indian mustard treatment could be due to fungi being stimulated by root exudates. Plant roots release carbon and other organic materials into the soil by secreting mucilage, exudates and senescence of root epidermal cells (Wu *et al*., 2019). It is known that some cover crops, such as sorghum-sudan grass, release exudates containing carbon and secondary metabolites, which stimulate fungal activity (Paudel *et al*., 2021).

Oilseed radish suppresses *Pratylenchus* spp., *Meloidogyne hapla* (Melakeberhan *et al*., 2008; Ploeg *et al*., 2008) and *Helicotylenchus, Rotylenchus, Trichodorus and Paratrichodorus spp*. (Waisen *et al*., 2022). In the current study, oilseed radish reduced *Pratylenchus* abundances in two trial sites (Isles of Scilly and Perth) before cover crop incorporation. This may reflect that it is a poor host or a partial biofumigant, continually releasing low levels of GSLs from the roots during growth (Ngala *et al.* 2015). The influence of brassica biofumigants on fungivore and bacterivore nematodes has been explored (Forge *et al*., 2003; Wang *et al*., 2004; Georgieva *et al*., 2005). Non-plant parasitic nematodes were less affected by brassica isothiocyanates (ITCs) than PPNs (Stirling and Stirling, 2003). However, in another study done in Belgium by Valdes *et al*. (2012), soil amendments using

Indian mustard increased bacterivore and fungivore nematodes but reduced PPN abundance.

French marigold reduced *Pratylenchus* spp. abundance in all three experiments. French marigolds confer nematicidal effects on PPNs, including root lesion and root knot nematodes (Oostenbrink *et al*., 1957). *Tagetes* spp. release a compound called α-terthienyl, which, upon UV-light activation, generates biocidal reactive oxygen species (Gommers & Bakker,1988). French marigold may reduce PPNs by being a non/poor host and trap crop (Wang et al., 2001), which may work simultaneously for additive effects. These results concur with previous field and greenhouse experiments (Miller, 1978; Kimpinski *et al*., 2000; Pudasaini *et al*., 2006). French marigold is grown in rotation (Ploeg, 1999), applied as plant extracts (Mateeva and Ivanova, 2000), as green manure (Siddiqui and Alam, 1987) or intercropped with cash crops (Abid and Maqbool, 1990). A previous study in the Isles of Scilly was inconclusive due to the absence of *Pratylenchus* in control experimental plots (Tompsett, 2004). This is the first report of French marigold reducing *Pratylenchus* spp. abundance in a UK agricultural/horticultural system.

The present study reports that Japanese oats and *Phacelia* neither reduce nor increase the abundance of *Pratylenchus* spp., even after incorporating the cover crops. This aligns with previous reports that classify Japanese oats and *Phacelia* as maintenance hosts for *P. penetrans* (Forge *et al*., 2000; Knoetze *et al*., 2023; Taning *et al*., 2024; Van Himbeeck *et al*., 2024). Growing alfalfa for three months reduced the abundance of *P. crenatus*, which agrees with a previous study that after five months, *P. crenatus* does not reproduce on alfalfa under field conditions (Willis *et al*., 1982). Other studies have rated alfalfa as a poor host to *P. penetrans* (Thies *et al*., 1995; Neupane and Yan, 2023). The mechanism behind the resistance of alfalfa varieties to *P. penetrans* is unknown, but some authors suggest an accumulation of tannins in root cells in response to nematode infection (Vieira *et al*., 2019). Previous greenhouse experiments in Chapter 3 rated forage chicory as a poor host for *P. penetrans,* which could be the case with *P. crenatus*; as in this study, under field conditions, a significant reduction of *P. crenatus* was observed after growing the cover crop for at least three months. Literature on the interaction of this nematode with forage chicory is unavailable.

Pratylenchus spp. abundance increased after three months under Indian mustard, suggesting it is a good host. Similarly, several studies have reported that Indian mustard is an excellent host to *Pratylenchus* spp. (Rudolph *et al*., 2017; LaMondia, 2021; Neupane and Yan, 2023). Brassica cover crops, including Indian mustard and oilseed radish, are known for their biofumigation effects on PPNs, including *Meloidogyne incognita* (Oliveira *et al*., 2011), *Globodera pallida* (Lord *et al*., 2011; Ngala *et al*., 2015), and *P. neglectus* (Potter *et al*., 1999). At cover crop termination, the stems and foliage of the brassicas are chopped and incorporated into the soil; myrosinase converts stored glucosinolates into nematicidal isothiocyanates. However, even after incorporation in this study, there was no evidence of biofumigation in the Indian mustard treatment. Biofumigation effects of brassica cover crops may be masked if the selected species support nematode reproduction and multiplication (Vervoort *et al*., 2014; Grabau *et al*., 2017).

Although *Narcissus* is known as a host of *D. dipsaci*, the species was not observed at any of the trial sites. This nematode species feeds on stems and bulbs, so it could be possible that their abundance in the soil is reduced to undetectable levels once bulbs are removed. The PCR diagnostic used in this experiment claims detection of as low as a single nematode (Jeszke *et al*., 2015); assuming this is applicable across different soil types, *D. dipsaci* was absent at all the sites.

Seed bulbs undergo hot water treatment every season before planting; could the method be so effective that the problem with *D. dipsaci* is diminishing? Data from the past ten years provided by the Grampian Growers Cooperative in Scotland showed that for their sites, the incidence of *D. dipsaci* is decreasing; in contrast, *Aphelenchoides* spp. is increasing (Supplementary Table 3.1). The USA, a significant export market for the UK *Narcissus* sector, has zero tolerance for *Aphelenchoides* spp.; therefore, any stock that tests positive for *Aphelenchoides* spp. will be rejected, a problem currently faced by growers in Scotland. *Aphelenchoides* is a genus of primarily mycophagous nematodes with a few facultative plant parasites, such as *A. fragariae, A. ritzemabosi* (Sànchez-Monge *et al*., 2017), therefore, it is possible that export bulbs are being rejected due to non-plant parasitic species.

3.4.1 Detection of *Pratylenchus* species using qPCR

The species with the greatest abundance at the two Scottish sites was *P. crenatus*; this species is the most abundant in Scottish potato fields (Orlando, 2020). The presence of nematodes is determined by soil type, present and previous crops, management strategies, and soil moisture (Castillo and Vovlas, 2007). The two study sites in Scotland were under *Narcissus* monoculture for over ten years. No records exist on the pathogenicity of *P. crenatus* on the *Narcissus* crop. *P. penetrans* was recorded in the Isles of Scilly (Hanks and Chastagner, 2018). In these fields, ‘soil sickness’, a root rot disease caused by *P. penetrans* and the fungus *Cylindrocarpon destructans*, is common (Lane, 1984).

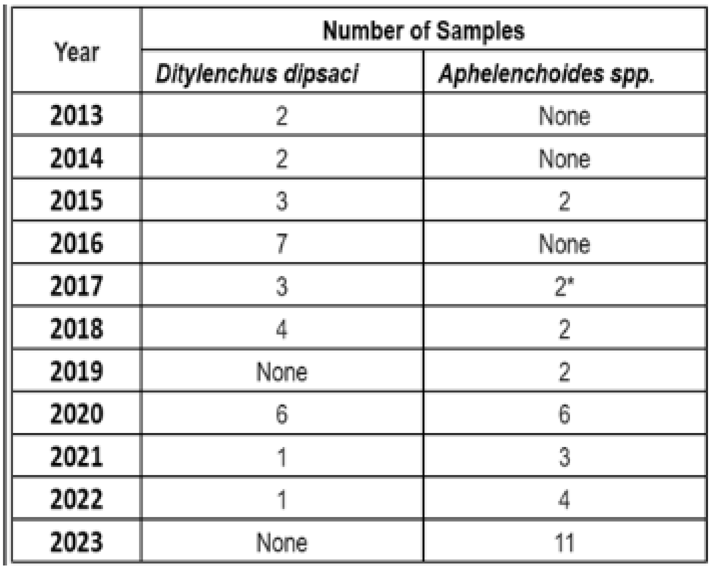
The qPCR detected fewer nematodes than observed under microscopy in Montrose and the Isles of Scilly; such discrepancies could be due to the presence of PCR inhibitors in the samples, leading to poor amplification of the total DNA or other untested *Pratylenchus* spp. (Braun-Kiewnick and Kienwnick, 2018). An overestimation of *P. crenatus* after qPCR compared to microscopy counts was observed in Perth. Similarly, Oliveira *et al*. (2017) recorded more nematodes using qPCR than microscopy counts in the four *Pratylenchus* spp. Previous studies report an overestimation of *Pratylenchus* spp. using qPCR compared to microscopy counts (Yan *et al*., 2013; Baidoo *et al*., 2017). The discrepancy between microscopy counts and qPCR data could be due to different levels of total DNA found in different growth stages of *P. penetrans* (Sato *et al*., 2011), with adults having more DNA than juveniles. There is also a difference between males and females, gravid and non-gravid females; males with sperm and egg in females have more DNA. Griffiths *et al*. (2006) suggest that, generally, larger nematodes contain more DNA than smaller nematodes. Additionally, an isolate of *P. crenatus* from Scotland exhibited more total DNA after qPCR than other tested isolates (Orlando *et al.,* 2024). The DNA copy number or D2-D3 fragment among different *P. crenatus* populations vary, and D2-D3 sequences of Korean *P. crenatus* populations have a 3.7% variation (Kim *et al*., 2019). The extraction methods used for microscopy and qPCR were different; therefore, comparing the abundances might not be as useful. Notwithstanding these issues, nematode diagnostics using DNA-based methods are essential to supplement morphological/microscopy methods to support nematode identification.

3.5 CONCLUSIONS

This Chapter explores the interaction of cover crops and soil nematodes under field conditions. Except for Indian mustard, Japanese oats and *Phacelia*, the tested cover crops support the hypothesis that cover crops suppress *Pratylenchus* spp. without exerting adverse effects on beneficial fungivore and bacterivore nematodes. These results can aid cover crop selection for managing *Pratylenchus* spp. and soil health improvement. In conclusion, growers could use French marigold and oilseed radish, chicory, and alfalfa as summer cover crops to reduce *Pratylenchus* spp. without harmful non-target effects on the beneficial nematodes. The cover crops should be grown for at least three months between June and September, between two consecutive *Narcissus* crops.

3.6. SUPPLEMENTARY INFORMATION

Supplementary Table 3.1. *Narcissus* bulb samples taken from Grampian Growers Cooperative stocks tested positive for *D. dipsaci* and *Aphelenchoides* spp. over the past ten years. (Jason Rumens supplied data from SASA). The total number of samples for each year ranged from 17-20.



Year	Number of Samples	
	Ditylenchus dipsaci	*Aphelenchoides spp.*
2013	2	None
2014	2	None
2015	3	2
2016	7	None
2017	3	2*
2018	4	2
2019	None	2
2020	6	6
2021	1	3
2022	1	4
2023	None	11

CHAPTER 4

ASSESSING THE RESPONSE OF SOIL NEMATODE COMMUNITIES TO COVER CROPS USING NEXT-GENERATION SEQUENCING

4.1 INTRODUCTION

Cover crops affect nematode communities during plant growth and after incorporation (Canarini *et al*., 2019; Liu *et al*., 2021). Although the interaction between certain cover crops on plant parasitic nematodes is known (Valdes *et al*., 2012; Grabau *et al.,* 2017; Taning *et al*., 2024), less information is available about their effects on non-target beneficial nematodes. Past studies have evaluated the influence of cover crops on soil nematode communities (Treonis *et al*., 2018; Li *et al*., 2023; Garba *et al*., 2024; van Himbeeck *et al*., 2024). The impact of cover crops on nematode groups in the soil can persist for a few months (Gruver *et al*., 2010) and sometimes for years (Forge *et al*., 2003). Some studies have reported that cover crops can effectively reduce plant parasitic nematodes without compromising nematode community structures (Gruver *et al*., 2010; Waisen *et al*., 2022). Soil amendments using yellow mustard (*Sinapis alba*) increased bacterivore nematodes (Valdes *et al*., 2012). Conversely, green manuring with *Brassica juncea* and *Sinapis alba* significantly reduced beneficial entomopathogenic nematodes (Ramirez *et al*., 2009). The responses of soil nematode communities to cover crops are species-dependant (Wardle *et al*., 2004; Waisen *et al*., 2022); for instance, oilseed radish (*Raphanus sativus*) was found to be better for improving beneficial nematodes and nutrient enrichment compared to brown mustard (*Brassica juncea*) (Waisen *et al*., 2022). These varying dynamics demonstrate the need to assess the influence of cover crops on non-target nematode groups in the soil.

Cover crop benefits were demonstrated in Chapter 6, where there was an increase in beneficial bacterivore and fungivore nematodes. Therefore, understanding the responses of soil nematode communities to cover cropping helps growers tailor their management practices for optimum ecological services. Cover crops for nematode management should have suppressive effects on plant parasitic nematodes without deleterious effects on soil nematode communities. This Chapter assesses the non-target effects of cover crop treatments that reduced *Pratylenchus* spp. (Chapter 3) on soil nematode composition and soil food web indices using Next-Generation Sequencing of the *18S rRNA* gene. The cover crop treatments used for sequencing across the three sites were French marigold, oilseed radish, forage chicory and alfalfa compared to fallow control.

4.1.1 Objectives

This study aimed to assess the effects of cover crops on the abundance and diversity of soil nematode communities at the three field sites using Next-Generation Sequencing.

The specific objectives were:

1. To assess the effects of cover crops on nematode trophic groups and soil food web indices.
2. To assess the effects of cover crops on soil nematode communities (Alpha and Beta diversity).

4.1.2 Hypotheses

H_1: Cover crops affect nematode trophic groups and soil food web indices.

H_1: Cover crops affect soil nematode communities (Alpha and Beta diversity).

H_0: Cover crops do not affect nematode trophic groups and soil food web indices.

H_0: Cover crops do not affect soil nematode communities (Alpha and Beta diversity).

4.2 MATERIALS AND METHODS

4.2.1 Experimental sites and cover crop treatments

The materials and methods section of Chapter 6 of this thesis describes the three experimental sites. Table 4.1 shows the cover crop treatments from which nematode communities were assessed. French marigold and fallow treatments were used at the Montrose site, and the same treatments plus oilseed radish were used in the Isles of Scilly. In Perth, the treatments included fallow, French marigold, oilseed radish, forage chicory, and alfalfa. These treatments were mainly chosen because they reduced *Pratylenchus* spp. without reducing non-target beneficial nematodes when assessed using microscopy counts (Chapter 3).

Table 4.1. Cover crop treatments used for sequencing across the three experimental sites.

Site	Cover crop	Species	Family	Cultivar	Seeding rate kg ha^{-1}
Montrose Scotland	French marigold	*Tagetes patula* L.	Asteraceae	French marigold	8
	Fallow control	-	-	-	-
Isles of Scilly, Cornwall	French marigold	*Tagetes patula* L.	Asteraceae	French marigold	8
	Oilseed radish	*Raphanus sativus* L.	Brassicaceae	Contra	30
	Fallow control	-	-	-	-
Perth, Scotland	French marigold	*Tagetes patula* L.	Asteraceae	French marigold	8
	Oilseed radish	*Raphanus sativus* L.	Brassicaceae	Contra	30

Alfalfa	*Medicago sativa* L.	Fabaceae	Artemis	25
Forage chicory	*Cichorium intybus* L.	Asteraceae	Commander	8
Fallow control	-	-	-	-

4.2.2 Nematode extraction

Nematode extraction was done from a 200 g soil sub-sample (Wiesel *et al*., 2015) via the modified Baermann funnel technique (Brown & Boag, 1988). Soil samples were gently mixed by hand, and a 200 g subsample was weighed into a labelled 5-litre plastic bucket. Water was added to the bucket until all the soil was covered and soaked overnight. The soaked soil was then fully suspended by jetting a fast stream of water into the bucket. This broke down any remaining soil structure and liberated any nematodes present to leave them suspended in the water. The samples were allowed to stand for 1 min before pouring the suspension through a 2 mm sieve into another bucket. The remaining soil and debris at the bottom of the bucket and any debris collected in the 2mm sieve were discarded. The soil suspension was sieved through a 250 µm sieve. The sieve contents (including nematodes) were placed into a labelled plastic 500 ml beaker, beaker A. The soil suspension was then poured through a 150 µm sieve, and any captured material (including nematodes) was washed off the sieve into the same beaker (beaker A). The beaker was set aside for a few minutes to allow the soil/debris to sediment. The process was repeated sequentially using 75 µm and 53 µm sieves, and the contents of both sieves were washed into a second labelled plastic beaker, beaker B. The recovered material from beaker A was poured through a 95 µm nylon support and then suspended in a glass funnel full of water. The recovered material from the second pair was poured into a 1 mm mesh nylon support on a glass funnel (lined with two-ply Kimtech Science tissues (Kimberly-Clark, product code 7557), suspended in a glass-filled funnel. The funnels were left for 48 hours to allow nematodes to pass through the mesh and migrate to the bottom of the funnels. After 48 h, 10 ml of the nematode suspension was collected from each funnel into a 50 ml glass vial. The vials were left to settle for 2 h before the water was slowly and carefully reduced from the top, using a pipette attached to a vacuum pump system until the final volume was 1.5 – 1.8ml. The nematode suspension was mixed, poured into a labelled 2 ml Eppendorf tube, and frozen at -20°C until completely frozen. The samples were freeze-dried and stored until use.

4.2.3 Genomic DNA extraction

DNA was extracted from freeze-dried nematode samples following a standard protocol using a Purelink 96 Genomic DNA kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Extracted DNA was eluted in 50 µl of 2X Purelink Pro 96 Genomic Elution Buffer.

4.2.4 PCR amplification

Genomic DNA was amplified using the 18s forward primer NF1: (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GGTGGTGCATGGCCGTTCTTAGTT) and reverse primer: 18Sr2b: (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-TACAAAGGGCAGGGACGTAAT) (Treonis *et al*., 2018). The PCR Master-Mix is shown in Table 4.2, and the cycling conditions are shown in Table 4.3 The PCR products were separated on 1% agarose gel and visualised under UV light to check the amplification and to verify the fragment size. The PCR was done in triplicate to concentrate the DNA and then combined.

Table 4.2. PCR Master-Mix volumes used for 18s amplification.

Reagent	Volume (µl)
Water	11.8
KAPA Hi-Fi Buffer	4.0
$MgCl_2$ (25 nm)	0.4
KAPA dNTPs (10 mM each)	0.6
Forward primer (0.3 µM)	0.4
Reverse primer (0.3 µM)	0.4
KAPA HiFi Hotstart polymerase	0.4
DNA template	2.0
Total volume	20.0

4.2.5 PCR clean up 1

AMPure XP beads (part number A63881, Beckman Coulter, Indianapolis, USA) were used to purify the PCR product by removing primers and primer dimers according to the instructions of the standard Illumina protocol.

4.2.6 Index PCR

The cleaned PCR products were indexed using the Nextera XT Index Kit (Set D, Illumina San Diego, USA). The cycling conditions and Index PCR Master-Mix are shown in Tables 4.3 and 4.4, respectively. The PCR products were separated on a 1 % agarose gel and visualised under UV light.

Table 4.3. Temperature, time and number of cycles for amplicon and index PCR of the 18S rDNA

	Temperature °C	Time	Units	
Amplicon PCR	Heated lid 112			
	95	3	minutes	
	95	20	seconds	25 cycles
	55	10	seconds	
	72	20	seconds	
	72	5	minutes	
	10	Hold		
Index PCR	Heated lid 112			
	95	3	minutes	
	95	30	seconds	8 cycles
	55	30	seconds	
	72	30	seconds	
	72	5	minutes	
	10	Hold		

Table 4.4. Reagents and volumes used in the indexing PCR

Reagent	Volume (µl)
Water	21.5
KAPA Hi-Fi Buffer	10
$MgCl_2$ (25 nm)	1
KAPA dNTP Mix (10 mM each)	1.5
KAPA HiFi Hotstart polymerase	1
Nextera XT Primer 1 (orange) 1-12	5
Nextera XT Primer 2 (white) A-H	5
Cleaned-up first-round PCR product	5
Total Volume	50

4.2.7 Index PCR clean-up

According to the manufacturer's instructions, the indexed PCR product was cleaned using AMPure XP beads. The DNA concentration of the final indexed amplicons was determined by Picogreen analysis. The DNA in all the samples was then pooled to equimolar concentrations before being sent for Illumina sequencing on the MiSeq platform at the James Hutton Institute.

4.2.8 Bioinformatics

Amplicon sequence data was imported into the High-performance computing cluster (UK Crop Diversity Bioinformatics HPC Resource) hosted by The James Hutton Institute, Dundee. All sequences underwent quality checking using FastQC, and Fastq-join was used to join the paired-end reads. Bioinformatics of the amplicon libraries was done through the QIIME 2 pipeline (Bolyen et al., 2019). The sequences were demultiplexed and filtered using the QIIME2-demux plugin to check if any samples produced very few sequences (<10 000) compared to the rest of the samples. All samples except negative controls produced read numbers ranging from 34 653 to 88 923. The primer sequences were removed using cutadapt (Martin, 2011). The sequences were trimmed and denoised to create paired-end reads using the QIIME2-DADA2 plugin (Callahan *et al*., 2016) using the DADA2 denoise paired function with default parameters and set to trim sequences after 220 bp. Approximately 80% of the input sequences passed the denoising and chimera filters. The Amplicon Sequence Variants (ASVs) were assigned taxonomy using the classify-sklearn and using trained Nemataxa_V1 as the classifier (Baker *et al*., 2023). All ASVs assigned to other Phyla except Nematoda or other Kingdoms such as Bacteria, unclassified or Unknown, were filtered out. These accounted for 0.7% of the total processed sequences. In the end, 80% of the sequences for the 18S remained for subsequent analysis (2038 ASVs).

4.2.9 Statistical analysis

4.2.9.1 Effects of cover crops on nematode trophic groups and soil food web indices

All nematode genera were assigned to five trophic groups (Yeates *et al*., 1993): herbivores/plant parasites, fungivores, bacterivores, omnivores and predators using NINJA (Sieriebriennikov *et al*., 2014) software (https://sieriebriennikov.shinyapps.io/ninja). The NINJA online web platform was used to calculate the enrichment index (EI), structure index (SI), maturity index (MI), and channel index (CI) (Ferris *et al*. 2001). The EI indicates the number of bacterial and fungal-feeding nematodes that respond quickly to carbon and nitrogen sources (Biswal, 2022). SI indicates soil food web connection and length by assessing the relative abundances of high-trophic group nematodes. CI is the fungivore: bacterivore ratio, which indicates primary decomposition pathways. The MI is the weighted average cp-value for nematodes in a sample minus plant parasitic nematodes and dauer larvae, which ranges from 1 to 5 (Bongers, 1990). However, the samples from this study include dauer larvae as they cannot be distinguished by the DNA metabarcoding methods used. To test the effects of the cover crops on trophic groups and food web indices, a linear mixed effects model was fitted with cover crop and time of sampling as fixed effects and block as a random factor. Estimated marginal means and statistical differences were calculated using emmeans (Lenth, 2022) and multcomp (Hothorn *et al*., 2008) with Benjamini-Hochberg correction for pairwise comparison. All data analysis was performed using R software (version 4.1.3) (R Core Team, 2022).

4.2.9.2 Effects of cover crops on soil nematode communities

The ASV, taxon tables and sample metadata were merged into a phyloseq object (McMurdie and Holmes, 2013). A standard data frame was obtained from this object for subsequent statistical analysis. Two standard Alpha (within) diversity indices, Shannon Weaver and Simpson, were computed on rarefied samples in phyloseq using the *estimate_richness* function. The Shannon index assumes all individuals are present and are randomly sampled. Simpson index focuses on the common or dominant species; therefore, a few rare individuals in the community will not impact the diversity. Thus, Shannon and Simpson are species richness and dominance indices, respectively (Dejong, 1975). A linear mixed-effects model was used to determine the effect of the cover crop treatment and time of sampling on the calculated indices. The assumptions of normality and homogeneity of variances were tested; when they were not met, data was log transformed, and alternative non-parametric tests were considered. ANOVA was performed on the models to test for significant differences in the indices across the cover crop treatments and time of sampling. When significant differences were detected, Tukey HSD test was used for post hoc pairwise comparisons of the treatments.

Beta diversity (between-sample dissimilarity) was assessed using Euclidean distance matrices (straight line between two samples in a multidimensional space). The vegan package (Oksanen *et al*., 2018) and *vegdist* function were used to compute the distance matrices. Principal coordinates analysis (PCoA) was done to determine differences between nematode communities based on the Euclidean resemblance matrix. *Betadisper* function from the *vegan* package was used to perform a dispersion test to test the consistency of the variability within each factor. A Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2005) was performed to test the effect of cover crop treatments and sampling time on the nematode communities. Post hoc analysis of the PERMANOVA was carried out whenever there was a significant effect using the *Adonis* pairwise function from the vegan package. Where significant differences in the communities were observed, similarity percentage analysis (SIMPER) was performed to understand which taxa mainly contribute to nematode community dissimilarity between treatments. All analyses were conducted in the vegan package of R using *metaMDS* and *simper* functions (Oksanen *et al*., 2018).

4.3 RESULTS

4.3.1 Nematode genera detected across the experimental sites

Table 4.5 lists the nematode genera detected across the three experimental sites. A total of 76 different taxa were identified at the genus level, with most taxa being bacterivores (28 genera), plant parasites (21 genera), predators (11 genera) and fungivores (10 genera). Omnivores were the least diverse trophic group, represented by only six genera. Sixty-five per cent of the genera were common at all three sites. In Montrose, *Rhabditis* (69%), *Mesorhabditis* (3%) and *Diphtherophora* (2%) were the most abundant genera, while at the Isles of Scilly, the abundant genera were *Rhabditis* (25%), *Pungentus* (11%) and *Pristionchus*, (9%). At the Perth site, *Rhabditis* (70%), *Pristionchus* (9%) and *Aphelenchoides* (2%) were most abundant.

Table 4.5. Nematode taxa detected from soil samples collected at Montrose, Isles of Scilly and Perth, their coloniser-persister (c-p) classification and feeding types according to Yeates *et al*., 1993 and Bongers, 1990. Y is present, and N is absent.

Genus	c-p scale (1-5)	Montrose	Isles of Scilly	Perth	Feeding type
Aglenchus	2	N	Y	N	Plant parasites
Axonchium	5	Y	N	N	Plant parasites
Basiria	2	Y	Y	Y	Plant parasites
Bitylenchus	3	Y	Y	Y	Plant parasites
Meloidogyne	3	N	Y	N	Plant parasites
Boleodorus	2	Y	Y	Y	Plant parasites
Coslenchus	2	Y	Y	Y	Plant parasites
Criconemoides	3	N	Y	Y	Plant parasites
Dorylaimellus	5	N	Y	Y	Plant parasites
Ecphyadophora	2	Y	Y	Y	Plant parasites
Helicotylenchus	3	Y	Y	Y	Plant parasites
Merlinius	3	Y	N	Y	Plant parasites
Irantylenchus	2	Y	Y	Y	Plant parasites
Lelenchus	2	Y	Y	Y	Plant parasites
Longidorus	5	Y	Y	Y	Plant parasites
Neopsilenchus	2	Y	Y	Y	Plant parasites
Paratylenchus	2	Y	Y	Y	Plant parasites
Pratylenchus	3	Y	Y	Y	Plant parasites
Rotylenchus	3	Y	N	Y	Plant parasites
Trichodorus	4	Y	Y	Y	Plant parasites
Tylenchus	2	Y	Y	Y	Plant parasites
Aphelenchoides	2	Y	Y	Y	Fungivores
Aphelenchus	2	Y	Y	Y	Fungivores
Aprutides	2	Y	Y	Y	Fungivores
Diphtherophora	3	Y	Y	Y	Fungivores
Bursaphelenchus	2	N	Y	N	Fungivores
Paraphelenchus	2	Y	N	Y	Fungivores
Ditylenchus	2	Y	Y	Y	Fungivores
Filenchus	2	Y	Y	Y	Fungivores
Tylencholaimellus	4	N	Y	Y	Fungivores

Tylopharynx	1	N	Y	Y	Fungivores
Acrobeles	2	N	N	Y	Bacterivores
Acrobeloides	2	Y	Y	Y	Bacterivores
Alaimus	4	Y	Y	Y	Bacterivores
Amphidelus	4	Y	N	Y	Bacterivores
Anaplectus	2	Y	Y	Y	Bacterivores
Bastiania	3	Y	Y	Y	Bacterivores
Bunonema	1	N	N	Y	Bacterivores
Cruznema	1	Y	Y	Y	Bacterivores
Cylindrolaimus	3	N	N	Y	Bacterivores
Domorganus	3	N	N	Y	Bacterivores
Eucephalobus	2	Y	Y	Y	Bacterivores
Eumonhystera	2	Y	Y	Y	Bacterivores
Heterocephalobellus	2	N	Y	N	Bacterivores
Heterocephalobus	2	Y	Y	N	Bacterivores
Mesorhabditis	1	Y	Y	Y	Bacterivores
Metateratocephalus	3	Y	Y	Y	Bacterivores
Monhystera	2	N	Y	Y	Bacterivores
Odontolaimus	3	Y	Y	Y	Bacterivores
Panagrolaimus	1	Y	Y	Y	Bacterivores
Plectus	2	Y	Y	Y	Bacterivores
Prismatolaimus	3	Y	Y	Y	Bacterivores
Prodesmodora	3	Y	Y	Y	Bacterivores
Protorhabditis	1	N	N	Y	Bacterivores
Rhabditis	1	Y	Y	Y	Bacterivores
Rhabditophanes	1	Y	Y	Y	Bacterivores
Rhabdolaimus	3	Y	Y	Y	Bacterivores
Steinernema	1	Y	Y	Y	Bacterivores
Achromadora	3	N	Y	N	Bacterivores
Anatonchus	4	Y	Y	Y	Predators
Clarkus	4	Y	Y	Y	Predators
Discolaimus	4	N	Y	Y	Predators
Mylonchulus	4	Y	Y	Y	Predators
Nygolaimus	5	Y	Y	Y	Predators
Paravulvus	5	Y	Y	Y	Predators
Prionchulus	4	N	N	Y	Predators

Pristionchus	1	Y	Y	Y	Predators
Seinura	2	Y	N	Y	Predators
Stenonchulus	3	Y	Y	Y	Predators
Tripyla	3	Y	Y	Y	Predators
Aporcelaimellus	5	N	Y	Y	Omnivores
Aporcella	5	Y	Y	Y	Omnivores
Prodorylaimus	4	Y	Y	Y	Omnivores
Pungentus	4	Y	Y	Y	Omnivores
Rhyssocolpus	4	N	N	Y	Omnivores
Thonus	4	Y	Y	Y	Omnivores

4.3.2 Effects of cover crops on nematode trophic groups and soil food web indices

At all sites, there was a significant difference in the relative abundance of different trophic groups ($p<0.001$). However, except for a single marginal effect ($p<0.06$) in the Simpson index for Montrose samples), there were no significant differences in nematode trophic groups between cover crop treatments or time of sampling (Figure 4.2) or alpha diversity (Shannon and Simpson indices; Tables 4.6, 4.7 and 4.8) across all sites ($p>0.05$). The Montrose site was highly nutrient-enriched soil dominated by opportunistic bacterivorous nematodes and a well-structured and matured soil food web (Figure 4.1). ANOVA showed that cover crop treatments and sampling times did not affect the MI, CI, EI and SI indices ($p>0.05$).

The Isles of Scilly was matured, N-enriched with bacterial-driven decomposition. The channel index was unaffected by cover crop treatments. Significant differences in EI ($p=0.01$), SI ($p=0.04$), and MI ($p=0.01$) were observed between the cover crop treatments. The highest EI was observed after incorporating oilseed radish and French marigold, while the highest SI was observed before incorporation of the same cover crops. At the same time, MI was reduced by incorporating oilseed radish, French marigold, and a fallow plot.

The plots in Perth had predominantly maturing, N-enriched bacterial decomposition food webs, with a few plots having a disturbed, N-enriched, and conducive food web (Figure 4.1). No effects of cover crop treatments were observed for all indices.

Table 4.6. Average Shannon and Simpson, Maturity (MI), Channel (CI), Enrichment (EI) and Structure (SI) indices ± standard error for the cover crop treatments at planting and three months after planting (pre-incorporation) and 6 weeks post-incorporation stage in Montrose, Scotland. There were no significant differences in nematode abundance between the individual plots at planting (by microscopy counts); therefore, the treatments were compared to the whole field at planting to save costs.

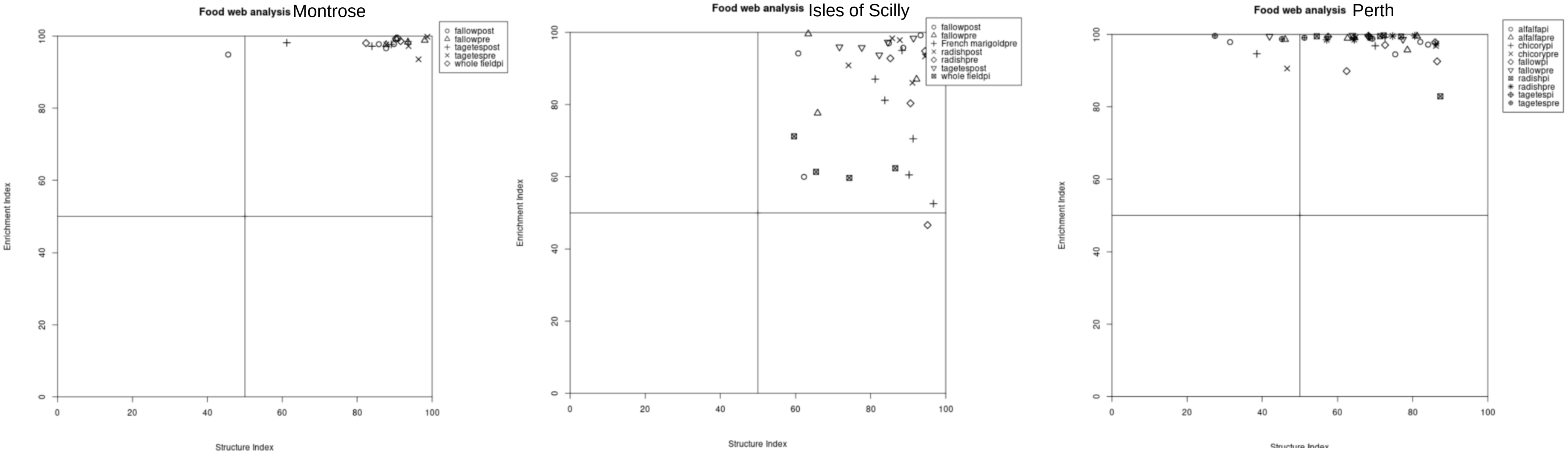
Cover crop	Time	Shannon	Simpson	MI	CI	EI	SI
Whole field	pi	1.45 ± 0.2	0.46 ± 0.1	1.29 ± 0.1	0.31 ± 0.2	98.81 ± 0.6	88.79 ± 4.3
Fallow	pre	1.89 ± 0.3	0.63 ± 0.1	1.60 ± 0.4	0.47 ± 0.4	98.52 ± 0.7	92.44 ± 4.4
	post	2.27 ± 0.1	0.78 ± 0.0	1.47 ± 0.2	1.05 ± 0.5	96.75 ± 1.4	77.23 ± 21.1
French marigold	pre	2.15 ± 0.4	0.68 ± 0.1	1.93 ± 0.7	1.7 ± 2.0	97.06 ± 2.6	94.16 ± 4.7
	post	2.13 ± 0.1	0.73 ± 0.1	1.42 ± 0.2	0.55 ± 0.48	97.87 ± 0.6	81.96 ± 14.3
	p-value	0.46	0.06	0.21	0.48	0.27	0.21

Table 4. 7. Average Shannon and Simpson, Maturity (MI), Channel (CI), Enrichment (EI) and Structure (SI) indices ± standard error for the cover crop treatments at planting and three months after planting (pre-incorporation) and 6 weeks post incorporation stage in Isles of Scilly, England. Different letters on MI, EI and SI indicate differences compared to the whole field at planting after the Tukey HSD test at p≤0.05. There were no significant differences between nematode abundances in the individual plots at planting (by microscopy counts); therefore, the treatments were compared to the whole field at planting to save costs.

Cover crop	Time	Shannon	Simpson	MI	CI	EI	SI
Whole field	pi	2.72 ± 0.1	0.87 ± 0.1	2.54 ± 0.4b	39.93 ± 7.3	63.65 ± 5.1a	71.47 ± 11.7a
Fallow	pre	2.33 ± 0.4	0.83± 0.1	1.98 ± 0.9ab	9.48 ± 12.1	88.08 ± 11.0b	73.81 ± 16.0ab
	post	2.25 ± 0.1	0.78 ± 0.0	1.70 ± 0.5a	16.18 ± 30.7	89.21 ± 16.5ab	77.95 ± 15.4ab
Oilseed radish	pre	2.48 ± 0.2	0.84 ± 0.0	2.80 ± 0.8b	13.84 ± 22.1	81.66 ± 20.4ab	92.13 ± 4.3b
	post	2.40 ± 0.3	0.82 ± 0.1	1.99 ± 0.7ab	5.41 ± 5.6	93.28 ± 5.1bc	86.65 ± 7.8ab
French marigold	pre	2.46 ± 0.1	0.83 ± 0.0	2.85 ± 0.8b	24.36 ± 24.1	74.47 ± 16.2ab	88.61 ± 5.5ab
	post	2.26 ± 0.1	0.80± 0.0	1.51 ± 0.2a	3.80 ± 2.7	96.21 ± 1.8bc	81.51 ± 7.4ab
	p-value	0.10	0.80	0.01	0.11	0.01	0.04

Table 4. 8. Average Shannon and Simpson, Maturity (MI), Channel (CI), Enrichment (EI) and Structure (SI) indices ± standard error for the cover crop treatments at planting and three months after planting (pre-incorporation) and 6 weeks post-incorporation stage in Perth, Scotland. Due to missed post-incorporation stage, individual plots at planting and pre-incorporation (3 months after planting) were compared.

Cover crop	Time	Shannon	Simpson	MI	CI	EI	SI
Fallow	pi	2.22 ± 0.2	0.75 ± 0.1	1.59 ± 0.3	3.75 ± 3.1	94.33 ± 3.8	76.89 ± 11.6
	pre	1.22 ± 0.0	0.50 ± 0.0	1.07 ± 0.1	0.30 ± 0.2	99.31 ± 0.5	62.87 ± 15.0
Oilseed radish	pi	1.48 ± 0.4	0.8 ± 0.1	1.35 ± 0.7	1.57 ± 3.3	96.21 ± 7.5	72.46 ± 11.9
	pre	1.37± 0.2	0.8 ± 0.1	1.08 ± 0.0	0.30 ± 0.3	99.06 ± 0.6	69.18 ± 10.5
French marigold	pi	1.26 ± 0.1	0.52 ± 0.1	1.07 ± 0.0	0.14 ± 0.1	99.33 ± 0.2	64.63 ± 5.1
	pre	1.18 ± 0.1	0.48 ± 0.1	1.07 ± 0.0	0.14 ± 0.1	99.05 ± 0.4	48.31 ± 17.3
Forage chicory	pi	1.83 ± 0.1	0.66 ± 0.0	1.28 ± 0.2	2.29 ± 1.7	97.03 ± 1.9	66.84 ± 20.1
	pre	1.90 ± 0.3	0.69 ± 0.1	1.39 ± 0.2	3.53 ± 3.8	96.01 ± 3.8	70.98 ± 19.1
Alfalfa	pi	1.85 ± 0.3	0.65 ± 0.1	1.35 ± 0.2	2.51 ± 1.6	96.87 ± 1.6	68.23 ± 24.8
	pre	1.73 ± 0.0	0.73 ± 0.0	1.19 ± 0.2	1.80 ± 1.4	98.20 ± 1.7	67.13 ± 16.2
	p-value	0.45	0.31	0.18	0.12	0.39	0.51



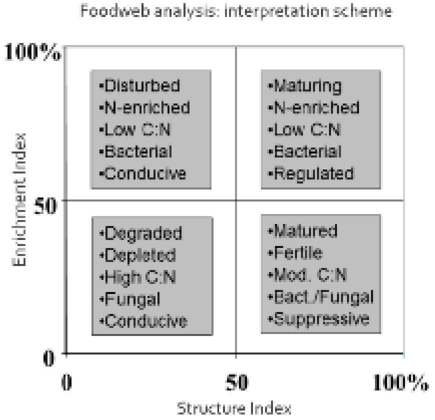


Figure 4.1. Illustration of the enrichment and maturity of the soil food web in Montrose, Isles of Scilly and Perth. The enrichment index (EI) is on the y-axis, and the structure index (SI) is on the x-axis. Each graph is divided into four quadrants that represent the level of nitrogen enrichment and system disturbance, according to Ferris *et al*. (2001). Quadrant food web interpretation is shown in the bottom right, representing the following food webs: 1) disturbed and N-enriched, 2) maturing and N-enriched, 3) degraded and N-depleted, and 4) mature and fertile.

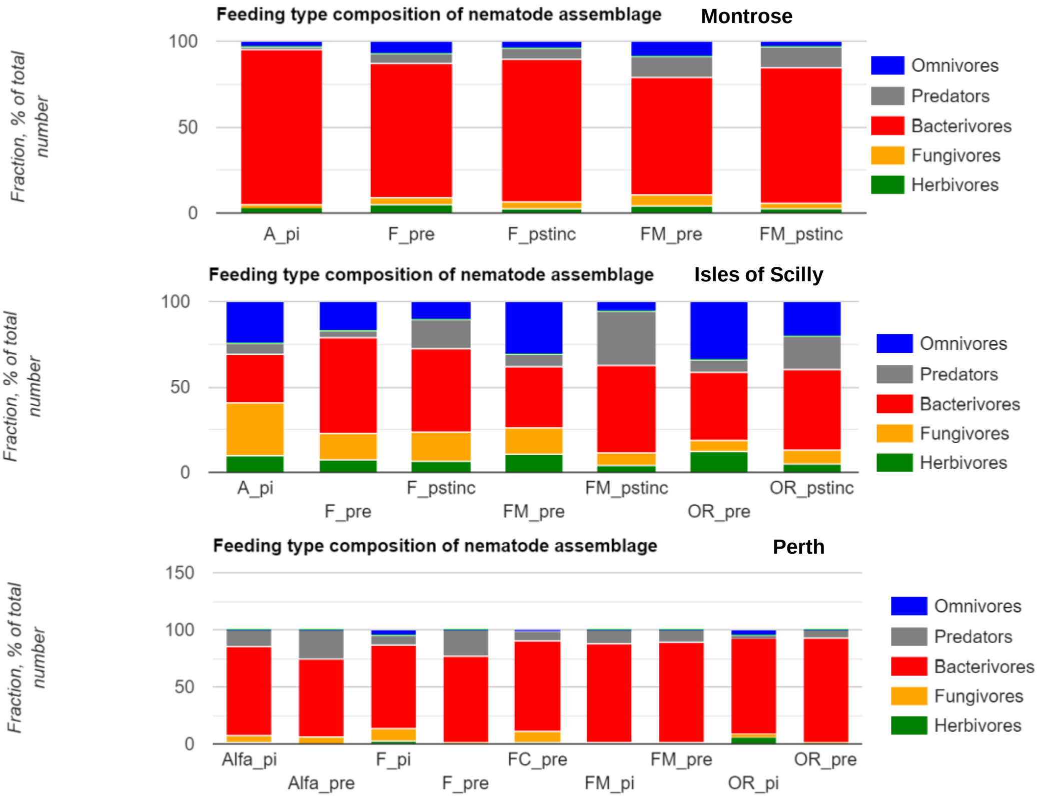


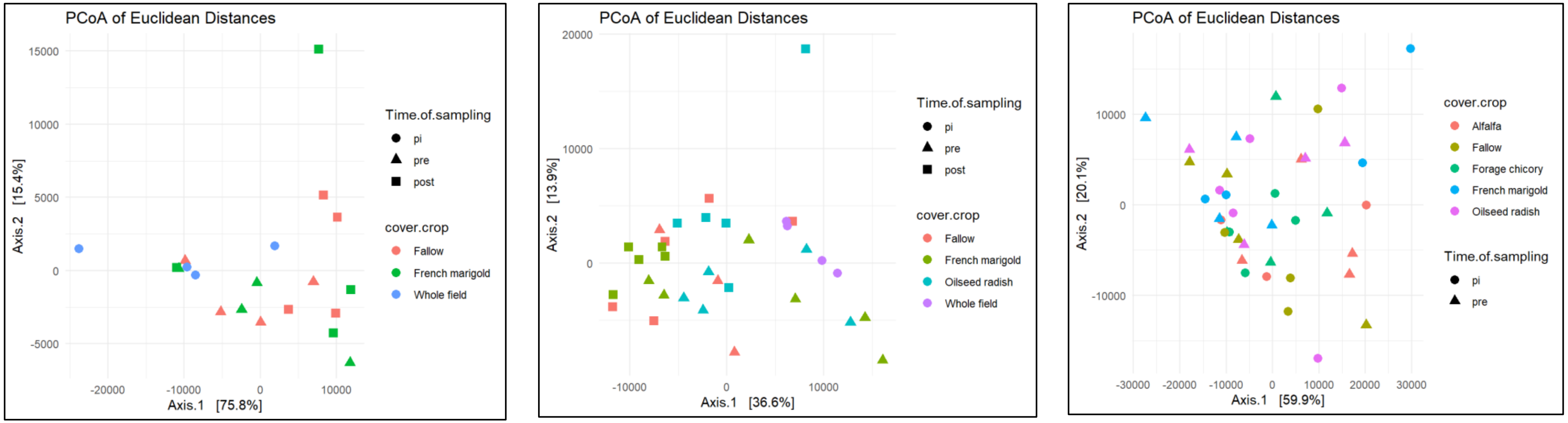
Figure 4.2. The abundance of different nematode trophic groups across cover crop treatments and time of sampling in Montrose, Isles of Scilly and Perth. A_pi= At planting, pre= pre-incorporation stage, pstinc= post-incorporation. Pi= at planting, F= Fallow, FM= French marigold, OR= oilseed radish, FC= forage chicory, Alfa= alfalfa.

4.3.3 Effects of cover crops on nematode communities (beta-diversity)

PCoA was used to visualise the patterns of nematode community composition based on Euclidean distance matrices. In Montrose, the first and second principal coordinates (PCo1 and PCo2) explained 75.8% and 15.4% of the variation without clear clusters or groupings (Figure 4.3). PERMANOVA showed a marginal significant effect of the cover crop treatment (p=0.06) and no effect of time of sampling (p=0.08) on the nematode communities.

In the Isles of Scilly, PCo1 explained 36.6% of the variation in the community, while PCo2 explained 13.9% (Figure 4.3). Analysis by PERMANOVA provided evidence that nematode communities were significantly affected by sampling time (p=0.02) but not by cover crop treatment (p=0.19). Pairwise comparisons revealed that nematode communities significantly differed at the planting, pre-, and post-incorporation stages. SIMPER analysis showed that eight genera (*Rhabditis, Mesorhabditis, Diphtherophora, Neopsilenchus, Prodorylaimus, Plectus, Oxydirus* and *Pungentus)* contributed the most to dissimilarities between post- and pre-incorporation stages (71.5% dissimilarity), with *Rhabditis* contributing the most (19%). In contrast, *Pungentus* contributed the least (5.0%) (Table 4.9). Nine genera (*Rhabditis, Mesorhabditis, Aporcella, Diphtherophora, Prodorylaimus, Neopsilenchus, Plectus, Acrobeloides* and *Mylonchulus*) were most influential in dissimilarity between planting and post-incorporation stages (72% dissimilarity). *Rhabditis* contributed the most (20%), while *Mylonchulus* contributed the least (3.9%) (Table 4.9). Nine genera (*Rhabditis, Aporcella, Oxydirus, Diphtherophora, Pungentus, Mylonchulus, Plectus*, *Acrobeloides and Mesorhabditis*) were most influential in dissimilarity between pre-incorporation and planting stages (70% dissimilarity).

In Perth, the first and second principal coordinates (Pco1 and Pco2) explained 59.9% and 20.1% of the variation, respectively, with no clear patterns (Figure 4.3). The nematode communities were not significantly affected by cover crop treatment (p=0.5) and time of sampling (p=0.3) as determined by PERMANOVA analysis.


Montrose

Isles of Scilly

Perth

Figure 4.3. Euclidean distance-based ordination plots for different times of sampling and cover crop treatments at Montrose, Perth, and the Isles of Scilly.

Table 4.9. Results of SIMPER analysis of the genera that contributed most to the dissimilarity of soil nematode communities at different sampling times in the Isles of Scilly.

Post vs pre-incorporation (71.5% dissimilarity)

Genus	%contribution to difference	% relative abundance	
		post	pre
Rhabditis	19.3	36.3	42.9
Mesorhabditis	13.3	13.1	1.7
Diphtherophora	9.9	5.7	4.7
Prodorylaimus	6.1	5.7	0.4
Neopsilenchus	6.6	6.2	0.4
Plectus	5.9	3.3	2.6
Oxydirus	5.5	3.1	6.8
Pungentus	5.0	2.4	4.2

Post-incorporation vs planting (72% dissimilarity)

Genus	%contribution to difference	% relative abundance	
		post	pi
Rhabditis	19.9	36.3	19.8
Mesorhabditis	12.2	13.1	2.3
Aporcella	7.8	2.7	9.9
Diphtherophora	6.6	5.7	1.8
Neopsilenchus	6.0	6.2	1.3
Prodorylaimus	6.0	5.7	0.1
Acrobeloides	5.5	2.0	6.0
Plectus	5.1	3.3	2.5
Mylonchulus	3.9	0.1	3.8

Pre-incorporation vs planting (70% dissimilarity)

Genus	%contribution to difference	% relative abundance	
		pre	pi
Rhabditis	28.2	42.9	19.8
Aporcella	8.4	2.9	9.9
Oxydirus	6.2	6.8	2.7
Diphtherophora	6.1	4.7	1.8
Pungentus	4.7	4.2	0.6
Mylonchulus	4.5	0.4	3.8
Plectus	4.3	2.6	2.5
Acrobeloides	4.2	2.9	6.0
Mesorhabditis	3.5	1.7	2.3

4.4 DISCUSSION

This study aimed to assess the effects of cover crops on the abundance and diversity of soil nematode communities at three field sites. In this study, bacterivore nematodes were the most abundant across all sites. These results are similar to observations from a meta-analysis in which bacterivores were the most abundant in arable fields (Van Den Hoogen *et al*., 2019). Ecological indices were analysed to further understand the nematode community structure and the associated soil food webs. The results indicate that soil nematode communities (beta diversity), alpha diversity indices (Shannon and Simpson), trophic groups, and food web indices (MI, CH, EI and SI) were unaffected by cover crop treatments. Incorporating cover crops was associated with a higher EI and lower SI and MI. The SI increased when French marigold and oilseed radish cover crops were grown for at least three months, but the SI decreased after incorporation. At the same site, soil nematode communities (beta diversity) were unaffected by cover crop treatments; instead, they were influenced by the time of sampling, which is reflected by changes in relative abundances of 8-9 nematode genera, particularly bacterivores, predators. These results agree with and support the hypothesis that cover crops have no adverse effects on soil nematode communities but instead shift the soil food web status to an enriched bacterial-dominated decomposition pathway due to the incorporation of plant material.

In this study, cover crop incorporation did not influence the abundances of nematode trophic groups at all sites, which aligns with the observations of Li *et al*. (2023). Indices help to reflect the shift in nematode communities, which sometimes, like in this study, were not visible from just trophic group abundances. Nematode maturity indices combine the life history strategies and functional roles in the food web (Bongers, 1990). Short life cycles and high fecundity with higher nutritional requirements are characteristic of "*colonisers*" or *r*-strategist nematodes with low coloniser-persister scores (c-p 1-2). On the other hand, long-lived, low fecund nematodes with low nutritional requirements are termed "*persisters*" or *K*-strategists with higher c-p values (c-p 3-5) (Bongers, 1990; Bongers and Ferris, 1999). Maturity, channel, enrichment, and structure indices are used to measure the ecological succession of soil nematode communities (Ferris *et al*., 2001). MI is scaled from 1-5 (Bongers, 1990), and it measures the sensitivity of nematode taxa to stress or disturbance in the agroecosystem (Freckman and Ettema, 1993). In this study, the MI was unaffected by cover crop or time of sampling in two of the sites, Montrose and Perth. In contrast, in the Isles of Scilly, a reduction in the MI and SI was observed after the incorporation of French marigold and oilseed radish. Low MI and SI values indicate a less structured nematode community with fewer persister nematodes with higher cp-values. Cover crop incorporation is mechanical and disturbs the soil, and persisters are sensitive to these disturbances (Ferris *et al*., 2001). The low MI values associated with the incorporation of cover crops indicate high disturbance of the soil ecosystem. In contrast, the MI values were high at planting and before incorporation, indicating persistent nematodes and low disturbance (Yeates and Bongers, 1999).

The three sites were predominantly N-enriched, low C: N, maturing, bacterial decomposition channels and regulative characteristics. In the Isles of Scilly, incorporating oilseed radish and French marigold increased the EI, meaning the cover crop's plant matter was sufficient to enrich the soil and activate the bacterial decomposition pathway. EI is responsive to bacterivore/fungivore opportunistic nematodes. Cover crop incorporation adds organic material to the soil, which increases bacteria, allowing coloniser nematodes with low c-p values to colonise it (Freckman and Ettema, 1993). For instance, oilseed radish strongly stimulates several bacterial families, such as Pseudomonaceae and Erwiniaceae (Cazzaniga *et al*., 2023). The findings align with previous studies (DuPont *et al*., 2009; Puissant *et al*., 2021; van Himbeeck *et al*., 2024) in which cover crops stimulate bacterivorous nematodes. Cover crops were expected to change the diversity and abundance of different nematode trophic groups, as crops are known to affect the structures of nematode communities (Ettema and Bongers, 1993; Neher, 1999). Cover crops add organic matter to the soil, increasing the abundance of all feeding groups (Nahar *et al*., 2006).

The EI and SI revealed that all the sites are N-enriched, maturing, and bacterivore-dominated food webs due to cover crops. EI can indicate an increase in soil fertility status after adding organic matter (Forge *et al*., 2003). The cover crop residues most likely had a low C: N ratio and stimulated bacterial microbes, which serve as substrate to bacterivorous nematodes; residues with high C: N are associated with fungal decomposition (Lundquist *et al*., 1999). Before the incorporation of cover crops, the SI was higher than fallow, suggesting that growing cover crops stabilises the soil and maintains a structured food web with more linkages. The shifting of nematode communities to higher c-p values indicates a transition from colonisers to persisters (Bongers and Bongers, 1998). However, disturbance at incorporation reduced the SI, but it was not as low as the fallow control. These results indicate that growing cover crops benefits soil health and nutrient cycling capacity. Generally, soil nematode communities associated with fields that have cover crops have a higher diversity, resulting in a healthier and more stable soil (Gruver *et al*., 2010, Wang *et al*., 2011). In previous studies, fallow plots had fewer bacterivores compared to cover crop treatments (Wang *et al*., 2006; DuPont *et al*., 2009).

The channel index (CI) is the ratio of fungivorous nematodes to the sum of fungivores and bacterivores. In this study, CI was unaffected by cover crop treatments or sampling time. CI > 50% means the soil is dominated by fungal decomposition, while <50% means domination of bacterial decomposition. At all three sites, the channel index was <50%; therefore, it can be concluded that the sites were predominantly characterised by bacterial decomposed soil food web channels (Ferris *et al*., 2001).

The nematode community composition differed at different times of sampling. The dissimilarity was due to differences in the abundance or absence of the mainly opportunistic bacterivores *Rhabditis, Mesorhabditis, Plectus, Oxydirus*, the fungivore *Diphtherophora*, and the predators *Prodorylaimus* and *Pungentus.* Fungivorous nematodes were less diverse than bacterivores and plant parasites, and the dominating genera were *Diphtherophora*, *Aphelenchus*, and *Aphelenchoides*. These fungivore genera dominate most arable fields in temperate zones (Grabau *et al*., 2017; van Himbeeck *et al*., 2024). This study was short-term; therefore, the long-term effects of cover cropping might differ from the current observation (Chapagain *et al*., 2020; Finney *et al*., 2017).

In Chapter 3, attempts to identify *A. subtenuis* and *D. dipsaci* using conventional PCR yielded inconclusive results. *Aphelenchoides* and *Ditylenchus* ASVs were blasted against the NCBI nucleotide database, and none of them were positive for either *A. subtenuis* or *D. dipsaci*. This provides confidence to the negative PCR results in Chapter 3 and adds evidence that both species, which are pathogens of *Narcissus,* were absent at the three field sites.

4.5 CONCLUSIONS

Growing cover crops impacts the soil nematode communities by favouring a shift towards an N-enriched bacterial-dominated decomposition. Cover crops are species-specific, but from the results of this study, no adverse effects on non-target nematodes were observed after growing alfalfa, oilseed radish, French marigold or forage chicory for a minimum of three months before incorporation. Testing the non-target effects of other cover crop species for their impact on nematode community structure and conducting similar studies over longer timescales are recommended.

CHAPTER 5

BEHAVIOURAL RESPONSE OF *PRATYLENCHUS PENETRANS*, AFTER EXPOSURE TO ROOT EXUDATES OF FORAGE CHICORY (*CICHORIUM INTYBUS*) AND ALFALFA (*MEDICAGO SATIVA*) UNDER *IN-VITRO* CONDITIONS

5.1 INTRODUCTION

Plant parasitic nematodes use amphids to perceive their environment, being attracted and repelled by beneficial and harmful stimuli, respectively (Curtis, 2008); a behaviour driven by chemotaxis. Chemotaxis is important in host-finding, mating, and predator avoidance (Zheng et al., 2014). Soil organisms, including PPNs, have co-evolved with plants and can recognise various chemical compounds produced by plant roots (Bais *et al*., 2006). The exudates affect the chemotaxis of nematodes, possibly attracting the nematodes towards the host plant or repelling them (Curtis, 2008; Yang *et al*., 2016) and, in some instances, components of exudates are nematicidal (Zasada *et al*., 2007). In more complex cases, other pathogens, such as the Tobacco Rattle Virus, modify the composition of root exudates of plants infected by *Trichodorus* nematodes (Van Griethuysen *et al*., 2024). Root exudates comprise amino and organic acids, volatile organic compounds and enzymes (Delory *et al*., 2016). The quantity and distribution of these compounds vary with plant growth stage and species (Sikder & Vestergård, 2020). The chemotaxis of root lesion nematodes to root exudates is an under-studied area, as most research focuses on root-knot and cyst nematodes (Atkinson *et al*., 1987; Rasmann *et al*., 2012; Wang *et al*., 2018; Bell *et al*., 2019; Sikder & Vestergård, 2020). However, the chemotactic response of *Pratylenchus penetrans* to several phenylpropanoids, monoterpenoids and alkaloids has been assessed (Wuyts *et al*., 2006). It was shown that *P. penetrans* was not responsive to these treatments compared to *Meloidogyne incognita* and *Radopholus similis*.

The greenhouse experiments in Chapter 2 established that forage chicory and alfalfa were poor hosts to *P. penetrans*. However, the study did not determine how the two cover crops affected nematode multiplication. Poor host cover crops may limit nematode multiplication through various mechanisms. The reduction in nematode numbers could have been due to 1) nematode repellence by root exudates or lack of attractive compounds during the host finding phase (Sikder and Vestergård, 2020), 2) failure to invade the plant roots due to physical barriers (Wuyts *et al*., 2007), 3) direct killing of nematodes due to existing metabolites within the plants or those released after nematode infection or, 4) arrest of nematode reproduction by the plant due to resistance genes (Hooks *et al*., 2010; Vestergård, 2019).

Forage chicory and alfalfa were classified as poor hosts to *Pratylenchus penetrans* under greenhouse conditions in Chapter 2 and reduced *Pratylenchus* spp. in field experiments (Chapter 3). To better understand how these cover crops reduced nematode multiplication of *P. penetrans*, further *in vitro* experiments were undertaken. Forage chicory and alfalfa were chosen for these experiments because they are known to reduce animal parasitic nematodes. In recent years, forage chicory (*Cichorium intybus*) and alfalfa (*Medicago sativa*) have received increased attention as raw materials for tannin-rich, anti-parasitic forage cover crop mixtures in livestock production (Peña-Espinoza *et al*., 2018; Ishaq *et al*., 2023). Both cover crop species are part of commonly sold cover crop mixes in the UK; however, their interaction with PPNs is unknown. Resistant alfalfa cultivars have higher levels of a phytoanticipin medicarpin in the roots, and *in vitro* assays with 55-75 µg ml^{-1} of pure medicarpin showed inhibition of *P. penetrans* motility (Balbridge *et al*., 1998). The crops have the potential for dual use, deworming livestock (Valente *et al*., 2021) and reducing root lesion nematodes.

Root exudates mediate the initial phases of nematode-plant interaction in the soil. Specifically, nematodes use their chemotactic sensory system to locate the roots of their plant hosts before root invasion (chemotaxis). The effect of root exudates on nematodes is likely to differ with plant age due to changes in biochemical composition (Wang *et al*., 2021); hence, root exudates from younger (three weeks) and older plants (six weeks) were used for the experiments. Previous reports have shown that exudates can paralyse nematodes. Some exudates might not paralyse the nematodes but would reduce the ability of nematodes to migrate during host finding; hence, the effects of exudates on nematode migration were tested.

5.1.1 Objectives

This study aimed to investigate the mechanisms through which cover crops reduce the multiplication of *Pratylenchus* nematodes *in vitro*. The specific objectives were:

1. To evaluate the effect of roots exudates and plant age of alfalfa and forage chicory on the motility, migration and chemotaxis of *P. penetrans.*

5.1.2 Hypotheses

H_1: Root exudates and plant age of forage chicory and alfalfa affect the motility, migration and chemotaxis of *P. penetrans.*

H_0: Root exudates and plant age of forage chicory and alfalfa do not affect the motility, migration and chemotaxis of *P. penetrans*.

5.2 MATERIALS AND METHODS

Plastic pots (1 L) were filled with soil from the trial site in Perth (Scotland, 56.62405, 3.17936) (73% sand, 23% silt, 4% clay, pH=5.3-5.5) and amended with 25% river sand to avoid soil slumping. Seed of forage chicory *cv*. Commander (Barenbrug, UK) _ and alfalfa *cv*. Artemis was supplied by Barenbrug, UK and Joordens, Netherlands, respectively. The forage chicory cultivar used in previous greenhouse experiments was not available at the seed supplier; therefore, a different cultivar was used. Seeds were planted in pots and thinned seven days after planting to achieve the recommended sowing rates: 8 and 25 kg ha^{-1} for forage chicory (3 plants per pot) and alfalfa (4 plants per pot), respectively. Four pots of each cover crop were sown as replicates for exudate collection. Irrigation and hand-weeding were regularly done throughout the experiment. The experiments were maintained in a greenhouse at the James Hutton Institute at 17 ± 2°C mean day and 15 ± 2°C mean night-time temperatures with a photoperiod of 16 h, between June and July 2023.

5.2.1 Root exudate collection

Root exudates were collected at three- and six weeks post-planting by the soil-leaching method (Ngala *et al*. 2021). Pots were saturated with water, and at field capacity, 100 ml of water was added before suspending the pots on 1 L beakers and allowing them to leach for 1h until 100 ml of the leachate was collected. For this chapter, this leachate is referred to as root exudates, as the organic components were not analysed. The beakers were standing on ice during exudate collection to minimise changes in exudate chemistry. Soil and root debris were removed from the exudates using a Whatman 0.45 mm filter paper and kept at -20°C until use.

5.2.2 Motility assays

Motility experiments were done to determine the effects of root exudates on mixed life stages of *P. penetrans.* Nematode cultures were multiplied and maintained as described in Chapter 2.2.1. Approximately 50 nematodes were incubated in 1 ml of root exudates at 20°C in each well of a 12-well plate. After 24 h, the nematodes were examined under a microscope at 20x magnification and rated as motile or non-motile. Nematodes were considered non-motile if no movement was observed after probing with a picking needle. The experiments had four replicates per cover crop, and from each, four replicates plus a sterile water control were used for the assay.

5.2.3 Nematode migration assay

Nematode movement was assessed in 5 cm diameter Petri dishes containing 4 ml of 2% Phytagel (Sigma Aldrich, UK). Nematodes were soaked in root exudates for 24 h and rinsed before use. Water-soaked nematodes were used as the control. Approximately 30 nematodes in 5 µl of water were pipetted in the centre of the Petri dish, pre-divided into three sections (zone 1= 0.5cm, zone 2 = 1 cm and zone 3 = 2.5 cm radius) (illustration presented with results graph Figure 5.3A). The Petri dishes were incubated in the dark at room temperature (20 °C) for 24 h before counting nematode distribution in the different zones under a 40x stereomicroscope (Leica, Germany). The treatments comprised exudates from younger and older plants (three and six weeks) of chicory, alfalfa, and water control. The experiments had four replicates per cover crop, and from each, four replicates plus a sterile water control were used for the assay.

5.2.4 Chemotaxis assays

Chemotaxis assays were conducted on a three-dimensional plastic column, as described by Laloum *et al*. (2020) and shown in Figure 5.1. An aliquot of 700 µl of 2% Phytagel (Sigma Aldrich, UK) was pipetted onto the central well of the column and allowed to set for 1 hr. Afterwards, 300 µl of root exudates/test solution were added to the test areas (zone A and B) before pipetting approximately. 30 mixed life-stage nematodes and left in the dark for 24 h. A known attractant (0.5 M $CaCl_2$) and a known repellent (0.25 % acetic acid) (Wuyts *et al*., 2005) were included in the experiment as negative and positive controls. Root exudates from 6-week-old tomato (cv Marmande, Sutton Seeds, UK) plants were also used as a positive control, as tomato is an excellent host to *P. penetrans* (Mbiro *et al*., 2024). Water on both the treatment and test areas was included as an additional control. Nematodes in each zone were counted under a 40x microscope. A chemotaxis index (CI) was calculated based on the number of nematodes which chose between the treatment and control wells (Laloum *et al*., 2020): $CI = [(A+B)/(A+B+T)] \times 100$, where T represents the number of nematodes in the neutral zone. A positive CI indicates attraction, while a negative CI indicates repulsion. CI values closer to ±1 indicate strong attraction or repulsion. Values between ±0.2 signify a lack of response to the test compound. For each plant age the treatments list was as follows: 1) chicory leachate/exudate, 2) alfalfa leachate/exudate, 3) a known attractant ($CaCl_2$), 4) a presumed attractant, tomato leachate/exudate, 5) a known repellent (acetic acid) and 6) sterile water (water on both sides (H_2O-H_2O).

5.2.5 Data Analysis

A generalised linear model with binomial distribution was fitted to test the effect of the root exudates on nematode motility. The R package emmeans package in R (Hothorn *et al*.,

2022) and Tukey HSD test post hoc analysis was performed whenever significant differences were observed ($p<0.05$). A generalised linear model with a Poisson distribution was fitted to test the effect of the type and age of the plant on the nematode distribution across the three zones. Post hoc tests were done using the Sidak-Holm method ($p<0.05$) (Holm,1979). A two-way analysis of variance (ANOVA) was done to evaluate the effects of the type of root exudates and the age of the plant on the chemotaxis index. Post hoc analysis was performed using the Tukey HSD test whenever there was significance. All statistical analyses and graphs were computed in R version 2023.12.1 (R core team, 2022).

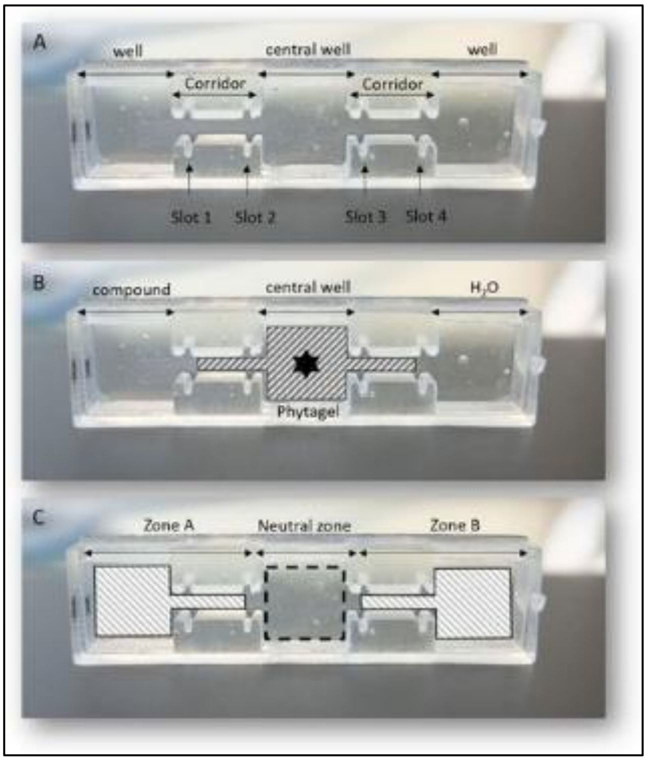


Figure 5.1 An image of the plastic column used for chemotaxis assays. Removable plastic walls were inserted into slots 1-4 (A), and 700 µl of 2% Phytagel was pipetted onto the central well and allowed to set for 1 hr. After that, the walls were removed, and 300 µl of test solutions and distilled water control were added to zones A and B, respectively. Approximately 30±5 mixed life-stage nematodes were added to the central well (B) and incubated in the dark for 24 h at room temperature. Nematodes in the different zones (C) were counted under a 40x stereomicroscope. Nematodes that chose either the treatment or control wells were calculated as $CI = [(A+B)/(A+B+T)] \times 100$, where T represents the number of nematodes in the neutral zone. Image published by Laloum *et al*. (2020).

5.3 RESULTS

5.3.1 Effect of root exudates on nematode motility

Root exudates from 3 or 6-week-old alfalfa and chicory plants did not affect the motility of the nematodes. For three-week old plants, the proportion of non-motile nematodes was 2.3, 2.2, and 2.4 % in the alfalfa, chicory, and water control and were not significantly different (p=0.35). Nematodes soaked in six-week-old exudates had 1.9, 2.2 and 2.1 % immobility in the alfalfa, chicory and water control, respectively and were not significantly different (p>0.05). There was no difference in the immobility of nematodes between the age of the plant (p=0.4) (Figure 5.2).

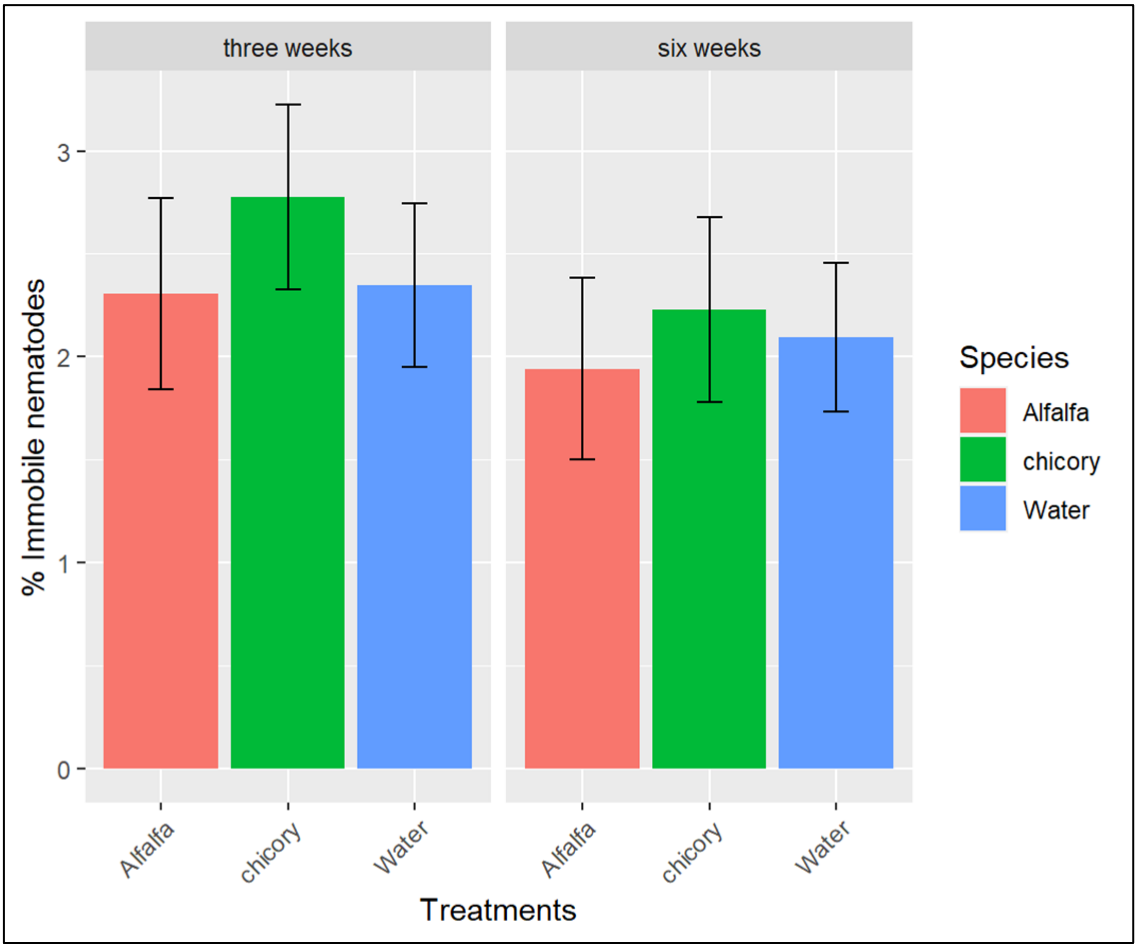


Figure 5.2 The average percentage of non-motile *P. penetrans* ± standard error after exposure to root exudates of three and six-week-old alfalfa and forage chicory plants for 24 h. The mean is averaged over four biological and four technical replicates. No significant differences were observed between the treatments.

5.3.2 Effect of root exudates on nematode migration behaviour

Root exudates from 3 and 6-week-old alfalfa and forage chicory plants had no impact on the migration behaviour of the nematodes. The nematodes incubated with root exudate from three-week old alfalfa plants were scattered across the three zones with a 31.6, 34.4 and 34 % distribution in zones 1-3, respectively. Similar observations of 33.4, 33.2 and 33.4 % distribution were observed for nematodes incubated in root exudates from three-week old chicory plants. In water control, an even distribution of nematodes occurred in all zones

(Figure 5.3). No significant differences (p=0.1) in nematode numbers were observed between the zones, type or age of the plant in all treatments.

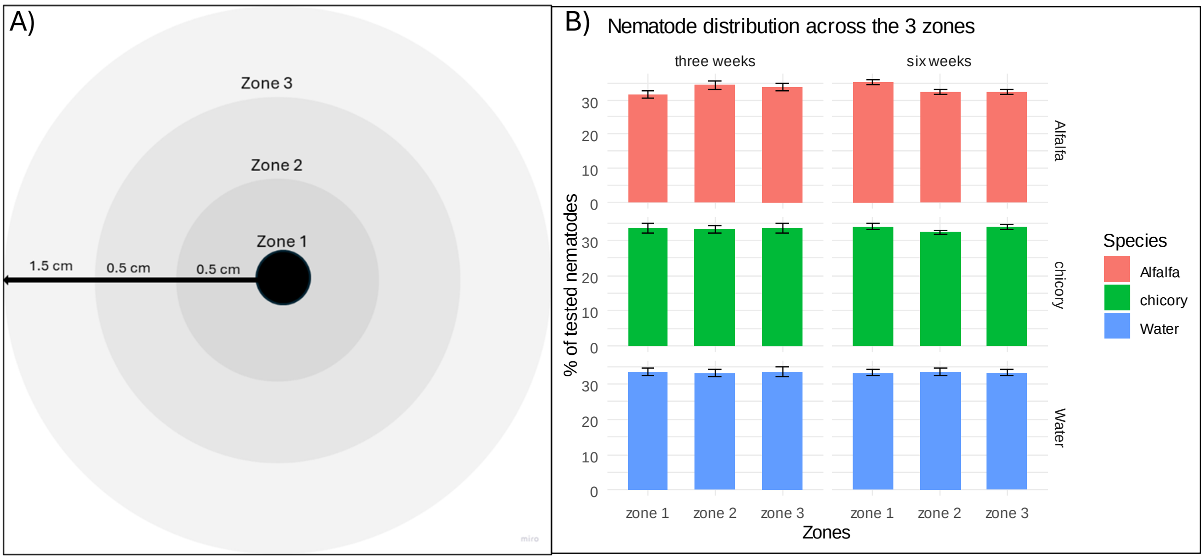


Figure 5.3 Distributions of nematodes across the three circular areas on a Petri dish after incubating them in root exudates from three- and six-week-old alfalfa and forage chicory plants for 24 h and placing them on the centre of agar for another 24 h. A) illustrates the different zones in the Petri dish used for the experiment (radius 0.5, 1, and 2.5 cm, respectively). B) shows the mean percentage ± SE of nematodes recorded in the different zones after 24 h. Means were obtained from four biological and four technical replicates. No significant differences were observed between the treatments. Error bars represent the standard error of the mean of % tested nematodes.

5.3.3 Effect of root exudates on nematode chemotactic behaviour

The chemotactic response of *P. penetrans* exposed to root exudates from three- and six-week-old alfalfa and forage chicory plants was compared to the water-negative control. As expected, 0.25% acetic acid was a repellent (CI=-0.8 and -0.9) for three and six weeks, respectively, and was significantly different ($p \leq 0.05$) from the other treatments (Figure 5.4). Tomato, alfalfa, forage chicory and water on both sides control (H_2O-H_2O) treatments induced no chemotactic response (Figure 5.4). $CaCl_2$ was an attractant (CI=0.38) significantly different from the other treatments (Figure 5.4). Plant age did not affect the chemotaxis index (p>0.05).

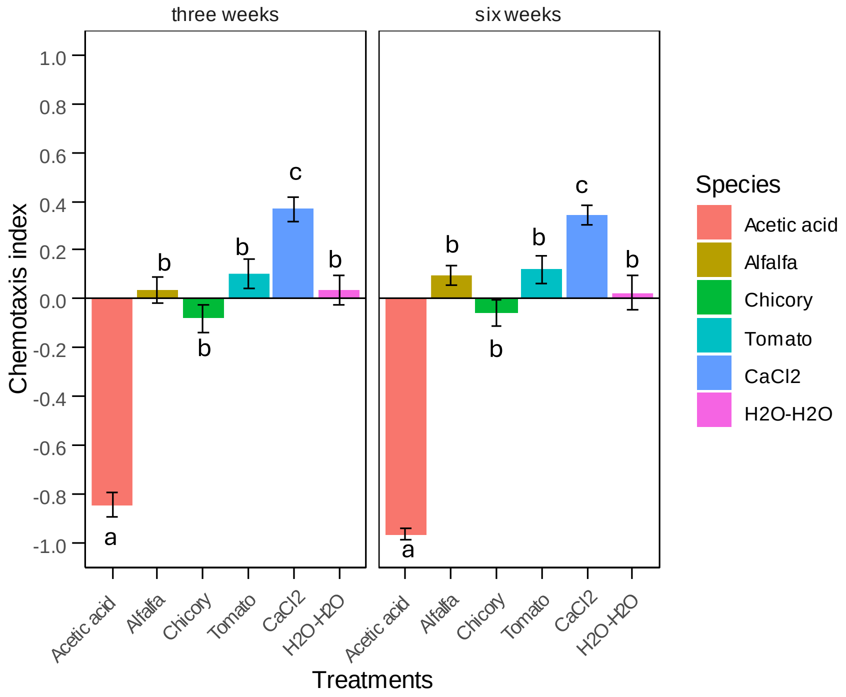


Figure 5.4 Chemotactic response of *Pratylenchus penetrans* to root exudates of three and six-week-old alfalfa and forage chicory plants. The y-axis shows attraction (positive) or repulsion (negative) CI indexes. Treatments included known repellent 0.25% acetic acid, known attractant 0.5 M $CaCl_2$ and presumed attractant (tomato root exudates), H_2O-H_2O (water on both sides control) and three and six-week-old root exudates of alfalfa and forage chicory. Bars with different letters are significantly different after Tukey HSD test ($p<0.05$). Error bars represent the standard error of the mean.

5.4 DISCUSSION

This study aimed to understand the impact of alfalfa and forage chicory root exudates on *P. penetrans* behaviour under *in vitro* conditions. None of the exudates/leachate used in this study impacted nematode motility, migration behaviour, or chemotaxis. This is the first study to explore the root exudate-nematode interaction between *P. penetrans* and forage chicory and alfalfa, and the observations of this study suggest that root exudates do not play a direct role in the poor host status of these two crops to *P. penetrans*. The composition of root exudates is known to change during plant growth (Wang *et al*., 2021); however, the age of the tested plant species did not affect the behaviour of nematodes to root exudates in this study. Tomato is a known host to *P. penetrans* (Mbiro *et al*., 2024), and as such, its root exudates were expected to attract the nematodes. Although the variety (Marmande) used in this experiment is known to host *P. penetrans* (Kutywayo and Been, 2006), the lack of response to root exudates observed could have been due to environmental conditions, as the chemistry of root exudates differs with different environments (Dietz *et al*., 2020).

Research on the interactions between plant parasitic nematodes (mainly *Meloidogyne* spp.) and root exudates is advancing, but the findings remain inconsistent. For instance, root exudates from a susceptible tomato variety were found to attract *M. incognita*, whereas those from a resistant cultivar repelled *M. incognita* (Yang *et al*., 2016). However, limited information is available on *P. penetrans*. A previous study found that putative good hosts, such as maize and bean, were most attractive to *P. penetrans*, while moderate hosts like potato and carrot were the least attractive (Pudasaini *et al*., 2007). However, their study was conducted using whole potted plants rather than isolated root exudates, and attraction varied with plant age, with younger plants being more attractive than older ones.

Flavonoids, tannic acids, fatty acids, lipophilic molecules, and specific volatile organic compounds play a crucial role in nematode host-finding abilities (Chitwood, 2002; Rasman *et al*., 2012). *Meloidogyne incognita* is attracted to methyl salicylate (Kihika *et al*., 2017; Murungi *et al*., 2018), zeatin (Kirwa *et al*., 2018), as well as α-pinene, limonene, and tridecane exuded by the roots of *Capsicum annuum* (Kihika *et al*., 2017).

Root exudates consist of a complex mixture of organic compounds that likely interact, therefore, conclusions about host status should not be drawn based on a single compound. Evidence suggests that resistant tomato cultivars produce higher concentrations of certain repellent compounds, such as dibutyl phthalate (C), following infection by *Meloidogyne incognita* (Yang *et al*., 2016). Earlier studies proposed that nematode chemotaxis was influenced by the ratio of attractants to repellents rather than the presence of a single compound (Wiesel, 1956; Castro *et al*., 1989). This was further supported by research on potato root exudates, which were fractionated into attractant, non-responsive, and repellent components. The movement of *G. pallida* and *G. rostochiensis* juveniles (*J2*) was found to depend on the balance of attractant-to-repellent semiochemicals in the exudates (Devine & Jones, 2003).

5.5 CONCLUSION

This Chapter explored the interaction of *P. penetrans* with root exudates from two growth stages of forage chicory and alfalfa. Such knowledge is essential in deciphering the poor host status of these crops to *P. penetrans* to understand signalling mechanisms between *P. penetrans* and forage chicory and alfalfa. If *P. penetrans* had shown a repulsion or attraction chemotactic response to forage chicory and alfalfa root exudates, the next step would have been to identify the semiochemicals in the exudates. Host invasion assays are recommended as the next step to determine how root-lesion nematodes invade forage chicory and alfalfa roots. Nematode-resistant plants suppress while hosts stimulate egg hatch (Dutta *et al*., 2012). This could be explored with root lesion nematodes, although egg separation is cumbersome. Investigating the defence-related genes could shed light on the molecular interactions between *P. penetrans* and these cover crops. Certain alfalfa cultivars are resistant to *P. penetrans*, for example, cultivar MNGRN-16, in which resistance genes are highly expressed before nematode infection (Hafez *et al*., 2006; Vieira *et al*., 2019).

CHAPTER 6

THE IMPACT OF BRASSICA-DERIVED ISOTHIOCYANATES ON *PRATYLENCHUS PENETRANS IN VITRO*

Chapter modified from:
Chekanai, V., Neilson, R., Roberts, D., Edwards, S., & Back, M. (2024). *In vitro* nematicidal efficacy of brassica-derived isothiocyanates against the root lesion nematode, *Pratylenchus penetrans*. *Nematology*, *1*, 1-10. https://doi.org/10.1163/15685411-bja10347

6.1 INTRODUCTION

Isothiocyanates are products of hydrolysed glucosinolates (GSLs) after chopping and incorporating brassica plant tissues into the soil (Lazzeri *et al.,* 1993; Matthiessen and Kirkegaard, 2007; Bellostas *et al.,* 2007; Ntalli and Caboni, 2017). Over 200 GSLs have been identified and grouped into aliphatic, aromatic or indole; determined by varying R side chains (Blažević *et al*., 2019). Glucosinolate concentration varies with brassica species, cultivar, plant organ, developmental stage, and soil and environmental factors (Mattner *et al.,* 2008; Ngala *et al.,* 2015). Under field conditions, the biomass accumulated, the concentration of GSLs in plant tissues, soil and environmental conditions at the time of and method of incorporation determine the success of biofumigation (Kirkegaard and Sarwar, 1998; Bellostas *et al.,* 2007; Kruger *et al.,* 2013).

Indian mustard (*Brassica juncea*), Ethiopian mustard (*B. carinata*), black mustard (*B. nigra*), rocket (*Eruca sativa*), turnip (*B. rapa*), canola/oilseed rape (*B. napus*), oilseed radish (*Raphanus sativus*), and field mustard (*B. campestris*), are some of the common biofumigants (Fourie *et al.,* 2016) and sometimes used as seed meals, or trap crops (Dossey, 2010; Oliveira *et al*., 2011). Pure ITCs, as a biofumigation additive, successfully suppressed *Meloidogyne hapla* (Dahlin and Hallmann, 2020). Indian mustard suppressed *P. penetrans* (Mazzola *et al.,* 2001), *M. chitwoodi* (Mojtahedi *et al.,*1993) and *M. javanica* (Rahman and Somers, 2005) under field conditions. Contrastingly, biofumigation of *M. javanica* (Stirling and Stirling, 2003) or *P. penetrans* (Korthals *et al.,* 2014; Vervoort *et al.,* 2014; Grabau *et al.,* 2017) was unsuccessful. The decrease in nematode densities was caused by physical disturbances or a lack of host plants, instead of biofumigation. The biofumigant cover crops were good hosts, which boosted the nematode abundances, and the ITC concentrations were insufficient to be toxic (Vervoort *et al.,* 2014).

Indian mustard contains 2-propenyl glucosinolate (sinigrin) hydrolysed to Allyl ITC. Allyl ITC is toxic to root lesion nematodes *P. penetrans, P. neglectus* (Zawadzki, 1996; Yu *et al.,* 2007), potato, beet and soybean cyst nematodes *(G. pallida, G. rostochiensis* (Wood *et al.,* 2017)*, Heterodera schachtii, H. glycines*) (Lazzeri *et al.,*1993)*,* root-knot (*Meloidogyne hapla, M. incognita*) (Zasada *et al.,* 2009) *and* stubby root nematodes (*Trichodorus/Paratrichodorus)* (Mwangi *et al.*, 2024).). Moreover, Benzyl and 2-Phenylethyl ITC were nematicidal to *Meloidogyne* spp. (Zasada *et al.,* 2009) and *G. pallida* (Wood *et al.,* 2017), *H. glycines* (Schroeder *and* MacGuidwin, 2010), *Trichodorus*/*Paratrichodorus* spp. (Mwangi *et al.,* 2024*), Ditylenchus dipsaci* and *D. gigas* (Musa, 2022)*.*

Brassica ITCs inhibit juvenile hatch, reduce egg viability (Lord *et al.,* 2011; Oliveira *et al.,* 2011), reduce mobility hindering host-finding ability (Wu *et al.,* 2011), induce oxidative stress (Murata *et al.,* 2000) and shrink the size of the nuclear dorsal gland (Dossey, 2010). ITCs permanently damage nematode enzymes, making them non-functional and disrupting metabolic and cellular reactions (Avato *et al.,* 2013).

Various UK-grown crops, including potatoes, carrots, peas, cereals, beans, and permanent pasture (Southey, 1959; Boag, 1979, 1980; Boag and Lopez-Llorca, 1989; Oliveira *et al.,* 2017; Orlando *et al.,* 2020) are parasitised by *P. penetrans*. The control of *P. penetrans* previously relied on chemical fumigants that are now banned owing to environmental hazards and risks (Warnock *et al.,* 2017), crop rotations are impractical due to a lack of land (Lambin and Meyfroidt, 2011) and broad host range (Kratochvil *et al*., 2004). Alternative methods are needed for *P. penetrans* management; biofumigation has the potential, but choosing the right brassica biofumigants based on ITC profiles requires preliminary *in vitro* screening assays.

The leaves of *B. Juncea* can produce 90 µmol g^{-1} (dry weight) while *B. nigra* produces 100 µmol g^{-1} (dry weight) of sinigrin GSL from which Allyl ITC is released after hydrolysis (Ngala *et al.,* 2015; Bellostas *et al.,* 2007). The roots of *Brassica rapa, B. carinata, B. nigra, B. napus, B. juncea* and *R. sativus* produce 14-25 µ*mol* (g dry weight) $^{-1}$ of gluconasturtiin GSL from which 2-Phenylethyl ITC is produced after hydrolysis (Gimsing and Kirkegaard, 2006; Bellostas *et al.,* 2007). Benzyl ITC is produced by the hydrolysis of glucotropaeolin GSL from the roots and shoots of *Sinapis alba and B. napus* (Gimsing and Kirkegaard, 2006; Couëdel *et al.,* 2018). These brassica biofumigants produce GSL levels adequate to release >50 µg ml^{-1} of ITCs concentration under field conditions (Gimsing and Kirkegaard, 2006; Ngala *et al*., 2015).

Greenhouse experiments (Chapter 2) revealed that oilseed radish reduces *P. penetrans* as a poor host, and field experiments (Chapter 3) showed that *Pratylenchus* spp. are reduced after growing oilseed radish. Oilseed radish is a biofumigant that contains GSLs, which are hydrolysed to various products, including 2-Phenylethyl ITC. To understand the mechanisms through which oilseed radish reduces *Pratylenchus* spp., *in vitro* assays were conducted to test the effects of brassica ITCs on *P. penetrans*. Benzyl and Allyl ITCs are hydrolysis products of GSLs found in commonly grown brassica biofumigants such as Indian and white mustard (*B. juncea* and *Sinapis alba*).

Pilot experiments with concentrations higher than 50 µg ml^{-1} ITCs were initially performed and caused 100% mortality. Thus, 50 µg ml^{-1} was selected as the maximum dose for these experiments. Lower doses were also used because they can be toxic if exposed to nematodes for more extended periods (Wu *et al.,* 2011; Ngala *et al.,* 2015). Previous work indicated that the duration of ITCs in soils can extend up to 60 h (Borek *et al.,*1995) and 72 h (Gardiner *et al.,*1999), while some reported four days after incorporation of brassica biomass (Morra and Kirkegaard, 2002). As a result, 72 h was set as the maximum exposure time for the current experiments.

6.1.1 Objectives

This study aimed to understand the mechanisms through which brassica cover crops reduce *Pratylenchus* spp., by testing the effects of brassica derived ITCs, specifically to:

a) Assess the influence of Allyl, Benzyl, and 2-Phenylethyl ITCs on the motility of *P. penetrans* (ED_{50}).
b) Determine the impact of time of exposure on *P. penetrans* motility.
c) Determine if the effects are non-reversible (mortality) and the $LD_{50.}$

6.1.2 Hypothesis

H_1: Isothiocyanates affect the motility and mortality of *P. penetrans.*

H_1: Time of exposure to ITCs influences the motility of *P. penetrans.*

H_0: Isothiocyanates do not affect the motility and mortality of *P. penetrans.*

H_0: Time of exposure to ITCs does not affect the motility of *P. penetrans.*

6.2 MATERIALS AND METHODS

6.2.1 Nematode cultures and ITCs

The motility and mortality assays were conducted using mixed-life-stage *P. penetrans* obtained from pure cultures maintained at Harper Adams Nematology Laboratory, as described in Chapter 2.2.1. Pure Allyl, 2-Phenylethyl, and Benzyl ITC stock solutions were acquired from Sigma-Aldrich (UK).

6.2.1 Motility and mortality assays

Approximately 45 ± 5 nematodes in double distilled water were pipetted into glass vials containing 1 ml of ITC solutions diluted to: 3.125, 6.25, 12.5, 25 and 50 µg ml^{-1}. The ITCs were diluted in 1% Dimethyl sulfoxide (DMSO); therefore, it was necessary to include DMSO as a negative control in addition to a double-distilled water control. The glass vials were sealed with airtight lids and incubated in the dark at 20°C. The treatments were replicated six times, and the experiment was repeated once. After 24, 48 and 72 h (repeated measures) the contents of the glass vials were poured into a counting dish, observed under a stereo microscope (40x magnification), and the number of motile and non-motile nematodes was recorded. Nematodes were considered non-motile if they remained immobile after continuously probing with a nematode-picking needle. After each counting stage, the nematodes were then returned to the glass vial and placed in the dark at 20°C.

Recovery assays were done after 72 h to assess the permanence of the non-motility (mortality). The nematodes were separated from the ITC solutions by washing them thrice with distilled water over a 20 µm mesh sieve. The nematodes were left in distilled water in glass bottles and left in the dark for 48 h. Mortality was assessed and confirmed if the nematodes remained immobile after pricking with a picking needle.

6.2.3 Data analysis

An F-test was conducted to compare the variances of the data from the two experiments and determine if a combined analysis was appropriate. Statistical analysis was done after combining the results from the two experiments (F-test, p=0.48). A generalised linear mixed effects model was fitted to test the influence of ITCs on motility and mortality using the 'lme4' package in R (Bates *et al.,* 2015; R core team, 2022). The ED_{50} of the ITCs after 24, 48 and 72 hours were computed by fitting dose-response curves. The influence of exposure time, type of ITC and concentration on mortality was tested by fitting a generalised linear model (GLM) (binomial distribution). Tukey HSD post hoc tests were done on emmeans ('multcomp' package in R (Hothorn, 2022). A correlation test determined the relationship between ITC concentration and mortality. The 'drc' package (Ritz *et al.,* 2015) determined ED_{50} and LD_{50}.

6.3 RESULTS

6.3.1 Influence of ITCs on the motility of *P. penetrans*

Nematode motility was significantly influenced by the type (p<0.001), concentration of ITC (p<0.001), and time of exposure (p<0.001). The numbers of non-motile nematodes in the distilled water and 1% DMSO controls were not significantly different (p>=0.05). At 50 µg ml^{-1} of Allyl ITC, the percentage of non-motile nematodes increased from 62% after 24 h to 100%

after 72 h (Table 6.1). Based on the ED_{50}, Benzyl had the highest potency (3.1 μg ml^{-1}) at 72 h while Allyl had the least (8.8 μg ml^{-1}) and all ED_{50} were significantly different ($p<0.001$) (Figure 6.1 A-C).

Table 6.1 Non-motile nematodes (%) ± SE after exposure to Allyl, Benzyl, 2-Phenylethyl ITC concentrations for 24, 48 and 72 h. The mean is the average of 12 replicates shared between two independent experiments. An asterisk* shows treatment which significantly differ from the control (Tukey HSD test, p≤005).

ITC treatment	**Concentration (μg ml^{-1})**	**24 h**	**48 h**	**72 h**
Control	1%DMSO	2 ± 0.4	2 ± 0.0	2 ± 0.4
	Water	2 ± 0.3	2 ± 0.4	2 ± 0.4
Allyl	3.125	2 ± 0.4	3 ± 0.3	3 ± 0.3
	6.25	8 ± 1.1	15 ± 2.4*	37 ± 2.3*
	12.5	14 ± 1.0*	30 ± 1.0*	54 ± 3.2*
	25	35 ± 3.1*	66 ± 3.1*	95 ± 1.7*
	50	62 ± 1.8*	100 ± 0.0*	100 ± 0.0*
2-Phenylethyl	3.125	7 ± 0.7	12 ± 1.2*	26 ± 2.8*
	6.25	18 ± 1.1*	44 ± 1.1*	61 ± 6.8*
	12.5	41 ± 1.4*	80 ± 2.0*	89 ± 3.3*
	25	80 ± 2.7*	98 ± 1.1*	100 ± 0.2*
	50	100 ± 0.0*	100 ± 0.0*	100 ± 0.0*
Benzyl	3.125	14 ± 1.4*	26 ± 1.2*	47 ± 1.3*
	6.25	32 ± 1.9*	67 ± 3.6*	98 ± 0.4*
	12.5	58 ± 2.5*	95 ± 0.8*	100 ± 0.0*
	25	99 ± 0.8*	100 ± 0.0*	100 ± 0.0*
	50	100 ± 0.0*	100 ± 0.0*	100 ± 0.0*
	p-value	**<0.001**	**<0.001**	**<0.001**

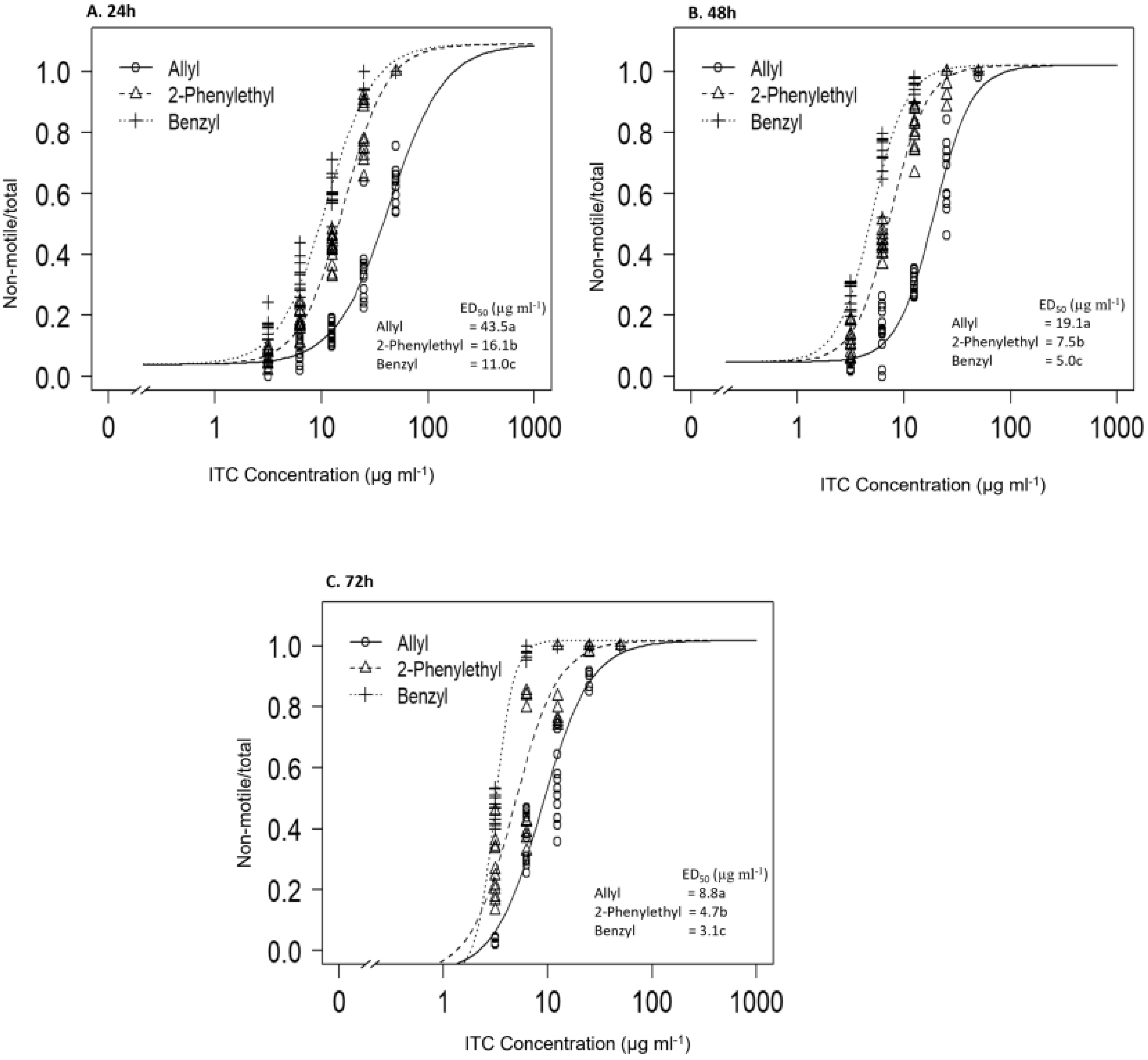


Figure 6.1 Dose-response curves and ED$_{50}$ obtained after exposing *P. penetrans* to Allyl, 2-Phenylethyl, and Benzyl ITCs and assessing nematode motility after 24 h, 48 h and 72 h. Different letters on the ED$_{50}$ indicate significant differences between ITCs based on Tukey HSD test ($p \leq 0.05$).

6.3.2 Influence of ITCs on *P. penetrans* mortality

Nematode mortality was significantly influenced by the type ($p<0.001$) and concentration of ITC ($p<0.001$). Excluding Allyl ITC at 3.125 μg ml^{-1}, all treatments had more dead nematodes than the water and 1% DMSO controls (Figure 6.2). There was a dose effect of the ITCs on nematode mortality, as demonstrated by a significant positive relationship between concentration and nematode mortality ($R=0.81$, $p<0.001$). Significant differences were observed between the LD$_{50}$ of the three ITCs ($p<0.001$). Benzyl had the lowest (LD$_{50}$=3.2 μg ml^{-1}), therefore most nematicidal, followed by 2-Phenylethyl (LD50=5.2 μg ml^{-1}) and lastly Allyl ITC (LD50=9.9 μg ml^{-1}) (Table 6.2).

Table 6.2 Mean lethal doses (LD_{50}), SE, lower and upper 95 percentiles for Allyl, Benzyl and 2-Phenylethyl mortality assays on *P. penetrans*. Means with different letters significantly differ after Tukey HSD tests ($p \leq 0.05$).

ITC	LD_{50} ($\mu g\ ml^{-1}$)	Std error	Lower 95 percentile	Upper 95 percentile	p-value
Allyl	9.90c	0.27	10.62	11.67	<0.001
2-Phenylethyl	5.20b	0.16	6.67	7.31	<0.001
Benzyl	3.20a	0.30	3.18	3.41	<0.001

6.3.3 Comparing motility and mortality

The impact of the ITCs on the nematodes was irreversible in both experiments. Comparisons of the ED_{50} (72 h) and LD_{50} from the mortality assays (Table 6.3) showed no significant differences ($p>0.05$).

Table 6.3 ED_{50} after 72 h and LD_{50} ± SE and p values (z-tests, $p \leq 0.05$)

ITC	ED_{50} ($\mu g\ ml^{-1}$) 72h)	LD_{50} ($\mu g\ ml^{-1}$)	p-value
Benzyl	3.1 ± 0.10	3.2 ± 0.30	0.59
2-Phenylethyl	4.7 ± 0.40	5.2 ± 0.16	0.47
Allyl	8.8 ± 0.90	9.9 ± 0.27	0.42

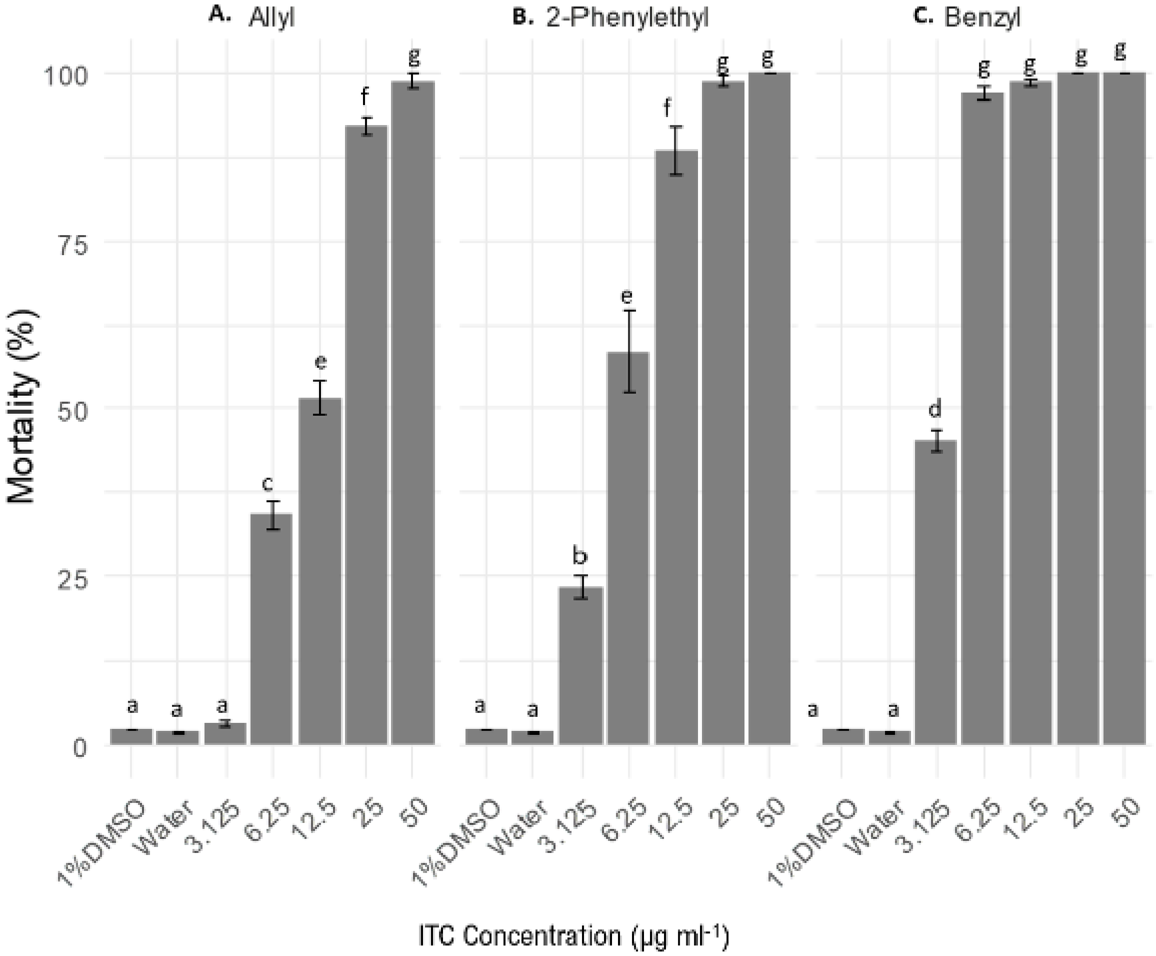


Figure 6.2 Percentage mortality +SE of *P. penetrans* after exposure to Allyl (A), 2-Phenylethyl (B) and Benzyl (C) ITCs across two experiments with six replicates each. Different letters indicate significant differences after Tukey HSD test (p≤ 0.05).

6.4 DISCUSSION

This Chapter revealed that ITC type, concentration, and time of exposure irreversibly affect *P. penetrans.* Benzyl ITC was the most toxic, followed by 2-Phenylethyl and lastly Allyl ITC. A similar pattern of potency was observed on *Heterodera glycines* (Schroeder *and* MacGuidwin, 2010), *M. javanica* (Zasada and Ferris, 2003), *Ditylenchus gigas*, *D. dipsaci* (Musa, 2022), *Trichodorus/Paratrichodorus* (Mwangi *et al*., 2024). The differences in toxicity of the ITCs are driven by the molecular structure (R-side chain) and lipid solubility properties. 2-Phenylethyl and Benzyl are lipophilic aromatic ITCs that are less volatile (due to large aromatic rings) and more toxic as they can permeate the nematode membrane (Lazzeri *et al*., 1993). Lipophobic aliphatic ITCs such as Allyl are typically highly volatile and less potent (Holst and Williamson, 2004; Matthiessen and Kirkegaard, 2007). Brassica roots tend to produce aromatic GSLs that are more toxic (Kirkegaard *et al*., 1996), compared to shoots, which produce aliphatic GSLs (Van Dam *et al.,* 2009).

In contrast to current findings, Allyl was more nematicidal than 2-Phenylethyl towards *M. javanica* (Wu *et al.,* 2011) and *M. hapla* (Dahlin and Hallmann, 2020). This variability in the toxicity of ITCs on different nematodes occurs because the nematicidal potential of ITCs is nematode-species specific. Table 6.4 summarizes the LD_{50} of the tested ITCs reported in different past studies on various important nematodes. This is the first attempt to test the nematicidal effect of 2-Phenylethyl and Benzyl ITC on *P. penetrans*. Although susceptibility varies greatly depending on nematode species, no plant parasitic nematodes have been reported to be completely tolerant to ITCs. *Caenorhabditis elegans*, a non-plant parasite, is tolerant to ITCs, and exposure to certain ITCs has been shown to prolong its lifespan (Qi *et al*., 2021). The varying toxicity of ITCs to nematodes is likely due to differences in nematode biology, with some studies attributing it to the differences in chemosensory receptors in the receptor binding sites or differences in the permeability of the nematode cuticle (Kampkotter *et al*., 2008; Qi *et al*., 2021). The mode of action of ITCs on plant parasitic nematodes is an understudied area (Eugui *et al*., 2022). Future research should focus on physiological and molecular ITC-nematode interactions, which will contribute to the development of novel brassica-based PPN management tools.

The toxicity of ITCs depends on the concentration and time of exposure to nematodes (Lazzeri *et al.* 1993). Mortality increases with increasing exposure time, consistent with the current observations. Minimal doses of ITCs have the potential to be nematicidal if exposed to nematodes in the soil for prolonged times (Mattner *et al.,* 2008). In a non-airtight system, the half-life of ITCs is 1 hour (Hanschen *et al.,* 2015); ITCs are released from plant tissues for 2-5 h (Gimsing and Kirkegaard, 2006) after incorporation and continue for up to 4 days (Morra and Kirkegaard, 2002). Some brassica cover crops, for instance *R. sativus* (Ngala *et al*., 2015), are partial biofumigants which release low concentrations of GSLs from the roots during growth, which can suppress nematodes. In previous greenhouse experiments in Chapter 2, the two different cultivars of oilseed radish tested might have been partial biofumigants, hence the poor host status observed. After the recovery assessment, the nematodes were alive but with limited mobility, which was previously observed on *D. dipsaci* and *D. gigas;* furthermore, their host-finding abilities were impeded (Musa, 2022). The infectivity of J2s and egg production of *M. incognita* were inhibited after exposure to sublethal doses of Benzyl ITC (Zasada *et al*. 2009).

The levels of 2-Phenylethyl GSL in *B. napus* roots influence its ability to resist *P. neglectus*; as such, cultivars with higher concentrations of glucosinolate are more resistant to nematode infection (Potter *et al*., 1999). *Brassica juncea, B. nigra*, *B. rapa*, *R. sativus,* and *S. alba* produce a minimum of 25 µg ml^{-1} of the tested ITCs (Gimsing and Kirkegaard, 2006; Ngala *et al*., 2015); therefore, they can potentially be used as biofumigants to suppress *P. penetrans*.

Table 6.4 *In vitro* studies assessing the effects of Allyl, 2-Phenylethyl and Benzyl ITCs on different plant parasitic nematodes

ITC	Target nematode	LD$_{50}$, (µg ml^{-1})
Allyl	*Pratylenchus penetrans*	16.0 (Zawadzki, 1996), 20.0 (Yu *et al.,* 2007)
	Pratylenchus neglectus	14.9 (Yu *et al.,* 2007),
	Heterodera glycines	10.3 (Yu *et al.,* 2007),
	Heterodera schachtii	30.8 (Yu *et al.,* 2007),
	Meloidogyne incognita	17.0 (Yu *et al.,* 2007),
	Meloidogyne hapla	24.1 (Yu *et al.,* 2007),
	Ditylenchus gigas	79.25 (Musa, 2022)
	Ditylenchus dipsaci	42.97 (Musa, 2022)
	Trichodorus/Paratrichodorus	10.67 (Mwangi *et al*., 2024)
2-Phenylethyl	*Ditylenchus gigas*	22.21 (Musa, 2022)
	Ditylenchus dipsaci	24.8 (Musa, 2022)
	Trichodorus/Paratrichodorus	6.91 (Mwangi *et al*., 2024)
Benzyl	*Ditylenchus gigas*	25.5 (Musa, 2022)
	Ditylenchus dipsaci	24.1 (Musa, 2022)
	Trichodorus/Paratrichodorus	4.6 (Mwangi *et al*., 2024)

6.4 CONCLUSION

The current study proves that Allyl, 2-Phenylethyl, and Benzyl ITCs are toxic to *P. penetrans in vitro*. According to previous studies, the tested concentrations are less than 50 µg ml$^{-1,}$ which can be produced during in-field biofumigation therefore, any cover crop which produces these ITCs can potentially manage *P. penetrans*. Further investigation into the sublethal effects of the ITCs on nematodes might provide insight into the mode of action of the tested ITCs to *P. Penetrans*.

CHAPTER 7

GENERAL DISCUSSION

As a leading world producer of *Narcissus* bulbs, the UK industry needs more environmentally friendly methods to contribute to integrated nematode management following recent bans on the use of nematicides. The shortcomings of hot water treatments, as discussed in Chapter 1, leave the industry vulnerable to potential yield loss due to plant parasitic nematodes. This thesis explored cover crops for managing PPNs in *Narcissus* in major growing regions during the fallow period between June and September, between *Narcissus* cropping cycles. The optimal cover crop should suppress the plant parasitic nematodes of economic importance without increasing other PPNs that affect both the current crop and those that form the typical cash crop rotation, while having no adverse effects on non-target nematode communities.

Pratylenchus penetrans is polyphagous and a significant pest of many crops in the UK (Orlando *et al*., 2020). Host suitability studies for *P. penetrans* have been extensively conducted on various cover crops worldwide (Castillo and Vovlas, 2007; Pudasaini *et al*., 2006; Neupane and Yan, 2023). However, there is limited equivalent information available on the host status of UK cover crops to this nematode species; therefore, this project directly contributes to this research gap. Initially, a controlled (greenhouse) experimental approach was adopted to screen cover crops commonly grown in the UK for host suitability to *P. penetrans* (Chapter 2). In addition, a few less common UK intercrops, e.g., African spinach/nightshade, were also screened. Ten of the 13 screened cover crops were poor hosts for *P. penetrans*, whereas *Phacelia* and Japanese oats were identified as maintenance hosts. Of the identified poor hosts, four (French marigold, oilseed radish, forage chicory, alfalfa) were selected to proceed for field trialling to ensure that the effects recorded under controlled conditions were replicated in the field over two seasons in Scotland (Montrose and Perth) and the Isles of Scilly (Chapter 3). Encouragingly, results under field conditions were consistent with the findings of the controlled experiments, with the abundance of *Pratylenchus* spp. reduced. Moreover, the selected cover crops had no adverse effects on beneficial nematodes, potentially improving nutrient cycling and soil health. This thesis further explored the impact of cover crops on soil food web indices and nematode communities (alpha and beta diversity) (Chapter 4). The results indicated that cover crop treatments significantly increased the enrichment index while reducing the maturity and structure indices. Cover crop treatments had no effect on soil nematode communities (beta diversity), but the time of sampling had a significant influence. Ecological indices (enrichment index) showed that cover crop incorporation increased bacterivorous nematodes (Freckman and Ettema, 1993), which suggests that cover cropping has no apparent adverse effects on soil nematode communities. The results agree with previous work (Puissant *et al*., 2021; van Himbeeck *et al*., 2024).

The results of this study align with previous research. For example, in this study, French marigold was found to reduce *Pratylenchus* spp. abundance, which is consistent with field studies by Kimpinski *et al*. (2000) and Evenhuis *et al*. (2004). In the Netherlands, French marigold is currently used as an agronomic intervention for managing *P. penetrans* in *Narcissus*. However, its adoption by UK growers may be challenging compared to other cover crops due to its limited economic value. Oilseed radish reduced *Pratylenchus* spp. at two of the trial sites in this study, supporting recent findings from the Netherlands (van Himbeeck *et al*., 2024) and Belgium (Taning *et al*., 2024). Similarly, alfalfa has been reported as a poor host for *P. penetrans*, consistent with previous studies (Neupane and Yan, 2024; Taning *et al*., 2024). However, contrasting results have been reported; Grabau *et al*. (2017) observed an increase in *P. penetrans* after growing oilseed radish.

The response of cover crops to plant-parasitic nematodes varies significantly depending on cultivar genetics, adaptability to local growing conditions, and planting time. For example, a recent field study in Dundee, Scotland, found that oilseed radish, Indian mustard, and cover crop mixes containing *Phacelia* and Japanese oats did not affect *Pratylenchus* spp. (Holland *et al*., 2021). This discrepancy with the current findings could be attributed to the fact that the cover crops in their study were grown between September and March as winter cover crops following barley. Furthermore, variation in oilseed radish performance was observed between the three tested sites in this study, a trend previously reported by Grabau *et al*. (2017), where the same cultivar had no effect at one site but led to an increase in *P. penetrans* at another. A 3-year temporal study of six spring sown cover crop treatments recently completed (autumn 2024) by the James Hutton Institute noted a slight increase in *Pratylenchus* abundance compared to a barley control towards the end of the growing season under *Phacelia*, and oilseed radish (cv. Defender) (*Neilson pers. comm*). Such high variability between studies highlights the influence of location, environmental conditions, and initial nematode population densities on *P. penetrans* response to cover crops (Miller, 1978; Kimpinski and Sanderson, 2004; LaMondia, 2006).

Most crops, including *Narcissus,* are susceptible to multiple pathogenic nematodes. The concept that a single cover crop could help manage multiple species of plant parasitic nematodes is potentially viable, as previous studies have shown that certain cover crops, such as French marigold, can effectively reduce root-knot and root lesion nematodes (Dutta *et al*., 2010). However, French marigold also serves as a good host for several ectoparasitic nematodes, including *Paratrichodorus teres* and *Hemicycliophora similis* (Lehman, 1979).

The likelihood of finding a cover crop that simultaneously suppresses nematodes of *Narcissus* is low because most species such as *P. penetrans* species are highly polyphagous. Therefore, on-farm recommendations should be based on initial soil sampling to identify the most dominant nematode species. This information can then guide the selection of cover crops based on their suitability as hosts for the identified nematodes.

A mechanistic understanding was sought regarding how cover crops reduce the abundance of *P. penetrans*. This was explored by chemotaxis and mortality assays using alfalfa and forage chicory root exudates (Chapter 5). Results showed that root exudates from these two cover crop species did not affect *P. penetrans.* Even though alfalfa and forage chicory were shown to be poor hosts (Chapter 2), their root exudates were not found to have any influence on the motility, migration or chemotaxis of *P. penetrans* under *in vitro* conditions.

Pure ITCs were used for mortality assays of *P. penetrans in vitro* (Chapter 6). Brassica isothiocyanates (Allyl, Benzyl and 2-Phenylethyl) are nematocidal to *P. penetrans*. The effects were irreversible, and the LD_{50}s are achievable under field conditions if common brassica biofumigants, for instance, oilseed radish and Indian mustard, are grown (Brown and Morra, 1997; Ngala *et al*., 2015). The irreversible nematocidal effect of ITC on *P. penetrans* aligns with results from other important plant parasitic nematodes such as *M. javanica* and *Trichodorus/Paratrichodorus* nematodes (Wu *et al*., 2011; Mwangi *et al*., 2024).

Data from this thesis strongly suggest that Indian mustard was a host to *Pratylenchus* spp. Most farmers already use Indian mustard as a cover crop, potentially increasing the abundance of the nematodes. Incorporation of the cover crop at the peak flowering stage can bring the nematode levels back to their initial levels, as observed in this study. UK growers already include cover crops in their production systems, so more information on the host status of cover crops will go a long way in preventing the use of cover crops that increase nematode abundance. For instance, Indian mustard (*Brassica juncea*) and ryegrass are typically grown as summer (spring sown) cover crops, although the benefits concerning nematode suppression in *Narcissus* are unknown. Nevertheless, both cover crop species have been reported as suitable hosts for *P. penetrans* (Neupane and Yan, 2023; Taning *et al.,* 2024). Common cover crops are sold as different cultivars; therefore, the cultivars identified in this study should be promoted for use by farmers, especially in the Isles of Scilly, where *P. penetrans* is dominant in *Narcissus* fields. The crop production system could immensely benefit from these results because *Pratylenchus* spp. are problematic in other important crops such as potatoes, carrots, onion, beans and sugar beet.

All three field experiments tested negative for *D. dipsaci,* and data from SASA for Grampian Growers cooperative over the past ten years suggest that the incidence of *D. dipsaci* is decreasing. In contrast, *Aphelenchoides* spp. are increasing in areas managed by industry partner Grampian Growers (Chapter 3, Supplementary Table 3.1). Attempts to obtain similar data from English growing areas (Lincolnshire and Cornwall) were unsuccessful. Such data could help to understand the complete picture of the occurrence of these nematodes from across the UK *Narcissus* industry, especially considering how the USA, a significant export market for the UK *Narcissus* sector, has zero tolerance for *Aphelenchoides* spp. and Europe has zero tolerance for *D. dipsaci*.

The main findings of this PhD project, i.e., the selection of suitable cover crop species to grow during the summer fallow, is arguably of immense agronomic importance to UK *Narcissus* growers. Agronomists, in general, could also benefit from such knowledge enabling better advice to be imparted to growers on using cover crops for nematode suppression. Industry interest in this study has been constant, with a keenness for the industry to increase their knowledge on the impact of cover crops under UK conditions. As a result, I have had the opportunity to share my results with several different groups of UK farmers and agronomists.

Although the project was successful, several challenges were faced during its delivery. Firstly, site selection should have occurred sooner so that a field infested with *D. dipsaci* could be identified and used for experiments. Secondly, attempts to culture *D. dipsaci* for *in vitro* or greenhouse experiments failed due to the incompatibility of the nematodes with carrot discs. Molecular confirmation of *Pratylenchus* species should have been done before the experiment was established, so that a field with higher densities of *P. penetrans* could have been selected. Based on the host suitability studies from Chapter 2, *Phacelia* was used as the susceptible positive control; however, it was not as good a host to *Pratylenchus* spp. as expected, making it less reliable for comparison with the rest of the evaluated cover crops. The same variety was tested by Taning *et al*. (2024) and was rated as a maintenance host. More than one positive control should have been used in the greenhouse host suitability experiments (Chapter 2) to make a suitable comparator available. The host suitability experiments used one initial population (Pi) density, yet the host status can vary with different initial populations of plant parasitic nematodes (Taning *et al*., 2024). Using a range of population densities, even a high and low Pi, would have made the host status results more reliable.

7.1 Future work

This project identified four cover crops (French marigold, oilseed radish, forage chicory and alfalfa) with the potential to suppress the abundance of *Pratylenchus* spp. To substantiate the findings, alfalfa and forage chicory must be tested again in field conditions. Cover crop host suitability for *D. dipsaci* (the *Narcissus* race) must be conducted under controlled conditions. The greenhouse experiments in Chapter 2 identified several poor hosts to *P. penetrans*; their modes of action should be explored. It is possible that the nematodes failed to invade the plant roots due to physical barriers (Wuyts *et al*., 2007). Therefore, invasion assays could be done using these cover crops.

The three sites used for experiments had varying levels of *Pratylenchus* spp. No records of damage threshold studies of this nematode exist. It is recommended to establish one and consider that it is associated with soil sickness; therefore, even low numbers could be harmful. This would be useful when deciding when to intervene with a control method and remove default management interventions, such as hot water treatments. *Aphelenchus subtenuis* is associated with *Narcissus* and reported to interact with *Fusarium,* causing rots in bulbs, but the understanding of pathogenicity is lacking. As we advance, research is essential to answer these questions. *Aphelenchoides* species are being rejected on samples in Scotland and must be identified. Communication with the USA Plant Health Services should be done to revise the “zero tolerance” for *Aphelenchoides* spp. if possible. Most species in this genus are not plant-parasitic; therefore, the regulations could focus on specific species. Species identification is required whenever *Aphelenchoides* are detected on bulbs, and then proceed to test the pathogenicity of that species on *Narcissus*.

Results in Chapter 6 demonstrated that brassica ITCs are toxic to *P. penetrans*; however, sublethal concentrations could also interfere with nematode growth, pathogenicity and reproduction. Subsequent tests of these low concentrations on nematode development and infectivity are recommended and might give insight(s) into how they cause mortality to *P. penetrans*. Oilseed radish reduced *Pratylenchus* spp. during the growing period, which might mean it is a partial biofumigant. Thus, there is a need to measure the glucosinolates and ITC produced by this cover crop under field conditions from planting to peak flowering.

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