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

Cover crops for managing plant parasitic nematodes in Narcissus

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1. Industry Summary

Plant parasitic nematodes cause significant direct and indirect yield loss in *Narcissus*, further exacerbated by limited management options due to the non-availability of nematicides and the shortage of land for rotation. Root lesion (*Pratylenchus penetrans*) and stem and bulb (*Ditylenchus dipsaci*) nematodes are serious plant parasites associated with *Narcissus* in the UK. Non-/poor-host, allelopathic, or resistant cover crops can potentially reduce soil nematode abundance in rotation with *Narcissus* crops when cultivated during the summer fallow. Three independent field experiments were conducted to assess the influence of *Phacelia*, oilseed radish, Indian mustard, Japanese oats, alfalfa, forage chicory and French marigold on the abundance of *Pratylenchus*, *Ditylenchus*, (fungivore) *Aphelenchus, Aphelenchoides* spp. and bacterivore nematodes. Fungivore and bacterivore nematodes were monitored because they are considered beneficial for soil health; an ideal cover crop should not adversely affect these non-target nematodes. Two sites were near Perth and Montrose, Scotland, while one was on the Isles of Scilly, England. Nematode abundances were assessed at planting, three months after planting (pre-incorporation), and six weeks post-incorporation of the cover crops. French marigold, forage chicory, alfalfa, and oilseed radish significantly reduced *Pratylenchus* spp. abundance. The highest reduction was observed in French marigold (37-70%), while the least was in oilseed radish (53%). Indian mustard significantly increased (>100%) the abundance of *Pratylenchus* spp., and *Phacelia* and Japanese oats maintained the abundance of *Pratylenchus* spp. Incorporating cover crops did not impact *Pratylenchus* spp., but they increased the abundance of *Aphelenchus*, *Aphelenchoides* spp. and bacterivore nematodes. There are four commonly found *Pratylenchus* spp.,namely *P. penetrans*, *P. crenatus, P. neglectus* and *P. thornei*. *Pratylenchus crenatus* and *P. thornei* were detected in samples from the Montrose site; only *P. crenatus* was detected from Perth samples, and *P. penetrans* and *P. crenatus* were detected from the Isles of Scilly. Cover crop treatment did not affect the abundance of individual *Pratylenchus* species in either Montrose or Isles of Scilly. However, forage chicory and French marigold significantly reduced *P. crenatus* abundance in the samples from Perth. Neither *Dityenchus dipsaci* nor *A. subtenuis* were present in the tested sites. Collating the results of the three experiments suggests that French marigold, oilseed radish, forage chicory, and alfalfa are potential options for managing *Pratylenchus* spp. in *Narcissus* fields

2. Introduction

The UK is the world's largest producer of *Narcissus* (4400 ha), a spring ornamental flower with an economic value of approximately £110 million (DEFRA, 2023). *Narcissus* is associated with the 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). No commercially available nematode-resistant varieties or nematicides are available on the market, the latter due to regulatory legislation. Increasing the length of crop rotations is not feasible due to limited land availability (Upcott *et al*., 2023), and 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). Thus, growers mainly rely on hot water treatment of *Narcissus* bulbs and in-field removal of infected plants, primarily for *D. dipsaci* management. There is, therefore, an urgent need to explore alternative chemical-free nematode management strategies. 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 without suppressive abilities can be avoided because they can potentially elevate nematode abundance (Neupane and Yan *et al*., 2023).

Cover crops confer various ecosystem services to soil and are grown during fallow periods to improve soil organic matter, biodiversity, soil fertility, reduce pathogens, weeds and soil erosion (Daryanto *et al*., 2018; Van Eerd *et al*., 2023). The UK government has incentivised sustainable soil management programs and adoption of cover crops to deliver "Greening measures" in England and Wales (Storr *et al*., 2019). Consequently, the awareness of farmers and agronomists of the benefits of cover crops has increased, with the choice of cover crop determined by the agronomic needs of their individual crop/livestock production system (White *et al*., 2016). *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 are known 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). Some cover crops employ more than one mode of action to reduce nematodes (Grabau *et al*., 2017).

The soil rhizosphere has a taxonomically and functionally diverse community of nematodes, with their composition and structure indicative of soil health (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 trophic group of nematodes, followed by plant parasites> fungivores>omnivores>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 fungal and bacterial populations in the soil (Thakur and Geisen, 2019). Bacterial-feeder and predatory nematodes directly and indirectly contribute 8-19% of nitrogen mineralisation under field conditions in conventional and integrated farming systems (Beare, 1997). They contribute by feeding on decomposer microbes and releasing ammonium through excreta and nitrogen immobilisation in live biomass (Ferris *et al*., 1998). Predatory nematodes feed on fungivore and bacterivore nematodes, channelling resources from low to high trophic levels, thereby regulating nitrogen mineralisation (Wardle and Yeates, 1993).

Cover crops may change or influence the composition of microbial communities within the rhizosphere, thereby influencing soil processes such as soil organic matter decomposition and nutrient cycling (Jin *et al*., 2019). Ideally, cover crops should suppress plant parasitic nematodes without reducing non-target beneficial nematodes. Thus, monitoring the abundance of beneficial nematodes such as fungivore and bacterivore nematodes is essential to develop cover crop schemes for sustainable nematode management.

The cover crops tested in this study were oilseed radish (*Raphanus sativus*), Japanese oats (*Avena strigosa*), French marigold (*Tagetes patula*), *Phacelia* (*Phacelia tanacetifolia*), Indian mustard (*Brassica juncea*), forage chicory (*Cichorium intybus*) and alfalfa (*Medicago sativa*). These cover crops were selected based on their poor host status as determined in greenhouse host status studies in Chapter 3, and suitability to climate and local growing conditions.

2.1. Objectives

The objectives of this chapter were i) to assess the impact of selected cover crops on the abundance of *Pratylenchu*s, *Ditylenchus*, *Aphelenchoides, Aphelenchus* spp. and bacterivore nematodes compared to a fallow control; ii) test for the presence of *P. penetrans, P. neglectus, P. crenatus* and *P. thornei*; iii) assess whether individual species respond to cover crop treatments using qPCR; iv) test for the presence of *A. subtenuis*; and v) *D. dipsaci* in the three field sites using conventional PCR.

3. Materials and methods

Six *Narcissus* fields with a history of pathogenic nematode infestation were selected in each region (Scotland or the Isles of Scilly) and sampled to assess the abundance of plant

parasitic nematodes before experiment establishment. Sites with the greatest abundances were chosen for the experiments. 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 Isles of Scilly), and in the second year (2023), only one site in Perth was established as a repeat of 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 recommended seeding rates detailed in Table 1. Brassica cover crop treatments received 100 kg ha-1 N and 25 kg ha-1 S fertiliser as Sulfan (24% N, 15% SO3) at planting.

Table 1. Cover crops sown across the three experimental sites

Cover crop (Cultivar)	Scientific name	Family
French marigold (French marigold)	*Tateges patula* L.	Asteraceae
Oilseed radish (Contra)	*Raphanus sativus* L.	Brassicaceae
Indian mustard (Caliente)	*Brassica juncea* L.	Brassicaceae
Phacelia (Angelia)	*Phacelia tanacetifolia* Benth.	Boraginaceae
Japanese oat (Pratex)	*Avena sativus* L.	Poaceae
Alfalfa (Artemis)	*Medicago sativa* L.	Fabaceae
Chicory (Commander)	*Cichorium intybus* L.	Asteraceae

3.1. 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 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 manufacturer'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 6 June 2022, and soil sampling was done to assess the initial nematode abundances (Pi). At the peak flowering stage (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. 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. 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 8 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 as no roller was available. The plots measured 3 x 1 m, and the treatments were arranged in a randomised complete block design with five replicates. After planting, the plots were watered using a watering can, and the cover crops reached peak flowering on 26 November 2022. Soil sampling was done to determine nematode abundance at the pre-incorporation stage. The cover crops were chopped and incorporated using a rotavator. Afterwards, the plots were rolled. Final nematode abundance was assessed on 4 February 2023 (8 weeks post incorporation) due to delays with the availability of machinery and labour.

3.3. Field Site 3

This experiment was an iteration of the experiments conducted in the first year, based on the cover crop treatments 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 29 June 2024. All seeds were hand-broadcast at the recommended seed rate due to the unavailability of drilling equipment. The treatments were arranged in a randomised complete block design with five replicates on 3 x 4 m plots. Approximately three months (29 September 2023) after planting, at the peak flowering stage, soil sampling was done to assess nematode abundance at the pre-incorporation stage. 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.

3.4. Soil sampling and nematode extraction

Soil sampling was performed at planting, three months after planting (pre-incorporation stage) and six weeks post-incorporation of the cover crops. 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 soil cores were homogenised 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 (Yeates et al., 1993) level under a compound microscope (Leica, Germany) at 40x magnification. The average of three subsamples was computed, and the total number of nematodes in the 200 g was calculated using the formula: *[total number of nematodes per 200 g soil sample = average number per subsample x 10 (total suspension volume in mL)].*

3.5. 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. 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 *µ*l of DNA elution buffer, and a 2 *µ*l 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 2. 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, as described previously (Orlando *et al*., 2024). The reaction mixture consisted of 10 µl of Taqman™ 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.

Table 2. 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. 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

3.6. 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. A composite soil sample was randomly collected from each experimental site, from which a 200 g subsample was extracted as previously described using a modified Baermann tray. Ten individual nematodes per site were handpicked from the extracted nematode suspension and placed in 10 µl of 10X PCR buffer (GoTaq, Promega, UK). 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 spun; 4 µl of proteinase K (100 µg ml^{-1}) and 1 µl of 10X PCR buffer 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 min. The primers used to amplify the mtCOI DNA region were forward: mtCOI-F

(CCTACTATGATTGGTGGTTTTGGTAA TTG) and mtCOI-R (GTAGCAGCAGTAAA ATAAGCACG) (Kanzaki and Futai, 2002). The reaction mixture was made up of 8.5 µl PCR grade water, 3 µl of 10X GoTaq buffer, 0.06 µl TaqPolymerase, 0.6 µl of 0.4 mM forward and reverse primer, 0.3 µl of 0.22 mM dNTP and 5 µl DNA template. The PCR reaction was as follows: 94 °C for 5 min, 42 cycles at (94 °C for 30 s, 51 °C for 30 s, 72 °C for 2 min), and 72 °C for 10 min (Sánchez-Monge *et al*., 2017). *Pratylenchus penetrans* was used as non-target control, while a known *A. subtenuis* positive control was unavailable. The PCR products were separated on 1% agarose gel using 6X GelRed loading buffer (Biotium) and visualised under UV light.

3.7. Molecular identification of *D. 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. Genomic DNA was amplified using ITS rRNA universal primers; Forward: CTGTAGGTGAACCTGC and Reverse: GACATCACCAGTGAGCATCG (Jeszke *et al*., 2015). The PCR reaction mixture was made up of 6.98 µl PCR grade water, 2 µl of 10X GoTaq PCR buffer, 2 µl of 0.4 mM forward and reverse primer, 1.82 µl of 0.22 mM dNTPs, 0.2 µl Taq polymerase and 5 µl of template DNA. PCR conditions were as follows: 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 63.5 °C, 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. The PCR products were separated on 1% agarose gel using 6X GelRed loading buffer (Biotium) and visualised under UV light.

3.8. 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 crop treatments and time on nematodes 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 provides an estimate of 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's HSD post hoc tests).

qPCR data was 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.

4. RESULTS

4.1. Montrose – Field Site 1 (Table 4)

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 had no effect on the abundance of *Pratylenchus* spp. ($p>0.05$). Incorporating all oilseed radish, French marigold and Indian mustard significantly increased the abundance of bacterivore nematodes, with the highest increase observed in oilseed radish (335%). Japanese oats, Phacelia and oilseed radish had significant effects on 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.

4.2. Isles of Scilly – Field Site 2 (Table 5)

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 ($p=0.01$). The abundance of *Pratylenchus* spp. was unaffected by fallow and *Phacelia* treatments ($p>0.05$). After incorporation, all cover crops significantly increased bacterivores ($p<0.05$). Oilseed radish and Indian mustard significantly increase *Aphelenchoides* spp. at the post-incorporation stage ($p<0.05$). Except for the fallow control, cover crops significantly increased the abundance of *Aphelenchus* spp. after incorporation ($p<0.05$).

4.3. Perth – Field Site 3 (Table 6)

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 4. 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 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 ($p<0.05$).

		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 5. 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 six weeks post-incorporation (Pf) of oilseed radish (*Raphanus sativus*), Indian mustard (*Brassica juncea*), French marigold (*Tagetes patula*) and *Phacelia tanacetifolia* at the field site on the Isles of Scilly, England. Significant differences 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 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) in oilseed radish (*Raphanus sativus*), French marigold (*Tagetes patula*), forage chicory (*Cichorium intybus*), alfalfa (*Medicago sativa*) and fallow control under field conditions at the field site near Perth, Scotland. Significant changes in nematode abundances relative to the 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

4.4. 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 tested positive for *P. crenatus* and *P. thornei*. The Isles of Scilly tested positive for *P. penetrans* and *P. crenatus,* while in Perth, they tested positive for *P. crenatus* only.

4.5. Montrose

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).

4.6. Isles of Scilly

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 individual species (p>0.05) differences between numbers.

4.7. Perth

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.

4.8. Molecular identification of *A. subtenuis* and *D. dipsaci*

Separation of the PCR products obtained from the samples on agarose gel yielded no visible band under UV light, indicating the absence of the target nematodes *A. subtenuis* and *D. dipsaci* at all three sites. However, due to the lack of positive PCR controls, this result only indicates the absence of these two species.

5. DISCUSSION

The key findings of this study support the hypothesis that certain cover crops effectively reduce *Pratylenchus* spp. abundance without exerting adverse effects on beneficial fungivorous and bacterivorous nematodes. Previous research has also shown that incorporating cover crops increased the abundance of beneficial non-plant parasitic nematodes whilst reducing the total number of plant parasitic nematodes (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, but an overall increase in the abundance of PPNs does not mean all the plant parasitic nematodes

present increase, as the response depends on the cover crop's host status. It is plausible that the cover crop treatments reduced some plant parasites we did not assess.

In the current study, the abundance of bacterivorous and fungivorous 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). One possible explanation is that adding plant material to the soil provides a substrate for fungal and bacterial microbes, 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, omnivore, and predator/carnivore nematodes (Dietrich *et al*., 2021). In this study, the shoot biomass of the different cover crops was not assessed at the pre-incorporation stage; therefore, a conclusion cannot be made to justify why oilseed radish had the greatest impact 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 has been reported to suppress plant parasitic nematodes, including *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, as previously reported (Ngala *et al.* 2015). Partial biofumigants constitutively release low levels of glucosinolates from the roots during growth; therefore, nematode suppression can be achieved before incorporation. Several studies have explored the effects of biofumigant cover crops on fungal and bacterivore nematode communities (Forge *et al*., 2003; Wang *et al*., 2004; Georgieva *et al*., 2005). Free-living/non-plant parasitic nematodes are considered less affected by brassica 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 fungal nematode abundance but reduced PPN abundance.

French marigold reduced *Pratylenchus* spp. abundance in all three experiments. French marigolds confer nematicidal effects on plant parasitic nematodes, including *Pratylenchus* and *Meloidogyne*

spp. (Tyler, 1938; 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 plant parasitic nematodes through several modes of action, including being a non/poor host and trap crop (Wang et al., 2001; Pudasaini et al., 2006), which may work simultaneously for additive effects. These results concur with previous field and greenhouse experiments (Miller, 1978; Kimpinski *et al*., 2000; Evenhuis *et al*., 2004; Pudasaini *et al*., 2006). French marigold has been grown as a cover crop in rotation schemes (Ploeg, 1999), applied as plant extracts (Mateeva and Ivanova, 2000), incorporated into the soil as green manure like in our study (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 agri/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). Our previous greenhouse experiments 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 that this cover crop 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, such as Indian mustard and oilseed radish, are known for their biofumigation effects on several plant parasitic nematodes, such as *Meloidogyne incognita* (Oliveira *et al*., 2011), *Globodera pallida* (Lord *et al*., 2011; Ngala *et al*., 2015), and *Pratylenchus 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. It is possible that insufficient ITCs were produced or the conditions were

not conducive to biofumigation, as previously observed (Vervoort *et al*., 2014). Alternatively, the biofumigation effects of brassica cover crops may be masked if the selected species support the reproduction and multiplication of the target nematode species (Vervoort *et al*., 2014; Grabau *et al*., 2017).

Although *Narcissus* is known as a host of *Ditylenchus 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. 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. Some of the *Aphelenchoides* species are facultative plant parasites (Sànchez-Monge *et al*., 2017), and it is possible that export bulbs are being rejected due to species misidentification. In this study, the sites tested negative for *A. subtenuis,* previously reported to be associated with *Narcissus*. The *Aphelenchus* and *Aphelenchoides* spp. counted were therefore assumed to be non-plant parasitic and fungivorous.

5.1. 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 several factors, such as 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 *Narcissus* crop. The presence of *P. penetrans* in the Isles of Scilly has been previously reported (Hanks and Chastagner, 2018). In these fields, ‘soil sickness’, a root rot disease caused by the interaction of this nematode with the fungus *Cylindrocarpon destructans*, is common (Lane, 1984).

The qPCR detected that fewer nematodes were observed under microscopy in Montrose and Isles of Scilly; such discrepancies could be due to the uneven distribution of the nematodes in soil samples due to their migratory nature (Yan *et al*., 2008). Secondly, some PCR inhibitors could have been 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. Similar results have been reported

by Oliveira *et al*. (2017), who also found higher numbers in qPCR compared to 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 inactive, dead nematodes and eggs being detected and quantified by qPCR, whereas microscopic counts only account for live/active adults and juveniles; hence, higher numbers of nematodes would be detected in qPCR. Different life stages of *P. penetrans* contain varying amounts of total DNA (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. Another reason could be extraction efficiency; only mobile nematodes are counted, and samples may differ in numbers due to their mobile lifestyle. *Pratylenchus* species can survive in an inactive, dehydrated anhydrobiotic state, which would otherwise be detected by qPCR. An isolate of *P. crenatus* from Scotland exhibited more total DNA after qPCR than other tested isolates (Orlando *et al.,* 2024). There is also intraspecific variation in the copy number or D2-D3 fragment among different *P. crenatus* populations. A 3.7% variation was observed in D2-D3 sequences of Korean *P. crenatus* populations (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.

This study provides insights into the effects of cover crops on 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 soil populations of *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.

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