Harper Adams University

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Harper Adams University

Management of stubby root nematodes (*Trichodorus and Paratrichodorus* spp.), associated with Docking disorder of Sugar beet (*Beta vulgaris* L) using brassica and non-brassica cover crops.

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Declaration.

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

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Abstract.

This study investigated the impact of utilizing cover crops in the suppression of stubby root nematodes (*Trichodorus* and *Paratrichodorus* spp.) — SRN, and the subsequent effects on quality and quantity of sugar beet (*Beta vulgaris*). The active compounds associated with some of these cover crops were also evaluated for their potential nematicidal/nematostatic effects on SRN in *in-vitro* assays. Pure Isothiocyanates (ITCs), associated with brassicas, namely 2-phenylethyl (PEITC), allyl (AITC), and sulforaphane (SITC) were tested at different concentrations (1.625, 3.125, 6.25, 12.5, 25, and 50 µg ml^{-1}). Effect on nematode mobility was evaluated after 24, 48, and 72 h, and mortality of SRN was assessed after 48 h of incubation in distilled water following ITC treatment.

The mortality for all ITCs at all tested concentrations was significantly higher than the controls, distilled water, and 1% DMSO. The concentration and type of ITC had a significant effect on SRN mobility and mortality, while an increase in exposure time did not significantly increase the immobility of SRN. The average 24-hour ED_{50} (dose resulting in 50% immobility) for SRN were 7, 5, and 44 µgml^{-1}, and the average LD_{50} (dose resulting in 50% mortality) after 48 h of recovery in distilled water was 7, 11, and 24.3 µgml−1 for PEITC, AITC and SITC, respectively. The efficacy of cover crops under field conditions was tested at three locations in England: Bury St Edmunds, Suffolk (site 1) and Docking, Norfolk (site 2) and Tibberton Grange, Shropshire (site 3).

Brassica and non-brassica cover crops were tested. The cover crops included Indian mustard (*Brassica juncea*), oilseed radish (*Raphanus sativus*), daikon radish (*Raphanus sativus* subsp.Longipinnatus), grass with endophyte (E+), grass without endophyte (E-) (*Festulolium loliaceum*), Italian rye grass (*Lolium multiflorum*), *Phacelia* (*Phacelia tanacefolia*), and opium poppy (*Papaver somniferum*), stubble turnips (*B. rapa*), strigosa oats (*Avena strigosa*), clover (*Trifolium alexandrinum*), vetch (*Vicia sativa)* and vitality mix. At site 1, plots sown with brassica cover crops, specifically Indian mustard, and oilseed radish, exhibited significantly lower SRN reproduction factor (Rf) ($P<0.05$) compared to the fallow control and daikon radish.

In site 2, plots sown with Italian rye grass, Indian mustard, grass without endophyte (E-), or left fallow and undisturbed had a significantly higher Rf ($P<0.05$) compared to plots with *Phacelia*, opium poppy, and disturbed or sterile fallows, while in site 3, clover had significantly higher multiplication rate of SRN compared to all the other cover crops. It was four times higher than the vitality mix, three times than radish and vetch and twice higher than oats and stubble turnips. The vitality mix had the lowest SRN multiplication rate. Results from assessment of sugar beet quantity and quality parameters post cover crops indicated that

sugar beet root fanging (%) and root soil tare (%) was significantly lower in cover crops and fallow plots with lower SRN Rf values, such as Phacelia, opium poppy, sterile fallow, and disturbed fallow. Environmental factors like rainfall and soil temperature also significantly impacted SRN densities at different sampling points, where SRN decreased with decreasing rain and increasing soil temperatures. The findings suggest that certain cover crops can impede SRN multiplication, despite SRN's polyphagous nature. Furthermore, factors such as weed occurrence, soil temperature, rainfall, and soil disturbance significantly affect SRN densities under field conditions.

Following the observed difference in SRN reproduction between grass with endophyte (E+) and grass without (E-) in the field trial, *in-vitro* experiments with shoot and root extracts were conducted to test the sensitivity of SRN to the associated compounds. Both E+ and E- extracts obtained from shoots and roots had the ability to immobilise SRN, despite the presence of the endophyte. However, a comparison of the LD_{50}values revealed that the presence of the endophyte significantly impacted the mortality of SRN. The LD_{50} values of E+ extracts were lower ($P<0.05$) than E- extracts across all ages. Specifically, the LD_{50} value for shoot extracts of endophyte grass (E+) was significantly lower at 8 weeks old compared to all other ages, being twice as low as 12 weeks, 11 times lower than 16 weeks, and six times lower than 20 weeks extracts.

The LD_{50} for E+ root extracts at 20 weeks were half that of 12 weeks, although not significantly different from 16 weeks. In contrast, the LD_{50} values of root extracts from grass without endophyte followed a different pattern, increasing with the age of the grass. The LD_{50} value for 20-week-old plants was five times higher than that of 12- and 16-week-old plants. No mortality was recorded in the 8-week-old root extracts of both E+ and E- grass. The LD_{50} values also revealed that root extracts from E+ grass were more potent than those from E- grass, with the LD_{50} values at 12 weeks being twice as low for E+ compared to E-, and nearly 50 times lower at 20 weeks when compared to E-.

Age of the grass significantly affected loline concentration in both shoots and roots, where the concentration increased with increasing age in both shoots and roots. On the other hand, the total flavonoid content (TFC) and total phenol content (TPC) in shoot extracts decreased with age in both E+ and E- grass, with no significant differences recorded between E+ and E- grass. A negative correlation between shoot biomass and TFC ($R = -0.94$), and between shoot biomass and TPC ($R = - 0.67$) and root biomass and TPC ($R = -0.79$) was recorded. Upon bruising and wounding of endophyte grass (E+), the change in composition and total concentration of lolines was recorded. Specifically, NFL, NAL, and NANL were present in the 3, 7, and 11 dpb extracts but absent in the 30 days post bruising (dpb)extracts and the

control. Total loline alkaloid content in the shoot extracts at 3, 7, and 11 dpb was significantly higher compared to the 30 dpb extracts and the control unbruised. This translated into lower LD_{50} values for shoot extracts from regrowth tissue at 3-, 7-, and 11-days post bruising (dpb) when compared to 30 dpb extracts and the control. In conclusion, this study has demonstrated potential of using brassica cover crops and non-brassica cover crops for managing SRN.

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Common Abbreviations.

SRN	Stubby root nematodes
AlCl3	Aluminium chloride
Na_2CO3	Sodium carbonate
NFL	N-formylloline
NAN	N-acetylnorloline
NA	N-acetylloline
CC	Cover crops
GSL	Glucosinolate
LD_{50}	Lethal dose at 50%
EC_{50}	Effective concentration at 50%
ITC	Isothiocyanates
TFC	Total flavonoid content
TPC	Total phenol content
N	Nitrogen
ND	Not detected
UK	United Kingdom

Research Outputs.

Oral presentations.

1. Population dynamics of SRN, associated with “Docking disorder” of sugar beet, in field rotations with cover crops in East-England, April 2024, 35th Symposium of The European Society of Nematologists, Cordoba, Spain.

2. Plant age influences nematicidal effects of Hybrid grass (*Festulolium loliceum*) on stubby root nematodes (SRN), September 2023, Harper Adams University Research Conference.

3. Potential of cover crops and their associated compounds in the management of SRN (SRN), May 2023, research seminar, Harper Adams University

Poster Presentations

1. Potential of cover crops in suppression of stubby root nematodes associated with Docking disorder in sugar beet (*Beta vulgaris*) May 2022, International Congress of Nematology, Antibes, France.

2. Potential of biofumigant cover crops in suppression of stubby root nematodes associated with Docking disorder in Sugar beet (*Beta vulgaris*), September 2022, Harper Adams University Research Conference.

3. Potential of cover crops in suppression of stubby root nematodes associated with Docking disorder in sugar beet (*Beta vulgaris*), December 2022, Association of Applied Biologists (AAB) - Advances in Nematology conference (Online)

Publications:

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3. Mwangi, N. G., Stevens, M., Wright, A. J., Watts, W. D., Edwards, S. G., Hare, M. C., & Back, M. A. (2025). Population dynamics of stubby root nematodes (*Trichodorus* and *Paratrichodorus* spp.) associated with ‘Docking disorder’ of sugar beet (Beta vulgaris L.), in field rotations with cover crops in East England. *Annals of Applied Biology*.

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Chapter 1: Literature review.

1.1 Introduction.

Plant parasitic nematodes, (PPNs) are important pests for many crops globally and result in crop losses which equate to US $80billion (Nicol et al., 2011). The sugar beet crop is no exception, being subject to infection by a variety of different PPN species such as beet cyst nematode (*Heterodera schactii*) (Wright et al., 2019), root knot nematodes—*Meloidogyne hapla* and *M. chitwoodi* (Griffin et al., 1982) and stubby root nematodes (SRN) —*Paratrichodorus* and *Trichodorus* spp. (Hafez, 1998; Whitehead & Hooper, 1970).

Stubby root nematodes are polyphagous ectoparasites and are widely distributed in light sandy soils (Cooke, 1973; Whitehead & Hooper, 1970; Winfield & Cooke, 1975). In East England, *Trichodorus* and *Paratrichodorus* spp. attack young sugar beet seedlings causing a condition known as Docking disorder, named after the parish “Docking”, where it was first recognized and described (Gibbs, 1959). These species have been isolated in 75% of samples collected in fields with docking disorder symptoms (Cooke, 1973).

Foliage of sugar beet suffering from Docking disorder appears to be deficient in nitrogen or magnesium and attacked roots have stubby lateral roots, which turn grey- brown and later black as they die and decay (Cooke, 1989; Whitehead & Hooper, 1970). In fields where the symptoms persist, root yield has been found to be 17.5 t /ha less and are more fangy — this is where the tap root ceases growing leading to thickening of the lateral roots than those from unaffected fields which yield on average 70–80 tonnes per hectare (t/ha) of clean beet (Cooke, 1973). Yield losses of up to 50 % have also been recorded due to the fangy root symptoms (Cooke, 1989). Docking disorder severity has been correlated with environmental and agronomic factors such as rainfall, physical conditions of the soil, previous cropping, rate and timing of fertilizer application/herbicides. Root damage is mostly evident at the end of May, coinciding with higher rainfall, while the symptoms on foliage are mostly visible in June (Cooke, 1973).

For many years, management of SRN relied upon the prophylactic use of pesticides including soil fumigants such as 1, 3 dichloropropene. Application was undertaken either in autumn before sowing or as a row application shortly after drilling sugar beet (Cooke & Draycott, 1971). In the UK, crops at risk from Docking disorder relied on treatment with the granular nematicide aldicarb, applied at drilling, to prevent root damage by SRN, but the expense and inconvenience limited its use (Cooke, 1989). Vydate (Oxamyl), another nematicide used by sugar beet growers, was no longer authorized for use in the UK in December 2020, leaving growers with no chemistry for the management of SRN. As the pressure to develop other active ingredients continues, other cultural and crop management strategies need to be evaluated for future use in the crop (Stevens, 2015).

The concept of cover crops has evolved over time, it was originally developed in US agriculture primarily for erosion control and green manuring but has since expanded to include additional benefits such as use as suppressive crops to pests and pathogens through exudation of toxic volatile compounds which can have direct effects to the pest causing mortality or indirectly by repelling the pathogen and limiting its host finding abilities. They also act as catch crops, where they trap the nematodes limiting the advancement of their life cycle (Couëdel et al., 2018, 2019). The use of cover crops has been shown to provide various ecosystem services and increase biodiversity. A new approach, known as multi-service cover crop (MSCC), has been introduced to

encompass these diverse benefits using agroecology principles When selecting species or mixtures for cover crops, it is essential to consider potential trade-offs, as various botanical families can be utilized, and interactions between cover crop species and main crops can occur (Couëdel et al., 2018).

The success of cover crop choices is underpinned by genotype-environment- management interactions. The presence of a growing plant cover affects light, nutrient and water fluxes, microclimate, and organism communities compared to bare soil. Growing cover crops provide substantial amounts of nutrients through root exudation and rhizodeposition processes, attracting and sustaining microorganisms that can suppress pathogens. Different cover crop species and legumes such as sole crops can enhance specific pathogen-suppressive microorganisms. Additionally, the combination of higher root tissue diversity and biomass in mixtures can lead to increased microbial mixtures could enhance soil organism diversity and activity, leading to increased disease suppression. Complementary effects of crucifer diversity and abundance in the rhizosphere, potentially improving control of pests and diseases (Couëdel et al., 2018).

The period between two cash crops is crucial for disrupting the cycles of pests, weeds and pathogens that are unable to survive without a suitable host for an extended period. It is important to select cover crop species that are not susceptible to pathogens, as they could otherwise serve as hosts for pathogens that would naturally decline during bare fallow periods. Brassicaceous plants are considered break crops, or non-hosts, for a variety of pathogens (Angus et al., 2015). However, despite releasing toxic compounds, they can also host or moderately host certain fungal pathogens (Lu et al., 2010) and nematodes (Ntalli and Caboni, 2017), which may diminish their efficacy as cover crops in specific conditions. Phytochemicals from widely used cover crops (CC) such as polythienyls and polyacetylenes from family Asteraceae, 2- dehydropyrrolizidine (PAs) from the families Asteraceae, Boraginaceae and Fabaceae, ITCs from Brassicaceae, saponins from Leguminosae and glucosides from Poaceae have also been shown to suppress nematodes (Thoden et al., 2009).

These nematicidal phytochemicals can be exploited through crop rotations, intercropping or use as green manures (Zhou et al., 2012), where they can be released either through volatilization, exudation, leaching from plant roots or through decomposition of plant residues (Dutta et al., 2019; Halbrendt, 1996). Alkaloids and secondary metabolites obtained from different plant species have been shown to have varied negative effects on nematodes such as causing mortality, paralysis, hatching inhibition, and repulsion, hence interfering with nematode host finding abilities. These compounds have been further exploited for the development of biopesticides for nematode management (Renco et al., 2014). This review focuses on the sugar beet crops, SRN parasitizing sugar beet crops, their distribution and pathogenicity and the potential of brassica and non-brassica cover crops with their associated phytochemicals in management of PPNs.

1.2 The sugar beet crop: origin and importance.

The sugar beet (*Beta vulgaris* L.) is believed to have originated in the Middle East, near the Tigris and Euphrates rivers, from where wild beets are thought to have spread west into the Mediterranean and north along the Atlantic Sea coast (CFIA, 2022). Sugar beet is the world's second most important sugar crop after sugar cane, accounting for 30% of global sugar production (Ahmad et al., 2017; Iqbal & Saleem, 2015; Nedomová et al., 2017; OECD, 2018). It is mainly grown in regions with temperate climates in North America, Europe and some areas north

Africa (Draycott, 2006; Fitters et al., 2017). In 2021, Russian Federation, Germany, Turkey, USA and France contributed to 52% of the 270 million tonnes world beet production (FAOSTAT, 2022). Sugarbeet is an economically important crop in East Anglia and the East Midlands areas of England, occupying 100,000 hectares of land, comprising 3,500 growers (British Sugar, 2019; Okom et al., 2017).

In the UK, sugar beet cultivation has a long history, with Norfolk hosting the first crop over 100 years ago (British Sugar, 2019). The crop covers 3.7% of total area under crops and supplies 55% of sugar consumed in the United Kingdom. (Tzilivakis et al., 2005). Despite, the suitability of 1.7 million hectares of arable land in England and Wales for sugar beet cultivation only a small percentage is under cultivation (Richter et al., 2006), where production in East England is confined within 30 miles from the main sugar beet factories in Newark, Wissington, Bury St Edmunds and Cantley (Fitters et al., 2017). The UK was globally ranked 10th with a total production of 7.4 million tonnes (accounting for 3% global production) from 95, 200 hectares (FAOSTAT, 2022). Sugar beet is typically grown in a rotation of 3–5 years or more, depending on local practices, soil conditions, climate, diseases, and weeds.

Prior to planting winter wheat, other crops such as corn, potato, soybean, alfalfa, and barley are often cultivated for one or more years. It is a biennial crop characterised by the development of a rosette of dark green, glossy leaves with prominent midribs and strong petioles (Elliott & Weston, 1993). The crop grows up to 120cm height and has three main parts namely: crown, neck, and cone shaped root system (Schulze-Lammers et al., 2015). The leaves emerge from the crown while the root may swell conspicuously forming the beet with the hypocotyl and the taproot may be branched. The leaves vary in size, shape, and color, often dark-green or reddish and shiny, and normally form a radicle rosette. The stems can be decumbent, ascending, erect or branched.

The flowers are arranged in small cymes and are hermaphrodite (OECD, 2006). Root growth and biomass accumulation occur concurrently up to the 8-10 leaf stage, after which root growth surpasses above-ground biomass (Elliott & Weston, 1993; Milford, 1973). Sugar beet roots can grow up to 1.5 m down the soil profile, regardless of water availability, with variations observed among varieties (Fitters et al., 2017; Stevanato et al., 2010). The crop has high levels of sucrose estimated on a fresh weigh basis to range from 12% to 21%, with the roots as storage organs. In addition to sugar, sugar beet by-products such leaves, molasses and pulp have other benefits i.e., molasses are used in alcohol production, leaves as fertilizer, pulp as animal feed and carbonation sludge provide soil nutrition.

Sucrose production occurs in the first year between the 5th and 8th weeks of planting (Stevanato et al., 2010). Sucrose is the primary sugar component, where 98% of extracted sugars in roots are sucrose (Trebbi & McGrath, 2003). The roots are made up of 75% water, 2.5% non-sugars, 17.5% sugar and 5% pulp. Sucrose makes up 70% of the root composition of a dry basis with the remaining 30% comprising of other components. The plant has a high soluble sugar content, along with high pectin and hemicellulose carbohydrate contents, and relatively low lignin contents, which can vary regionally and seasonally due to numerous interacting factors, including plant biology, location, agronomy, harvest, and post- harvest practices (Zicari et al., 2019).

1.3 Pests and pathogens of sugar beet.

Sugar beet yield losses associated with pests and pathogens are estimated to be 26% and exceed 80% in absence of crop protection measures (Oerke & Dehne, 2004). The crop is at risk of various pests and pathogens as highlighted in Table 1.1.

Table 1.1: Major pests and diseases affecting sugar beet crops.

Pest/disease	Vector /causal agent	Reference
Virus Yellows	Green peach aphid (*Myzus persicae*)	Stevens et al., 2005
Rhizomania	Slime mould (*Polymyxa betae)*	Stevanato et al., 2019
Beet Curly Top	Beet leafhopper (*Circulifer tenellus)*	Duffus & Ruppel, 1993
Cercospora Leaf Spot	Fungus (*Cercospora beticola*)	Skaracis et al., 1993
Powdery Mildew	Fungus (*Erysiphe polygoni*)	Whitney, 1989
Rhizoctonia root rot and crown rot	*Rhizoctonia solani*	Windels et al., 2009
Beet moth	*Scrobipalpa ocellatella*	Stevens et al., 2022
Beet Rust	*Uromyces betae*	Kaczmarek et al., 2019
Leaf spot	*Ramularia beticola*	Byford, 1975

Ongoing research in sugar beet has focused on exploiting genetic resistance to diseases and abiotic factors (Stevanato et al., 2019). Although this endeavor has not fully met expectations, in terms of covering all challenging pests and diseases affecting sugar beet, positive results have ensured the competitiveness and survival of beet cultivation in most regions. Efficient genetic resistance or tolerance is considered the best approach to mitigate damage from diseases or stress, eliminating the need for pesticide applications. The risk of pathogen compromise on resistance in sugar beet appears limited, as evidenced by few reports of resistance breakdown to rhizomania even after almost 20 years of cultivating resistant varieties (Stevanato et al., 2019).

In the United Kingdom, virus yellows is an economically important disease affecting the yield of the sugar beet crop (Dewar & Qi, 2021). It is a complex of three viruses transmitted by aphids namely: beet yellows virus (BYV), beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV). Beet mild yellowing virus has been identified as the most important of these viruses causing up to 30% yield reduction in surveys conducted over the last 20 years (Qi et al., 2004).

The latest virus yellows epidemic resulted from a combination of factors, including high overwintering survival of the principal vector, the peach potato aphid, *Myzus persicae*, following a mild winter, early migration of infective aphids into newly emerging beet crops in April, and the removal of neonicotinoid seed treatments by EU directive, which had previously controlled the disease for 26 years (Dewar & Qi, 2021). Furthermore, the depletion of alternative insecticide spray products, either due to other bans or the development of resistance in the vectors,

compounded the challenges faced by growers (Dewar & Qi, 2021). Figure 1.1 gives a summary of the major pests and pathogens received by the plant clinic at British beet research, where nematode cases represented 10% of the total cases reported.

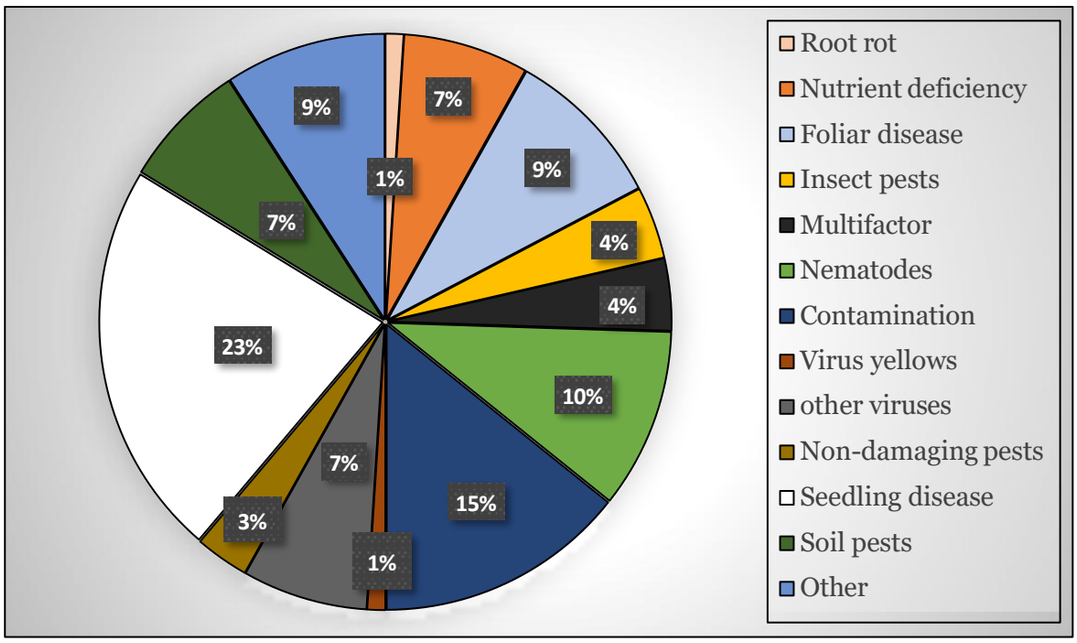

Figure 1.1: Plant Clinic cases from 2023 from British beet research organization (BBRO) lab (Oram, 2024).

1.3.1 Plant parasitic nematodes of sugar beet.

Various species of plant-parasitic nematodes have been documented in sugar beet around the world. Sugar beet has been reported to harbor 65 species of nematodes from 27 genera (Ashmit et al., 2021). The earliest record of nematode species parasitising sugar beet was *Heterodera schachtii*, in 1859, now known as the sugar beet cyst nematode, was recorded by Schacht on sugar beet plants in Germany, resulting in severe yield losses due to its rapid spread with frequent planting of sugar beet. This led to the closure of more than 20 sugar beet factories in Germany in 1876. Root-knot nematodes (*Meloidogyne* spp.) were first reported in sugar beet in 1885, in Germany and in 1911 they were reported in the United States (Bessey, 1911). The stem and bulb nematode (*Ditylenchus dipsaci)* were first reported to be associated with sugar beet damage in England, Germany, and the Netherlands in 1900 (Weischer & Steudel, 1972). In 1956, the false root-knot nematode, *Nacobbus aberrans*, was recorded on sugar beet in the United States (Thorne & Schuster, 1956).

Stubby-root nematodes (*Trichodorus* and *Paratrichodorus* spp.) and needle nematodes (*Longidorus* spp.) were recorded infesting beet in England, the Netherlands, Sweden, and Denmark (Andersson, 2018; Whitehead & Hooper, 1970). A race of the clover cyst nematode *Heterodera trifolii* was reported in 1970*,* on sugar beet in the Netherlands (Steele, 1984), it was later described as *Heterodera betae* (Wouts et al., 2001). Root lesion nematodes, *Pratylenchus* spp. have also been isolated from sugar beet fields but are rarely found to cause any above ground symptoms in the crop, which is considered as a poor host (Westerdahl et al., 2023). The attack of sugar beet by *Meloidogyne* spp*.,* is temperature dependent. In temperate regions *M. hapla, M. naasi, M. chitwoodi,* and *M. fallax*, are the most dominant species infecting sugar beet (Moens et al., 2009), while in warmer regions such as the southern parts of USA, the

predominating species attacking sugar beet are *M. incognita* and *M. javanica* (Weiland & Yu, 2003). *Meloidogyne* species also have different multiplication rates in sugar beet, where *M. hapla* was found to reproduce more on sugar beet than *Meloidogyne chitwoodi*. The tolerance limit in sugar beet to *Meloidogyne chitwoodi* and *Meloidogyne hapla* was determined as 2.8 and 0.6 eggs and juveniles per cubic soil, respectively (Griffin et al., 1982).

In Iran, 37 species of plant-parasitic nematodes have been identified in sugar beet including *Helicotylenchus* spp. (spiral nematode), *Heterodera* spp. (cyst Nematode), *Meloidogyne* spp. (root-knot nematode), *Paratylenchus* spp. (pin nematode), *Pratylenchus* spp. (root-lesion nematode), *Paratrichodorus* spp. (stubby-root nematode), and *Tylenchorynchus* spp. (stunt nematode). (Karegar, 2006). In Idaho and East Oregon, the predominant sugar beet nematodes were identified as beet cyst nematode (SCN) (*Heterodera schachtii*), root-knot nematode (*Meloidogyne* spp.), and stubby-root nematodes (*Paratrichodorus* spp.) (Hafez, 1998). The beet cyst nematode (*Heterodera schachtii*) is recognized as economically important sugar beet pest globally, has been found in forty different countries and seventeen states in the US with estimated yield losses ranging from 10-80 percent (Hafez, 1998), and an annual loss greater than US$95 million in the European Union (Müller, 1999).The common species of *Heterodera* attacking sugar beet are *H. schachtii* and *H. betae*, also known as the white and yellow beet cyst nematode, respectively. The species *H. betae*, has higher temperature requirements and is mainly found in sandy soils and has been found in Netherlands, Sweden, Germany, Switzerland, and Italy Sometimes the two species occur together as mixed populations (Wouts et al., 2001). In east England, BCN is considered as an economically important pest on sugar beet (Wright et al., 2019). Under UK conditions, BCN can have two to three generations per year (Wright & Bowen, 2020), although beet sickness is uncommon in England due to a history of crop rotation with brassicas, where some are non- hosts for *H. schachtii* (Wright et al., 2019).

Yield losses by BCN can be severe (30–40% on susceptible cultivars) (Blok et al., 2018) and have been calculated at as high as £3.8 million per year in the UK (Wright et al., 2017). However, the existence of tolerant modern sugar beet cultivars has minimized the yield losses, even though the BCN levels still build up (Wright et al., 2019). The stem nematode, *Ditylenchus dipsaci*, also attacks sugar beet seedlings causing galling, distortion of the petioles, midribs, and bloating. The symptoms are only visible in the autumn where they appear as cankers. Damage by *D, dipsaci* is seldom recorded in England, but in other EU countries it is a serious pest (Lane, 1999). In sugar beet infested fields with *D. dipsaci*, sugar content and root yield can be reduced by 50% or more (Hillnhütter et al., 2011).

Ditylenchus dipsaci is endoparasitic and produces fourth stage juveniles that can survive long periods of desiccation by clumping together into a “nematode wool” (IPPC, 2016). They multiply under favorable conditions and may have up to six generations in a year (Storelli et al., 2021). Wounds created in the hypocotyl by *D. dipsaci* create entry points for other pathogens such as *Rhizoctonia solani* and *Verticillium alboatrum,* leading to rotting of the crown later in the season. The disease complex between *Rhizoctonia solani* and *Ditylenchus dipsaci,* leads to extensive damage, as the fungus opportunistically penetrates the plant through wounds caused by the nematode, thereby saving energy on production of enzymes for lysis of the cellular barriers and therefore enabling it to easily attack the plant (Hillnhütter et al., 2011; Westerdahl et al., 2023).

Stubby root nematodes, *Trichodorus* and *Paratrichodorus* spp. (family Trichodoridae), have been reported to cause damage in sugar beet known as Docking disorder where the root system is

fangy, the tap root stops growing (Hafez, 1998). In the sandy soils in East of England, SRN attack sugar beet seedlings causing stubby/fangy appearance overall (Winfield & Cooke, 1975). Table 1.2 gives a summary of damage symptoms caused by PPNs on sugar beet.

Table 1.2: Damage symptoms caused by plant parasitic nematodes on sugar beet crop (Westerdahl et al., 2023).

Nematode species	Common name	Symptoms of sugar beet	References
Ditylenchus dipsaci	Stem and bulb nematodes	Swelling of the hypocotyls and distortion of the cotyledons	Hillnhütter et al., 2011
Heterodera schactii	Beet cyst nematode	Above ground: Yellowing and chlorosis on the leaves. Below ground: Bearding of the main root, where excessive amounts of extra roots are Formed	Muller, 1999
Naccobus abberans	The false root knot nematode	Above ground: Stunting and chlorosis Below ground: Galling of roots	Inserra et al., 1983
Meloidogyne spp. *(M. naasi), (M. hapla)*	Root knot nematodes	Above ground stunting and wilting of the beets	Lane et al., 1999
Longidorus. elongatus, *L. attenuatus*, and *L. macrosoma*	The needle nematode	Below ground: Causes destruction of root tips and lead to formation of hook-like galls	Andersson, 2018
Paratylenchus spp.	The pin nematode	Feeding on the taproots causing forking as other ectoparasitic nematodes on sugar beet	Ghaderi, 2019
Trichodorus and Paratrichodorus spp.	Stubby root nematodes	Above ground: Stunting and chlorosis of leaves Below ground: Thickening of lateral roots and death of tap root (Fangy root system).	White head 1970

1.4 Stubby root nematodes (SRN).

1.4.1 Taxonomy and classification of SRN.

The family Trichodoridae Thorne 1935, is composed of 109 species, plus one subspecies and six genera (Table 1.3): *Trichodorus* Cobb, 1913 (64 valid species), *Paratrichodorus* Siddiqi 1974 (26 valid species), *Nanidorus* Siddiqi, 1974 (seven valid species), *Monotrichodorus* Andrássy, 1976 (4 valid species), *Allotrichodorus* Rodriguez-Montessoro, Sher & Siddiqi, 1978 (six valid species) and *Ecuadorus* Siddiqi, 2002 (two valid species), (Asghari et al., 2018). Previously the family only contained one genus, *Trichodorus* sensu lato which was split into genera, *Trichodorus* and *Paratrichodorus* Siddiqi (1974). The genus *Trichodorus* was established by Cobb in 1913, when he described the species *Trichodorus obstutus*. The genus *Paratrichodorus* was also subdivided into three subgenera *Paratrichodorus, Atlantadorus* and *Nanidorus*.

Monodelphic species, were included in two new genera: *Monotrichodorus* for species closely related to *Trichodorus*. and *Allotrichodorus* Rodriguez-M., Sher & Siddiqi, 1978 for species closely related to *Paratrichodorus* (*Atlantadorus*) (Decraemer, 1980). *Dorylaimus primitivus* de Man 1880, was transferred to the genus *Trichodorus* spp. and *Trichodorus obstutus* made a synonym of *Trichodorus primitivus* Micoletzky, (1922) (Winfield & Cooke, 1975). They are didelphic-amphidelphic genera meaning that they have two genital tubes, and their uteri are opposed hence the vulva is located near the mid-body. *Monotrichodorus, Allotrichodorus* and *Ecuadorus* are monodelphic–prodelphic meaning that they have one anteriorly directed genital tube, and the vulva is located at 60% of the total body length. (Decraemer & Geraert, 2013).

Table 1.3: Taxonomic classification of stubby root nematodes (Decraemer & Robbins, 2007).

Phylum:	Nematoda
Class:	Enoplea
Subclass:	Enoplia
Order:	Triplonchida
Suborder:	Diphtherophorina
Superfamily:	Diphtherophoroidea
Family:	Trichodoridae
Genera:	*Trichodorus* Cobb, 1913, *Paratrichodorus* Siddiqi, 1974, *Monotrichodorus* Andrassy, 1976, *Allotrichodorus* Rodriguez-M, Sher and Siddiqi, 1978 and *Ecuadorus* Siddiqi, 2002.

1.4.2 Habitus and morphological characteristics.

Nematode species in the family Trichodoridae are small (0.5 to 1.5 mm long), cylindrical nematodes tapering at the anterior end. The body is thick, and males and females are short and bluntly round (Allen, 1957). They often have 'cigar shaped' bodies, especially males and females of *Paratrichodorus* and *Allotrichodorus* spp. The males of genera *Trichodorus* and *Monotrichodorus* are clearly ventrally curved (Decraemer, 1995). The cuticle is smooth and marked by lines or punctuations and described as a three-layered cuticle with thin outer layer, thick middle layer and thinner inner layer under a light microscope. Transmission electron microscopy has, however, revealed eight layers in the cuticle of *Paratrichodorus allius* (Decraemer, 1995). The oesophagus is tubular and slender, expanding gradually to form a conoid swelling at the base. The feeding structure known as onchiostyle is described as hollow, slender, and tripartite; it is not fully hollow in its length as there is a muscular sheath that lies in the base, which surrounds the new onchiostyle produced when the old one molts (Allen, 1957). The secretory excretory (SE) pore is present in all known species. Posterior location of the SE is regarded as an important diagnostic feature differentiating subgenus or genu (Decraemer, 1995). All known males and females have one pair of subterminal caudal pores (Allen, 1957; Decraemer, 1995), except for *P. weischeri* with two pairs located terminally or sub terminally. All species of *Trichodorus, Paratrichodorus, Monotrichodorus*, and *Allotrichodorus* are bisexual and have a spermatheca (Decraemer, 1995). In lateral view, the vulva varies in shape, where it may either be a pore, a transverse slit or a longitudinal slit. The vulva has been used as a distinguishing feature especially in the subgenera *Paratrichodorus* (small longitudinal slit), *Antlantadorus* (pore-like) and *Nanidorus* (small transverse slit). (Siddiqi, 1980). SRN males are monorchic meaning that they possess a pair of outstretched testes. Spermatids are stored in the *Vesicula seminalis* which is located posteriorly in the testis. The diagnostic features used to separate some genera are highlighted in Table 1.4 below aswell as the morphological features of species reported in the United Kingdom.

Table 1.4: Characteristics shared by the genus *Trichodorus* and *Paratrichodorus* spp. (with respective %) and the morphological characteristics of species reported in the United Kingdom (Allen, 1957; Hooper, 1962; Winfield & Cooke, 1975).

Body length	26% - 43%
Onchiostyle length	41% - 56.5%
Position of excretory pore	20.5% - 34.5%
Shape of vaginal sclerotization	64.5% - 21.5%
Spicule: -length	26.5% - 47.5%
-Shape	64.5% - 21.5%
-ornamentation	23.5% - 21.5%
Precloacal supplementary papillae: -number	35% - 34.5%
-position	73.5% - 34%
Number of ventromedian cervical pores	70.5% - 30%
Characters used only in one or the other genus (with respective %)	
Trichodorus spp.	*Paratrichodorus* spp.
Position of pre-cloacal papillae in relation to spicule	Tail shape: 30%
Shape of gubernaculum	Ventral overlap of pharyngeal glands :43%
Number and position of lateral body pores in female	Ventral view of shape of vulva :43%
Number of Ventro median cervical pores	Presence of males: 21.5%
Position of ventromedian cervical pores in relation to the excretory pore	Number of post cloacal papillae: 26%

Morphological characteristics of species in the United Kingdom and their co-occurrence.

Species	Male Features	Female Features	Co-occurring Species
T. primitivus	Three ventromedian cervical papillae; three tail supplements; unique papillae positions	Longer tail; ventral caudal pores; three lateral hypodermal pores near vulva	*T. cylindricus, T. similis, P. pachydermus*
T. cylindricus	Caudal alae, striated spicules, distinct spicule, and gubernaculum shape	Distinct vaginal shape; cutinized vulval structures; one pair of lateral hypodermal pores	*T. primitivus, T. teres*
T. similis	Two well-developed supplementary papillae, pronounced spicules, dorsally oriented gubernaculum	One pair of lateral pores; distinct vulval cutinized ring shape	*T. primitivus, P. pachydermus*
T. viruliferus	Three ventral cervical papillae (equidistant); spicule with narrow bend; longer keel-shaped gubernaculum	Thickened vulval pieces; distinct vagina shape; anterior excretory pore; oesophageal overlap	*T. primitivus, T. similis*
T. teres	Pronounced oesophagus-intestine overlap; longer body/onchiostyle	Longitudinal vulval slit; distinct vagina and vulval cutinized structure; anterior excretory pore	*T. cylindricus*

1.4.3 Distribution and occurrence of SRN.

Stubby root nematodes (SRN), mostly inhabit sandy soils; clay and silt particles are said to inhibit movement of trichodorids, (Winfield & Cooke, 1975). The ability of the nematode to move is dependent on the size of the soil pore as well as the free path, i.e., the distance a nematode can move without distraction; this is mostly important for longer nematodes like members of the Longidoridae. For shorter nematodes, like trichodorids, the body diameter is key as the nematodes are plump, which explains the relationship between the adult body diameter and their occurrence in coarse sandy soils where the pore size allows the nematode to move freely (Winfield & Cooke, 1975). The juveniles of *Heterodera* and *Trichodorus* and the early juvenile stages of Longidorus average 20 µm in diameter and are therefore unable to penetrate densely packed soils consisting only of clay, silt or the finer fractions of fine sand with particle diameters less than 50 µm (Jones et al., 1969). Evidence of the nematodes' ability to move in sandy soils with larger pore sizes was shown in experiments conducted using four different grades of sand and movement assessed along an 8.5 cm long glass tube. The distance travelled by the nematodes greatest in the 200-400µ sand fraction and slightly less in the 100-200 µ and 400-800 µ fractions; the least movement was in the 800-1400 µ fraction (Winfield & Cooke, 1975). Mechanical analysis of ten soils obtained from East Anglia in which Trichodorus nematodes were found to be abundant, had a profile of 32-60% coarse sand, 22-42% fine sand, 6-12% silt and 7-12% clay, however the two species T.primitivus and P.pachydermus have been recovered in clay soils , indicating that the two species can occupy diverse soil habitats (Seinhorst, 1963) In a survey conducted to determine distribution of SRN in the British Isles, SRN were mainly found (50% of infested sites) in soils with a sand fraction greater than 80% and a less than 10% silt. The remainder of the sites with SRN had sandy loamy soils. No SRN were obtained in clay or silt soils (Alphey & Boag, 1976).

In 98 fields with light sandy soils from eastern England, P. pachydermus Seinhorst occurred in thirty-five, T. primitivus (de Man) in twenty-nine, T. viruliferous Hooper in thirteen, T. similis Seinhorst in nine, T. cylindricus in eight and T. teres Hooper and T. anemones in two each, showing the wide distribution of *Trichodorus* spp. in sandy soils (Whitehead & Hooper, 1970). *Trichodorus velatus* was isolated from sandy soils with Sitka spruce seedlings and herbaceous plants while *Trichodorus variopapillatus* was isolated from moist sandy soil, planted with elder (Sambucus nigra L.). *Trichodorus hooperi* was isolated from sandy loam soils in mixed conifer woodland with herbaceous undergrowth (Loof, 1973).

In Scotland, SRN were detected in 75% of potato fields although spraing (Tobacco rattle virus), which is transmitted by trichodorids, was not detected in fields with high clay content. However, spraing was constantly detected in fields where certain potato varieties had been grown in sandy soils. The frequency of occurrence of *P. pachydermus* and *T. primitivus* varied on different soil series with *P. pachydermus* being found in ridges and raised beach soils, while in fluvioglacial soils it occurred only in the Boyndie series and was absent in till soils. *Trichodorus primitivus* on the other hand, occurred in raised beaches, till alluvial and fluvioglacial soils and rarely exceeded 50 nematodes in 200 grams of soil (Cooper, 1971).

In sites in East England where Docking disorder of sugar beet occurred, the soil had lower silt and clay fractions; Trichodorus spp. were recovered from 75% of soil samples (Cooke, 1973). In potato fields located in East and West Flanders of Belgium, *T. primitivus, T. similis* and *P. pachydermus* was isolated from sandy loam soils. However, T. primitivus was the most abundant

and widely distributed species found in this region (Decraemer et al., 1979). However, very coarse sand may limit distribution of *Trichodorus* spp. as reported in a study where the roots of *Ammophila arenaria* and *Desmoschoenus spiralis* were sampled in sand dunes; *Trichodorus* spp. were not found due to the very course nature of the sand (Yeates, 1967).

In Great Britain, different trichodorid species occur in some counties and are absent in others. For instance, *T cylindricus* Hooper, 1962, *T. viruliferous* Hooper, 1963, *T. similis* and *P. teres* Hooper, 1962 are the most common in the eastern counties. Species like *Paratrichodorus nanus* has only been recorded in Scotland whereas *T. hooperi* is restricted in the southwest of England. On the other hand, *T. primitivus* was reported as the most prevalent and cosmopolitan species occurring in majority of the sites followed by *P. pachydermus* (Alphey & Boag, 1976).

The prevalence of *T. primitivus* and *P. pachydermus* was also recorded in Eastern England, in sugar beet fields, where out of seven species of SRN reported, 35% were *P. pachydermus*, 30% were *T. primitivus*, 13% *T. viruliferous*, 9% *T. similis* Seinhorst, 8% *T. cylindricus*, 2% *T. teres* Hooper and 2% *T. anemones* (Whitehead & Hooper, 1970). The frequent occurrence of *T. primitivus* and *P. pachydermus* has also been reported in a soil sampling exercise conducted to determine the distribution of tobacco rattle virus and virus vector nematodes in Angus, Banff, Berwickshire and Kincardineshire in Scotland. *Trichodorus* spp was recorded in 133 out of 153 sites sampled. *Trichodorus primitivus* and *T. pachydermus* were the most predominant species, occurring in about 100 soils while *T. nanus* was recorded in only five sites. Although five *Trichodorus* spp. were found in Scottish soils, *T. nanus, T. cylindricus* were rare; the density of nematodes was low, and populations were restricted to soils in which commercial potato crops were rarely grown (Cooper, 1971). In studies conducted to determine the factors affecting Docking disorder in sugar beet in England, *Trichodorus* spp were found to be widely distributed, where they were recovered from 75% of samples collected (Cooke, 1973).

The vertical and horizontal distribution of SRN species in soil can vary enormously. Unlike other nematode genera, the distribution is influenced largely influenced by environmental factors such as soil moisture. When soil is at field capacity, the numbers of SRN can significantly increase especially when there is an abundance of host plant roots. Stubby root nematodes tend to move deeper in the soil during dry conditions, where population densities can decrease rapidly due to their high susceptibility to desiccation (Winfield & Cooke, 1975).

A total of fifteen field studies evaluating management of SRN and other plant parasitic nematodes, low numbers of SRN were recovered from 0-5cm depth, however when the soil was wet in rainy months, a greater number was recovered in the topsoil layer indicating a positive correlation between high numbers observed with accumulated rainfall (Cooke & Draycott, 1971). The ability of SRN to move when moisture levels are optimum was also experimentally demonstrated in a study conducted at four different moisture regimes, where highest numbers were recorded when soil pores were half full of water and least when soil was dry or waterlogged (Bor & Kuiper, 1966; Winfield & Cooke, 1975).

Under field conditions, sandy soils are free draining and unlikely to be subject to waterlogging for a sustained period. The topsoil in sandy soil dries out as water percolates deeper through the soil profile. Experiments investigating the effect of soil drying on PPNs population densities also reported that *Trichodorus* spp were more susceptible to desiccation than *Rotylenchus* spp and *Pratylenchus* spp (Rössner, 1971). Similar studies showed that the family Dorylaimida was more susceptible to high desiccation and osmotic stress when compared to family Tylenchida (Wyss,

1970). The free-draining characteristics in sandy soils also leads to leaching of nutrients such as nitrogen and manganese which are essential in root growth and development, and soils depleted in these elements have been associated with more Docking disorder symptoms and high densities of SRN (Whitehead & Hooper, 1970). Deficiency in copper has also been associated with distribution and occurrence of *Trichodorus* spp in different soils where *P. pachydermus* was shown to be sensitive to high copper and manganese exposure and had higher prevalence in copper deficient soils. On the other hand, *T. primitivus* was shown to be tolerant to high levels of copper and explains why this species is widely distributed in diverse environments compared to other SRN species (Cooper, 1971).

The vertical distribution of SRN is also determined by the species in question. Findings by Richter (1969), in Germany, showed that the males of *P. pachydermus* were found in deeper soil layers as compared to males of *T. viruliferous*. Similarly, the number *Trichodorus teres* was lower at 15-30 cm depth and increased deeper below 30cm (Kuiper & Loof, 1962). On the contrary, studies in England found no clear difference in the depth where *T. cylindricus* or *P. anemones* were found in the topsoil of infested sugar beet fields, except for one field where fewer *P. cylindricus* were found at 0-5 cm depth (Whitehead & Hooper, 1970).

1.4.4 Survival and Life cycle.

Soil moisture is an important factor that affects the survival of trichodorids. Increased nematode densities have been observed in wet months in England. A positive correlation was recorded in severe damage observed in young sugar beet seedlings and the high total rainfall in the month of May (Cooke, 1973). Stubby root nematodes are also very sensitive to mechanical injury associated with sampling and handling (Bor & Kuiper, 1966). This was shown in a study where soil carefully transported from field to laboratory yielded more *Paratrichodorus teres* (2240 nematodes l^{-1} soil) as compared to soil sent via post in a cardboard box which yielded 628 nematodes l^{-1} soil. The reduction was attributed to manual handling during transportation resulting in the death of a high proportion of nematodes.

In the same study, the diameter of the sampling auger was also shown to influence the numbers of SRN recovered during a sampling exercise. Sampling with a 10cm corer diameter yielded 2540 *P. teres* l^{-1} soil while 2cm and 1cm corer yielded 580 and 390 *P. teres* l^{-1} soil respectively; this was explained by the fact that a narrow corer exerts more mechanical pressure during sampling compared to a wider diameter corer. Finally, the effect of soil sample handling on the survival of the nematodes was also investigated in this study, where the effect of dropping soil from certain heights and mixing the soil was investigated. Significantly higher numbers of nematodes died when soil samples were dropped from 100-350 cm as compared to the control where soil was not dropped. In contrast, mixing of the soil did not cause significant mortality when compared the control (no dropping/mixing) (Bor & Kuiper, 1966).

Sensitivity of the nematodes to chemical compounds has also been shown in experiments using $CuSO_4$ or $MnSO_4$. Variability in nematode sensitivity upon exposure to three different concentrations, depended on the species. In this experiment, *P. pachydermus, T. cylindricus, T. primitivus* and other soil nematodes were compared. Results showed that SRN were more sensitive than other parasitic nematodes as none of them were mobile after 36 hours exposure time. *P. pachydermus* was the most sensitive to copper and manganese while *T. primitivus* was the least sensitive. The most copper sensitive species (*P. pachydermus*) was found to mainly occur in calcium and manganese deficient soils. However, further field tests to evaluate the

sensitivity of *Paratrichodorus* spp. by artificial application of cupric and manganous sulphate, did not reduce the densities of *Paratrichodorus spp* 16 months after application (Cooper, 1971). The lifecycle of SRN has four juvenile stages and an adult stage (male/female)— (Figure 1.2).

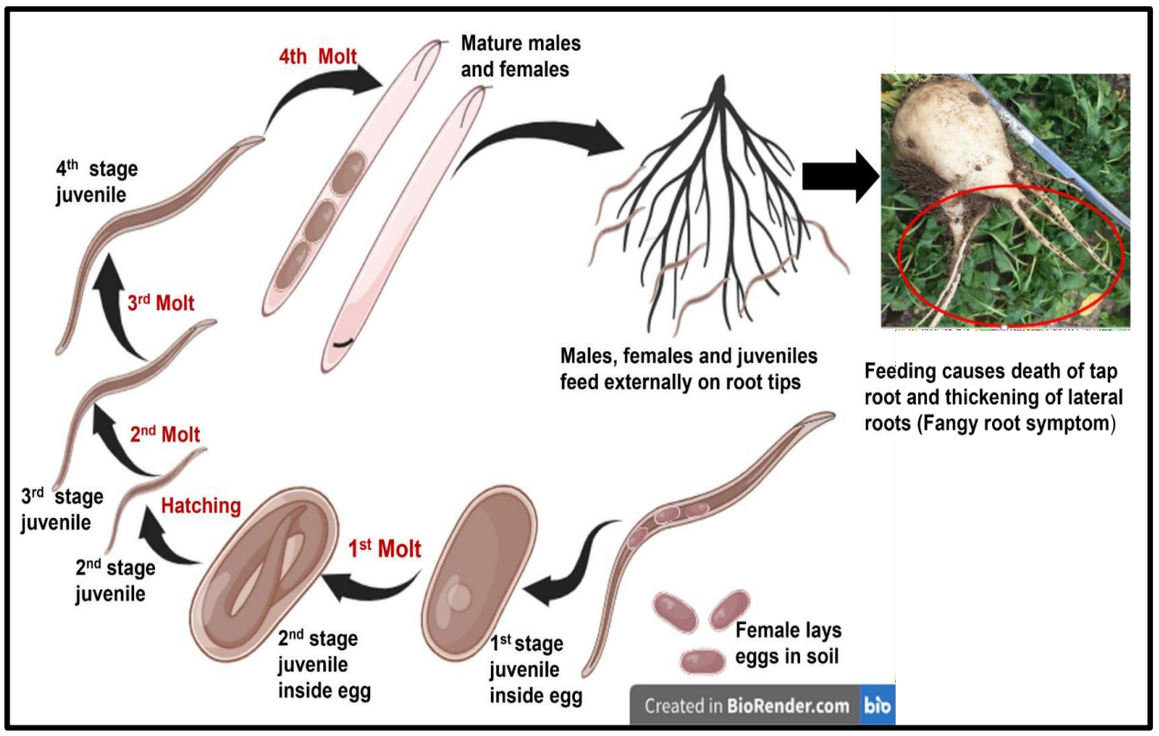

Figure 1.2: The lifecycle of stubby root nematodes (Created with BioRender.com).

The eggs are laid by the female in the soil. Embryogenesis studies for *Paratrichodorus christie* showed that the egg is laid in a single cell stage, and the first two cleavages are transverse and longitudinal. First juvenile stages occur after 96 hours while emergence from the egg is seen at 100-120 hours after being laid (Bird et al., 1968). The lifecycle takes 3-7 weeks depending on prevailing temperatures and the species involved. Optimum temperature for reproduction and development ranges from 16-24°C depending on the species and development of *Trichodorus* spp. Has been shown to be inhibited at 35°C (Rohde & Jenkins, 1957).

The overall length of the life cycle of *Trichodorus viruliferous* was recorded in laboratory experiments using apple seedlings grown in cylindrical tubes where 30 gravid females and 20 males were inoculated. Eggs and juveniles were first observed 5 and 19 days respectively after inoculation. After 35 days, the medium size juveniles were more predominant, while the first adults appeared after 45 days (Pitcher & Mcnamara, 1970).

The proportion of actively breeding females with developed oocytes of *T. viruliferous* increased rapidly on inoculation experiments with apple seedlings, this was however not the case of *T viruliferous* from field soil samples collected from a mature apple orchard, which was mostly composed of non-breeding females with undeveloped oocytes. Comparisons made between the roots of apple seedlings used in laboratory experiments and roots of established apples in orchards, showed that the fine fibrous root system found in young apple roots provided a more efficient substrate than the fine feeder roots found in mature apple trees (Pitcher & Mcnamara, 1970). Similar experiment showed that population increase of *Paratrichodorus christie* followed a sigmoid growth pattern when grown in pots with seedlings of *Lactuca sativa* (lettuce) where

populations densities doubled from 45 days to 90 days (Bird et al., 1968; Sykes & Brown, 1971). The degree of attractiveness of roots to SRN determines the densities found in the soils taken near the root system (Whitehead & Hooper, 1970).

1.4.5 Host range and damage symptoms.

The damage and subsequent economic importance of SRN to crops were first reported by Christie and Perry who described it as *Trichodorus christie* and named it stubby nematode due to the symptoms it caused to plant roots (Christie & Perry, 1951). Similar damage symptoms observed in other species now apply to all members of the genera *Trichodorus* and *Paratrichodorus* spp. (Winfield & Cooke, 1975). They feed externally as they are ectoparasitic and adhere closely to the roots causing injury to the root tips and stunting root growth (Christie & Perry, 1951; Whitehead & Hooper, 1970). The feeding is divided into four phases: exploration, penetration, salivation, ingestion, and withdrawal. During exploration, the lips of the nematode rub the plant cell wall to find a suitable cell. Once a suitable cell has been located, the lips are pressed against the cell wall and penetration involves several thrusts of the onchiostyle. After penetration, the rate of thrusting drops and the cell cytoplasm streams and accumulates into the feeding site. At this point, the nematode ingests large volumes of cytoplasm through retraction of the onchiostyle (Figure 1.3).

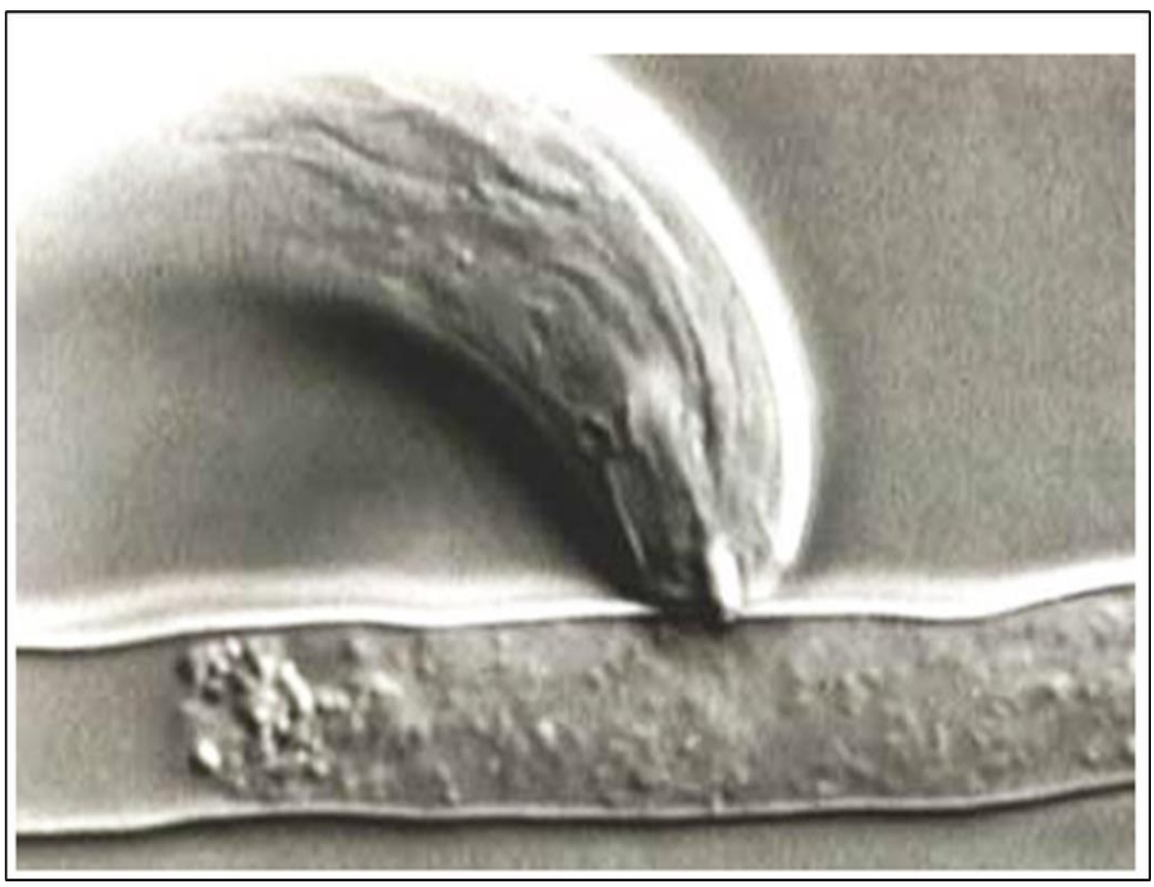

Figure 1.3: Stubby root nematode feeding on a root hair via a feeding tube. photograph (Wyss, 981), institute of phytopathology, Germany.

After ingestion of the cytoplasm, the nematode withdraws the onchiostyle, leaving behind a feeding tube attached to the cell wall (Wyss, 1981). Rasping during feeding has been reported for *P. minor* (Rohde and Jenkins,1957, Russell and Perry 1966). *Trichodorus. similis* was

observed feeding on up to 15 cells in an hour where it fed on an individual cell for few minutes and moved to the next cell. Males and females fed the longest with feeding times of 2h and 50 mins respectively (Wyss, 1981). Stubby root nematodes tend to aggregate, as has been observed with *Trichodorus viruliferous* on apple trees via direct observations and cinematography. The close adherence and aggregation of SRN has been observed to occur around the elongating zone of young roots (Pitcher, 1968). In young sugar beet seedlings monitored in East England, *Trichodorus* spp. were also reported to aggregate near the young roots (Whitehead & Hooper, 1970). Damage mainly occurs on the epidermal tissues of young seedlings, preventing the growth or causing the death of the tap root. The tap root may be replaced by lateral roots occurring near the surface, which thicken resulting in a poorly yielding misshapen (fangy) root at harvest. (Whitehead & Hooper, 1970). At Gayton, Thorpe, England, *Trichodorus* spp. especially *T. cylindricus* or *P. pachydermus* were common mostly around young seedlings (1500/Litre soil) than around large plants (600/Liter soil) (Whitehead & Hooper, 1970). The stubby lateral roots later turn grey- brown and then black as they die and decay (Christie & Perry, 1951).

As a result of damage to the root system, plants are unable to absorb enough nutrients, and the leaves may show symptoms of nitrogen or magnesium deficiency (Cooke, 1989; Whitehead & Hooper, 1970; Winfield & Cooke, 1975).The continuous thrusting and withdrawal of the stylet causes large brown lesions to form, while repeated feeding at the root tip inhibits further growth and leads to the formation of browning (Decraemer & Robbins, 2007). *Trichodorus proximus* was found to cause chlorosis to St. Augustine grass and reduce the plants root weight. An examination of infected roots by *T. proximus* showed that lesions were irregular in shape and were deeper in the root tissues (Rhoades, 1965). A study on the pathogenicity of *P. minor* on onions showed that symptoms were linked to a longitudinal and radial increase of the cortex and that earlier damage may have resulted from abnormal cell maturation near the apical meristems (Hoff & Mai, 1962). Although shallow root systems are associated with feeding damage caused by *Trichodorus and Paratrichodorus* spp, the effects differ because of the occurrence of secondary pathogens or soil conditions. Above ground, SRN infected crops may have a patchy appearance due to the stunted roots; weakly affected plants may recover resulting in a 'hen and chick' effect in the field (Winfield & Cooke, 1975).

The family Trichodoridae are polyphagous in nature, attacking crops from diverse plant families (Table 1.5). Host preference varies for different *Trichodorus* and *Paratrichodorus* spp., hence knowledge of species present and the host status of the crops in the rotation can assist in maximizing the yields of susceptible crops (Ayala et al., 1970). SRN densities are influenced by the previous crop or sequence of crops in a rotation, due to host preference and suitability for multiplication (Winfield & Cooke, 1975). In a series of rotations testing the effects of Bermuda grass or bahia (*Paspalum notatum*), *Paratrichodorus minor* was slightly favored by a continuous row-crop (cotton-maize-peanut) rotation where cotton and maize increased numbers while peanuts suppressed the densities. Low densities of SRN were recorded in a rotation sequence where cotton and maize did not follow each other after a grass ley (Andersen et al., 2016). Susceptibility of hosts differs with SRN and variations have even been reported within species from different populations. For instance, different isolates of *P. minor* exhibited different host-preferences in a host range test. Peas and spinach were rated as good hosts for *P. minor* Riverside isolate, while they were poor hosts for *P. minor* - Florida isolate. Cabbage was also an excellent host for the Florida isolate while it was ranked a good host for the Riverside isolate (Ayala et al., 1970).

Table 1.5: Host status of crops to different genera and species of Stubby root nematodes (SRN).

Host crop	*Paratrichodorus pachydermus*	*Paratrichodorus teres*	*Trichodorus primitivus*	*Trichodorus similis*	Reference
Beans	Excellent host	Moderate host	Excellent host	Unknown	https://www.best4soil.eu/database based on research from Wageningen University and research | Field crops, Lelystad.
Black salsify	Poor host	Moderate host	Unknown	Moderate host	
Cabbage (Incl. Cauliflower and Broccoli)	Unknown	Excellent host	Excellent host	Unknown	
Carrot	Moderate host	Moderate host	Moderate host	Poor host	
Chicory	Moderate host	Moderate host	Unknown	Moderate host	
Leek	Unknown	Poor host	Unknown	Poor host	
Onion	Non-host	Moderate host	Excellent host	Unknown	
Spinach	Excellent host	Poor host	Poor host	Unknown	
Italian ryegrass	Excellent host	Excellent host	Excellent host	Excellent host	
Perennial ryegrass	Excellent host	Excellent host	Excellent host	Excellent host	
Persian reversed clover	Unknown	Poor host	Unknown	Unknown	

Host crop	*Paratrichodorus pachydermus*	*Paratrichodorus teres*	*Trichodorus primitivus*	*Trichodorus similis*	Reference
Phacelia	Moderate host	Unknown	Poor host	Unknown	
Radish	Moderate host	Poor host	Excellent host	Moderate host	
Vetch	Unknown	Unknown	Excellent host	Unknown	
White clover	Unknown	Excellent host	Unknown	Unknown	
White mustard	Excellent host	Unknown	Excellent host	Excellent host	

Host crop	*Paratrichodorus minor* (Riverside isolate)	*Paratrichodorus porosus*	*Paratrichodorus allius*	Reference
Tomato	Excellent host	Good host		Ayala et al., 1970
Potato			Good host	
Sweet potato			Good host	
Tobacco	Non-host	Non-host		
Red pepper	Non-host	Non-host		
Egg plant	Excellent host		Excellent host	
Garden pea	Good	Good host	Excellent host	
Alfalfa	Excellent host	Excellent host	Good host	
Cowpea	Excellent host	Good host	Poor host	
Broad bean	Excellent host			
Bean, Bountiful	Excellent host			
Sweet pea	Excellent host		Excellent host	
Baby lima beans	Poor host			
White clover			Good host	
Ladino clover	Excellent host			
Upland cotton	Excellent host	Excellent host	Excellent host	

Host crop	*Paratrichodorus minor* (Riverside isolate)	*Paratrichodorus porosus*	*Paratrichodorus allius*	Reference
Okra	Excellent host			Ayala et al., 1970
Celery	Good host		Excellent host	
Carrot	Poor host	Good host		
Garden radish	Poor host		Poor host	
Peach		Poor host		
Strawberry			Excellent host	
Cherry		Good host		
Common apple		Good host	Excellent host	
Pear		Poor host		
Rye			Excellent host	
Wheat			Excellent host	
Sudan grass			Good host	

1.4.6 Docking disorder in sugar beet.

Damage caused by SRN can either be direct or indirect. Directly, SRN feed on roots causing mishappening of the roots and ultimate yield reduction (Christie & Perry, 1951). Indirectly, they can transmit three viruses belonging to the tobravirus group namely tobacco rattle virus (TRV) (Alphey & Boag, 1976), pea early browning virus (PEBV) (Hoff & Mai, 1962) and pepper ringspot virus (PRV) (Asghari et al., 2018; Gibbs & Harrison, 1963). All SRN life stages can transmit viruses and virus particles are selectively absorbed in the oesophagus lining. Dissociation occurs when the nematode saliva is injected into the host, without killing of the cells for the transmission to be successful. However, juveniles lose the virus after every moult. Therefore, juveniles need to acquire viruses to be able to transmit again while adults can retain the virus for a long period (Cooper, 1971).

In sugar beet in East England, *Trichodorus and Paratrichodorus* spp. attack young seedlings causing a condition known as Docking disorder, which later leads to foliage appearing to be deficient in nitrogen or magnesium (Cooke, 1989; Cooke et al., 1985; Whitehead & Hooper, 1970). Docking disorder is named after the parish where it was first recognised and described by Gibbs (1959). In studies done in 1963 and 1964, investigations on nematode transmitted viruses were conducted to establish whether the viruses were involved in the Docking disorder outbreaks. Studies conducted later by Whitehead et.al, (1970) established that nematodes in the genus *Trichodorus* and *Paratrichodorus* spp. were involved in stunting of sugar beet in fields with light sandy soils in Docking. Subsequent studies have shown that SRN i.e. *T. primitivus* and *P. pachydermus* are more prevalent in fields exhibiting Docking disorder (Cooke, 1989). Similar damage has also been reported from the Netherlands, where it is known as T-disease (Kuiper & Loof, 1962).

Docking disorder causes stunting of sugar beet in late May or early June. Affected beets may have symptoms of magnesium or nitrogen deficiency. The crops may recover through autumn/summer but the resulting roots at harvest are often fangy/misshapen. Where Docking disorder occurred, sugar beet roots presented more fanging and produced 17.5 t/ha less than those from unaffected fields (Cooke, 1973) — (Table 1.6).

Table 1.6: The proportion of the sugar-beet crop reported to be affected by Docking disorder for each factory area in England between 1967-1972; the areas from 1968 onwards are those reported affected in June (the month in which most Docking disorder is usually apparent). Source: Cooke, 1973.

	Estimated root yield loss (tonnes)					
Factory	1967	1968	1969	1970	1971	1972
Allscott	345	87	486	0	2428	75
Bardney	0	0	0	0	0	0
Brigg	1224	61	1828	0	0	364
Bury St. Edmunds	2715	372	5133	24	668	17
Cantley	323	0	1369	18	0	6
Cupar	0	0	0	0	0	0
Ely	24	0	97	0	49	0
Felsted	0	0	0	0	0	0
Ipswich	182	0	3096	10	243	0
Kidderminster	215	146	983	12	0	73
Kings Lynn	6313	389	11244	0	0	304
Newark	516	117	1748	0	0	0
Nottingham	0	109	5924	7	486	231
Peterborough	0	0	0	0	0	0
Selby	1942	560	4128	546	0	504
Spalding	5	19	7	0	0	0
Wissington	431	121	2556	32	237	182
York	4272	206	6167	0	73	129
Total	18507	2187	44766	649	4184	1885

A yield loss of up to 50 % has been recorded due to the fangy root symptoms where there is complete loss of the tap root and thickening of the lateral roots because of Docking disorder. Severity of the disorder is often worsened by environmental factors such as rainfall, previous cropping, physical conditions of the soil, rate and timing of fertilizer application/herbicides and other agricultural practices. Increased accumulated rainfall is ideal for the movement of SRN into the rhizosphere where they multiply and cause more damage to the roots. The host status of the previous crop also influences the severity in that if the previous crop was a good host of SRN, the densities at sugar beet planting are higher and this leads to attack of the young seedlings which are more vulnerable (Cooke, 1973).

Root damage is mostly evident at the end of May, coinciding with higher rainfall, while the symptoms of the foliage i.e. symptoms of magnesium and nitrogen deficiency are mostly visible in June (Cooke, 1973). Seedlings attacked by SRN may show typical damage symptoms with stubby lateral roots, which turn grey- brown and later black as they die and decay. Any new roots formed during development are also attacked. Often the tap root stops growing or is killed and lateral roots near the surface thicken and replace it resulting in a poorly yielding misshapen (fangy) root at harvest (Figure 1.4).

Figure 1.4: Fanged root system of sugar beet (left) versus healthy sugar beet root system (right).

1.5 Management options for SRN.

The management of SRN previously relied on prophylactic use of pesticides, i.e., use of soil fumigants. The British Beet Research Organisation (BBRO) recommends assessment of SRN densities before application of any nematicide. The set threshold for management measures i.e., nematicide application is 1000 trichodorid per litre of soil. Application was in the past done either in the autumn before sowing or as a row application shortly after drilling sugar beet (Cooke & Draycott, 1971) using volatile nematicides such as 1,2-Dibromo-3-chloropropane (DBCP), 1,2-Dichloropropane and 1,3-Dichloropropene (D-D) or ethylene dibromide (EDB), which have now been banned in Europe and the USA. Non-volatile nematicidal compounds such as aldicarb, carbofuran, fenamiphos and oxymyl have also been used previously; these active substances inhibit acetylcholine esterase at nerve synapses, limiting the host finding ability of SRN and preventing virus transmission (Pelsmaeker & Coomans, 1987).

Previously, sugar beet crops at risk from Docking disorder were treated via row application of granular nematicides, usually aldicarb, at drilling but the expense and inconvenience of these techniques limited their use (Cooke, 1989). Application of aldicarb in direct contact with the seed was reported to cause phytotoxicity on young sugar beet seedlings, which decreased the seedling numbers especially in dry soils following drilling. (Cooke & Holden, 1975; Maughan et al., 1984). However, consistent soil fumigation as an overall treatment in the autumn prior to planting sugar beet increased yields in nematode affected fields (Cooke & Holden, 1975).

An annual survey by British Sugar carried out in 1985, suggested that there was no decrease in

area of sugar beet showing symptoms resulting from nematode damage despite use of nematicides (Cooke et al., 1985). Nematode damage on the roots was still evident post application of carbamates, as the reversible nature of carbamate effect means that nematodes resume feeding once the active ingredient has been degraded or leached from the rhizosphere (Steele, 1977).

In other studies, SRN have been shown to be less susceptible to soil fumigants compared to other parasitic nematodes such as sting nematodes (Grabau et al., 2019). Field experiments carried out for two years to evaluate the efficacy of soil fumigants EDB + chloropicrin, and 1,3-dichloropropene (1,3-D) when applied independently or in combination in comparison to aldicarb on potato, *Solanum tuberosum* cvs. Atlantic and Sebago, for control of trichodorid nematodes and potato corky ringspot disease (CRS), showed that soil fumigation was ineffective in management of CRS in northeast Florida (Weingartner & Shumaker, 1990).

The ineffectiveness has been attributed to the ability of SRN to recover post treatment with fumigants, for instance, *Paratrichodorus christie* was shown to recover more quickly compared to other parasitic nematodes post application of a fumigant (Christie & Perry, 1951). The rate of recovery has been shown to vary considerably, where in a study investigating efficacy of EDB, DD and DBCP fumigants, *P. christie* was shown to multiply more on cabbage four months post fumigation with DD or EDB as compared to DBCP which was lower as it had more residual action (Rhoades, 1969).

Successful chemical fumigation using dichloropropene in SRN management was reported from trials conducted in Yorkshire, Northern England and in Norfolk, Eastern England. In Yorkshire, 93% *Paratrichodorus anemones* were killed during winter of 1965-67 where they were fewer when compared to unfumigated plots. In Norfolk, no recovery of *Paratrichodorus. teres* and *T. cylindricus* were observed after fumigation and a 99% kill was achieved during the period of 1966-67. It was also shown that the re-establishment of *T. cyindricus* and *P. pachydermus* was slow following fumigation (Cooke & Draycott, 1971).

Combined use of abamectin and azoxybitron in field experiments aimed at the management of *Trichodorus obstutus* on zoysiagrass, showed that the root weight of treated plants increased by 0.50 and 0.81 g respectively compared to untreated controls. (Shaver et al., 2016). With the recent disapproval of the last remaining nematicide oxymyl for use in SRN management in the UK in December 2020, farmers have only NEMguard for use in SRN management. As such there is need for development of more eco-friendly management strategies for recommendations to sugar beet growers (Stevens, 2015).

1.5.1 **Management of PPNs using cover crops.**

Cover crop use in the United Kingdom.

The use of cover crops in UK field crop rotations has increased due to increased awareness on managing soils sustainably, with farmers and the UK government acknowledging the significance of soil in providing ecosystem services and food (Storr et al., 2019). This is inspired by extensive research that has demonstrated that cover crops can have positive effects on various aspects of soil condition, including soil structure (Munkholm et al., 2013; Tonitto et al., 2006), soil biology (Reeleder et al., 2006; Roarty et al., 2017), soil erosion control (Magdoff & van Es, 2000), and nutrient management (Cooper et al., 2017; Wendling et al., 2016).

Sustainable management of soils and eradication of soil degradation by 2030, was proposed by

the "Safeguarding our Soils in England" strategy (DEFRA, 2009). The main soil challenges in England are soil erosion, compaction, loss of organic matter (DEFRA, 2009), hence DEFRA has continued to focus on achieving a sustainable, dependable and profitable food system while conserving the environment (AHDB, 2018; DEFRA, 2018). Cover crops stand out as a viable tool to address these challenges because they increase the soil organic carbon, increase available nutrients, preserve soil moisture due to reduced soil water evaporation and enhance nutrient cycling (Maetens et al., 2012; Posthumus et al., 2015; Williams & Weil, 2001).

The Common Agricultural Policy (CAP) was reformed in 2013 (Zinngrebe et al., 2017), and from 2015, the "Greening Measures" incentivized the use of cover and/or catch crops within Ecological Focus Areas (EFA) in the Basic Payment Scheme (BPS). The Rural Payments Agency in England regulates the guidelines for using cover and catch crop species. These regulations specify that cover and catch crops must be a visible mixture of at least two different crops from a prescribed list of eight species, where one species in the mixture must be a cereal and the other a non- cereal (Rural payment agency, 2016; Storr et al., 2019).

Additionally, cover and catch crops must be maintained for a specified period. In 2015/2016, 55,900 ha were planted with cover or catch crops as an EFA feature, representing a 45% increase from the previous season (DEFRA, 2017). Similarly, a survey showed that 56% of respondents had ≤3 years' experience using cover crops and 75% of respondents have used cover crops for <5 years. (Storr et al., 2019).

A current survey on cover crop use showed that 48% of farmers were using cover crops and 15% had previously tried using cover crops. However, there were 37% of farmers who had not used cover crops (Figure 1.5). This could be linked to farmers who have more spring cropping and on lighter soil hence being able to integrate cover crops easily. The three main aims of cover crops use by farmers from this survey were soil type driven and included improving soil structure, organic matter addition and prevention of nutrient leaching (Barratt, 2023).

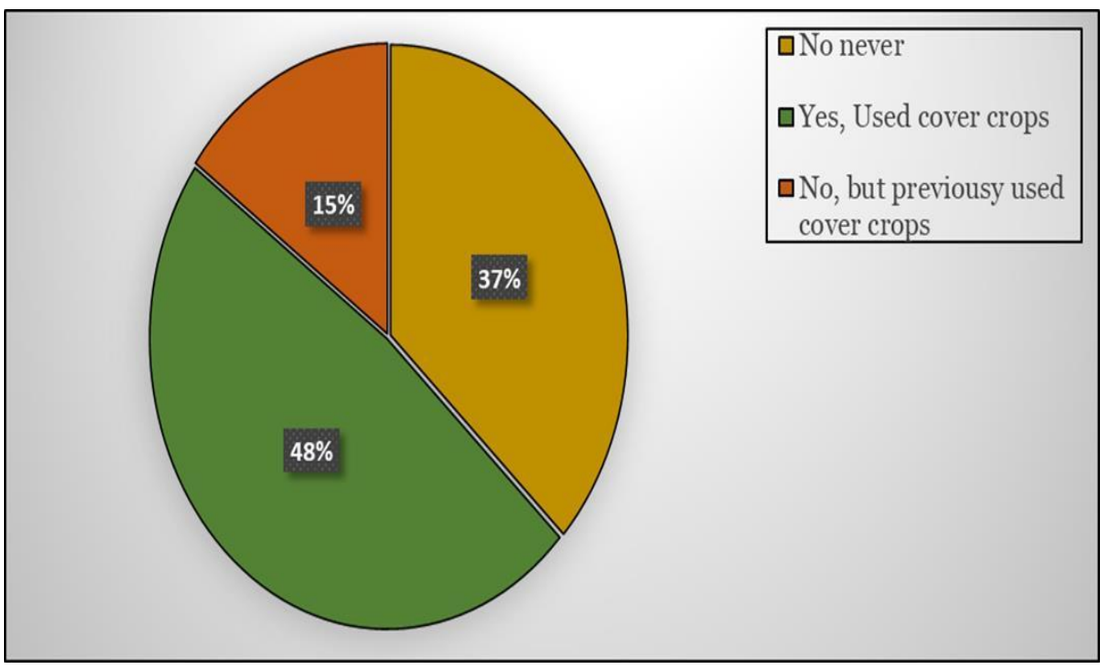

Figure 1.5: The split of farmers that use, have used but no longer do and have never used cover crops in the UK, in a survey with 295 respondents (Barratt, 2023).

The six most common cover crop species by farmers were found to be oilseed radish, *Phacelia*, vetch, black oat, white mustard and stubble turnips (Figure 1.6). The oilseed radish stands out as the most grown cover crop, however not on heavier soils due to obstruction by its large storage roots during drilling of the main crop. Vetch, *Phacelia* and black oats were popular also heavier soils (Barratt, 2023). In areas prone to wind or water erosion, winter or cover crops are necessary, along with conservation tillage, to prevent nitrogen leaching during winter.

Additionally, winter catch crops like *Sinapis alba* is commonly used to reduce the population of nematode cysts in the soil. Continuous cultivation of sugar beet in the same field leads to a rapid decline in yield due to increased disease inoculants, making it imperative to avoid this practice. Some of these cover crops species, namely radish and mustard, have already been used to suppress the beet cyst nematode (BCN) in the UK, and are marketed as Class 1 and Class 2, with class one offering up to 90% suppression of the BCN (Barratt, 2023).

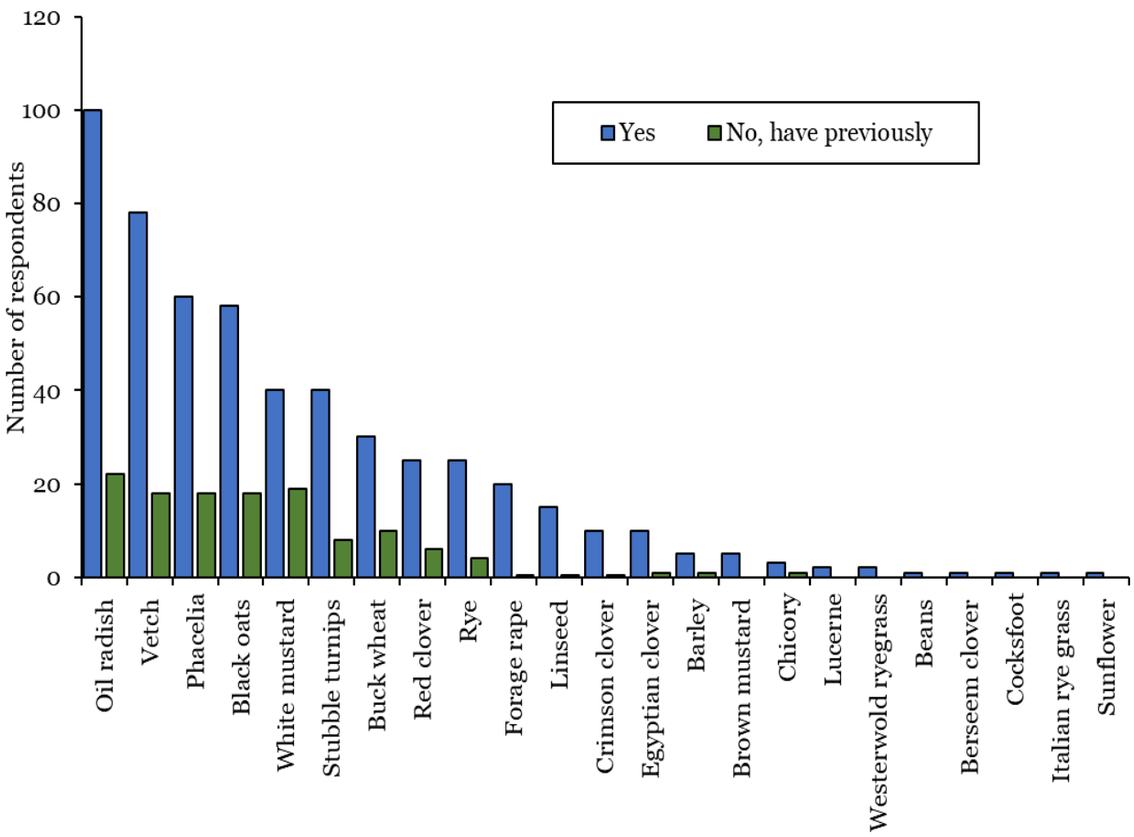

Figure 1.6: Common cover crops grown by farmers in the United Kingdom, in a survey conducted with 295 respondents (Barratt, 2023).

ii) Cover crops as a tool for plant parasitic nematodes management.

Cover crops have been shown to suppress PPNs densities through various mechanisms this includes: 1) acting as resistant hosts, poor hosts or non-hosts, 2) producing allelochemicals that are toxic or inhibitory, 3) providing an ecological niche for antagonistic flora and fauna and 4) trapping the nematode (Wang et al., 2002). In some instances, one or more mechanisms can be used by a single cover crop. For example*, Crotolaria juncea*, was shown to be a poor host to *Rotylenchus reniformis*, possessed allelopathic effects when leaves were incorporated in the soil and promoted antagonistic nematode trapping fungus namely *Monocosporium ellipsospora* and

Arthrobotrys dactyloides (Wang et al., 2001).

Similarly, *R. sativus* can serve as a cover crop, trap crop, cash crop or a biofumigant crop. As a trap crop to *H. schachtii*, it allows infection by the nematode but inhibits completion of the life cycle while as a biofumigant crop, it produces ITCs from hydrolysis of glucosinolates, that suppresses soil borne pests (Aydınlı & Mennan, 2018). *Eruca sativa* (Rocket or Arugula) acts as a trap crop in the management of the Northern Root Knot Nematode (*Meloidogyne hapla*), the nematodes are attracted to the roots but are unable to reproduce, which lowers the population densities (Aydınlı & Mennan, 2018). Selection of cover crops for pest and pathogen requires careful considerations such as host-status of the cover crop to different pests and pathogens, to avoid hosting pathogens that would otherwise decline during bare fallow periods, which can reduce their effectiveness as cover crops in specific conditions (Couëdel et al., 2019).

Other key considerations include the cover crop variety, rotation sequence and the nematode species /race involved (Mcsorley et al., 1994). Secondary metabolites, which are products released during plant growth and development, play a key role in defence against pathogens and pests (Thoden & Boppré, 2010). Compounds from widely used cover crops such as polythienyls and polyacetylenes from family Asteraceae, ITCs from Brassicaceae, alkaloids from Leguminosae and glucosides from Poaceae. 2-dehydropyrrolizidine alkaloids (PAs), particularly associated with plants belonging to the families Asteraceae, Boraginaceae and Fabaceae have also been reported in suppression of nematodes (Chitwood, 2002; Thoden et al., 2009). These secondary metabolites have been further exploited for the development of biopesticides for nematode management (Renco et al., 2014).

Nematodes from different genera and species exhibit differences in their preferences for cover crops from diverse plant families due to their differences in residues and resource utilization (Orwin et al., 2010; Sohlenius et al., 2011). For instance, brassica species have been known to stimulate microflora that is vital in residue decomposition which then favors the diversity of nematode communities (Collins et al., 2006). Radish has been shown to facilitate bacterial decomposition, while rape has been shown to enhance fungal decomposition processes (Bhan et al., 2010; Gruver et al., 2010).

The quality of the residue plays a significant role in influencing bacterial feeding and fungal feeding nematode communities. Some studies have suggested an increase in beneficial nematode communities, such as a twofold increase in bacterivores, following brassica incorporation (Engelbrecht, 2012; Valdes et al., 2012) , although fungivore nematodes decreased by 25% in specific experiments (Valdes et al., 2012). Notably, the toxicity of seed meals from Indian mustard were twice as biocidal to PPNs as compared to bacterial and fungal feeding nematodes (Yu et al., 2007). However, mustards have also been shown to have negative effects on entomopathogenic nematodes used in insect pests' biocontrol (Ramirez et al., 2009), which poses a challenge of using the biocontrol of insects through entomopathogenic nematodes and biofumigation processes (Jaffuel et al., 2017).

The presence of a growing plant cover has an impact on light, nutrient, and water fluxes, as well as microclimate, resulting in changes in organism communities compared to bare soil (Vukicevich et al., 2016). Growing cover crops provide substantial amounts of nutrients through root exudation and rhizodeposition processes, attracting and sustaining microorganisms including bacteria, non-pathogenic *Fusarium* species, S*treptomyces*, and other actinomycetes (Hinsinger et al., 2009; Wichern et al., 2007) which employ mechanisms such as competition,

antibiosis, parasitism, or by inducing systemic plant resistance in suppression of numerous pathogens (Audenaert et al., 2002; Rayns & Rosenfeld, 2006). The differences in the exudate composition, quantity and seasonality of different cover crops belonging to various plant families and species shapes the microbial structures (Broeckling et al., 2008; Buyer et al., 2010; Schweitzer et al., 2008) and this diversity when explored through cover crop mixtures can enhance disease suppression (Berg & Smalla, 2009; Legay et al., 2014). Cover crops residues when incorporated provide additional benefits such as slow release of nutrients from the residues, and nematicidal compounds associated with them are also released slowly over long periods of time, hence giving long term nematode suppression (Wang et al., 2002).

Additionally, cover crops can promote antagonistic flora and fauna, and these include some nematode antagonists such as fungal egg parasites, nematophagous fungi, nematode trapping fungi, endoparasitic fungi, plant health promoting rhizobacteria and obligate bacterial parasites (Venette et al., 1997). Several hypotheses on how cover crops enhance antagonistic microorganisms have been described. For example, Linford (1937) speculated that incorporation of biomass from cover crops promotes proliferation of bacteria during decomposition of the organic material, which become a source of food for microbiovorous nematodes and in turn become a food base for nematophagous fungi (Van Den Boogert & Deacon, 1994).

Incorporation of *Crotalaria juncea* (Sunn Hemp) increased the reproduction of the bacterivorous nematode *Acrobeloides bodenheimeri* which is a prey to nematophagous fungi *Hirsutella rhossilensis* (Venette et al., 1997). Incorporation of organic material has also been shown to promote mycostatis,which is a scenario where the inability of parasitic fungus to germinate facilitates proliferation of antagonistic fungus which in turn suppresses parasitic nematodes (Stirling, 1988). Microplots amended with Alfalfa were also shown to promote the proliferation of nematophagous fungi namely: *Arthrobotrys dactiloydes* and *Dactylellina elipsospora* (Van Den Boogert & Deacon, 1994).

The type of cover crop has been shown to determine the degree of fungal proliferation, where pea crops were shown to stimulate the proliferation of *Athrobotrys oligospora* to a greater degree as compared to crops like barley, and mustard (Persmark & Janson, 1997). In terms of pathogen suppression, organic matter has been shown to play a significant role in pathogen suppression at cover crop termination rather than the specific allelochemicals contained in different cover crop species, for example brassicas which contain biocidal glucosinolates had similar level of pathogen suppression with other cover crops at termination (Larkin, 2013; Zhou & Everts, 2007). Pathogen suppression by organic inputs has been mainly attributed to indirect effects of higher antagonist diversity and density, rather than a decrease in pathogen inoculum (Davis et al., 1996; Ennaïfar et al., 2005).

This biocontrol due to organic matter addition has been shown to last longer than the effects of specific allelochemicals such as ITCs and is often complementary with allelochemical suppression (Cohen & Mazzola, 2006; Mazzola, 2007; Motisi et al., 2009). Poor physical soil conditions such as inadequate drainage, poor aggregate stability, and high soil compaction can exacerbate the damage from soil-borne diseases and weeds (Hossain et al., 2012; Widmer & Abawi, 2000). The summary of mechanisms employed by different cover crops are summarised in Figure 1.7.

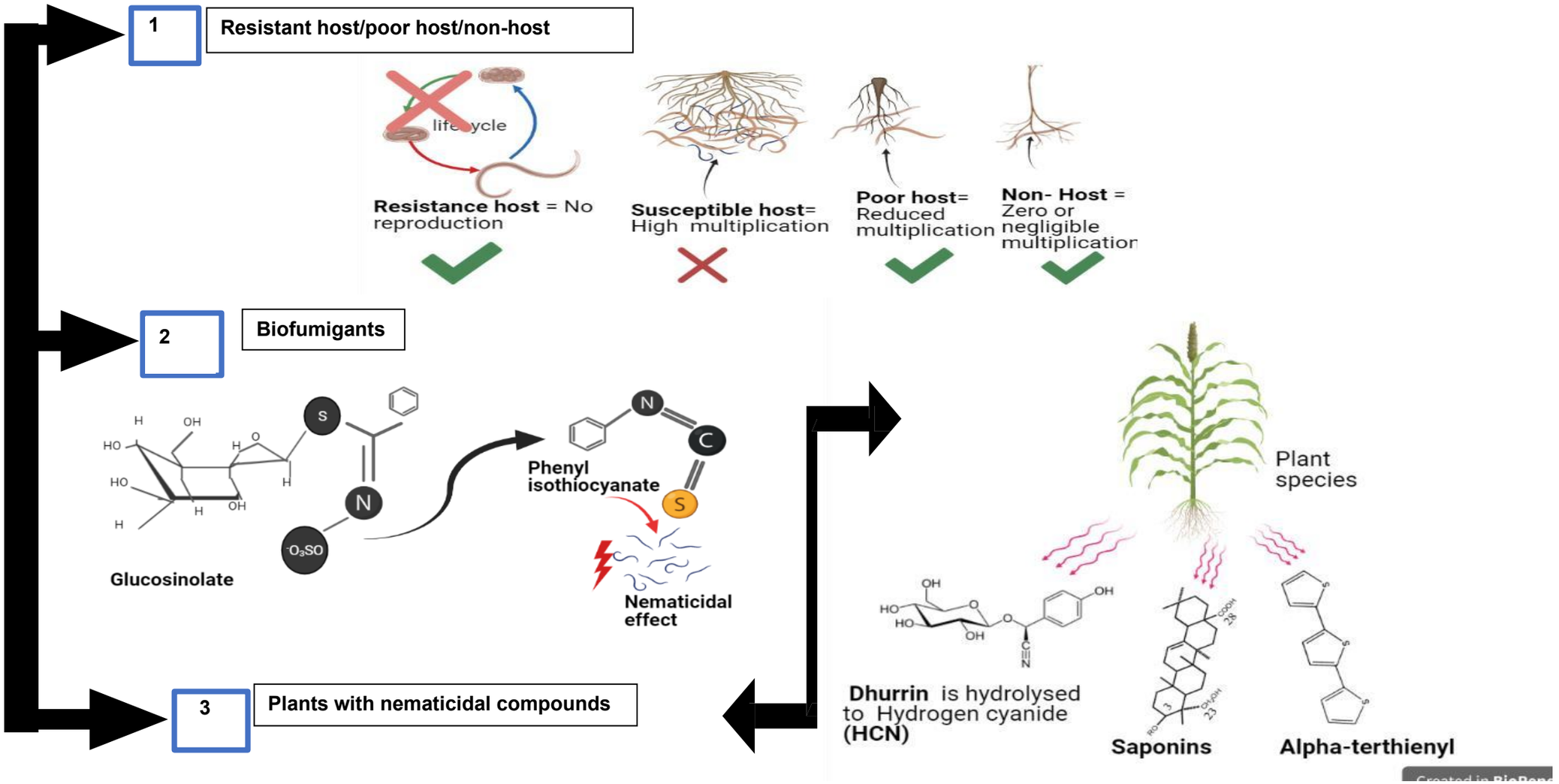

Figure 1.7: Mechanisms of actions used by different cover crop species to suppress plant parasitic nematodes (Created with BioRender.com).

1.5.2 Brassicaceous cover crops for PPN management.

Members of the brassica family are used in the management of PPNs either as green manures (Table 1.7) or as seed meals. Seed meals consist of the residual products of brassica seeds after oil extraction and are spread and incorporated in the soil as pellets (Zasada et al., 2009). Green manuring involves growing the brassicas to early flowering and then flailing and incorporating them, when the glucosinolate concentrations are highest (Lord et al., 2011; Zasada et al., 2009). Biofumigation involves the disruption of brassicaceous tissues to induce a chemical process that produces a range of bioactive compounds including ITCs within the soil (Lord et al., 2011; Ntalli & Caboni, 2017). The term biofumigation was first used to refer to the suppression of soil borne pathogens, weeds, and pests via the release of volatile compounds from incorporated brassica residues (Kirkegaard et al., 1993). Brassicas with biofumigant properties include *B. oleracea* (broccoli, cabbage, cauliflower, Brussels sprouts, kale), *B. napus* (rapeseed and canola), *B. rapa* (turnip), *Raphanus sativus* (radish), *B. campestris* (field mustard), *B. juncea* (Indian mustard), *S.alba* (white/yellow mustard), *B. nigra* (black mustard), *B. carinata* (Ethiopian mustard) and *E. sativa* (salad rocket) (Dutta et al., 2019). The process of biofumigation is explained by the fact that brassicas contain a class of thioglucoside secondary metabolites known as Glucosinolates (GSLs).

Glucosinolates were first described in the 17th century in research investigating the chemicals responsible for the bitter taste in mustards. Glucosinolates are limited to the order Capparale which includes the families Brassicaceae, Capparaceae, Resedaceaae and Moringaceae (Brown et al., 2003). Sinigrin (2-propenyl or allyl glucosinolates) and sinalbin (4-hydroxybenzyl glucosinolates) were the first GSL to be isolated from *Brassica nigra* (Black mustard) and *S. alba* (white mustard) respectively (Fahey et al., 2001). GSLs are sulphur containing metabolites, stored in the cell vacuole. Chemically, they exist as β-thioglucoside from amino acids and are categorized based on the structure of their side chain (R). Members of the Brassicaceae produce different quantities and types of GSLs. There may even be variation in GSL content within different cultivars of the same species grown in the same environment (Bellostas et al., 2004). For instance, the rapeseed variety Hyola 401 contains lower GSL content compared to the variety Dwarf Essex (Dutta et al., 2019).

The quantities and types of GSLs produced varies between plant organs, genetic makeup of the species, developmental stages, and exposure to environmental factors such as soil nutrients (nitrogen and sulphur), seasonal variations, or drought. For instance, variation in GSL concentration was observed in similar broccoli genotypes grown in different seasons and under distinct agricultural practices (Bhandari et al., 2015). The aromatic GSLs are highest in roots while the aliphatic GSLs are concentrated in seeds and the indole GSLs are more concentrated in the roots or shoots of tissues of most brassicas (Bhandari et al., 2015). There are more than 130 GSL that have been identified that are structurally different and are divided into different classes based on the structure of amino acid derived side chain (R) (Buskov et al., 2002). They are categorized into three classes namely: Aliphatic, aromatic and indole, with sinigrin usually being the predominant GSL being identified from Brassicaceae plants (Kruger et al., 2013). Aliphatic GSLs are known to be derivatives of 5 methionine, aromatic GSLs from tyrosine or phenylalanine, while indole GSLs are derivatives from tryptophan (Schonhof et al., 2004) and the latter do not produce ITCs and are therefore not relevant in biofumigation. Table 1.7 below illustrates the nomenclature, source and structure of different glucosinolates.

Table 1.7: Glucosinolates nomenclature, source, structure and acronyms (Wathelet et al., 2004).

Glucosinolate	Category	Source	Side chain	Acronym
Sinalbin		*S. alba*	4-hydroxybenzyl	SNB
Sinigrin		*B. juncea*	2-propenyl or allyl	SIN
Gluconapin		*B. rapa*	3-butenyl	GNA
Glucocapparin		*Capparis spinosa*	Methyl	GCA
Glucobarbarin		*Barbarea vulgaris*	(R)-2-hydroxy-2-phenelethyl	GBB
Gluconarsturtin		*Barbarea verna*	2-phenylethyl	GST
Glucolimnanthin	Aliphatic and Arylaliphatic	*Limnanthes sativum*	3-methoxybenzyl GLI	
Glucotropaeolin		*Lepidium sativum*	Benzyl	
Glucobrassicanapin		*B. rapa*	4-pentenyl	GTL
Protogoitrin	Hydroxylated aliphatic	*B. napus*	(R)-2-hydroxy-3-butenyl	PRO
Glucosisymbrin		*Sisymbrium loesilii*	2-hydroxy-1-methylethyl	GSY
Glucoringiin		*Conringia orientalis*	2-hydroxy-2-methylpropyl	GCN
Glucoceomin		*Conringia orientalis*	2-hydroxy-2-methylbutyl	GCL
Epi-progoitrin		*Crambe abyssinica*	2-hydroxy-3-butenyl	ePRO
Gluconapoleiferin		-	(R)-2-hydroxy-3-pentenyl	GNL
Glucobervirin	Thiofunctionalised	*Thlaspi sempevirens*	3-methiopropyl	GIV
Glucoiberin		*Iberis amara*	3-methylsulfinylpropyl	GIB
Glucocheirolin		*Cheirantus annus*	3-methylsulfonylpropyl	GCH
Glucoerucin		*E. sativa*	4-methiobutyl	GER
Glucoraphanin		*Broccoli*	4-methylsulfinylbutyl	GRA
Glucoraphasatin		*R. sativus*	4-methylthio-3-butenyl	GRH

Glucosinolate	Category	*Source*	Side chain	Acronym
Glucoraphenin		*R. sativus*	4-methylsufunyl-3-butenyl	GRE
Glucoalyssin			5-methylsufunylpentyl	GAL
Glucobrassicin			3-indolylemethyl	GBS
4-OH-Glucobrassicin			4-hydroxy-3-indolylemethyl	4-OHGBS
4-OMe Glucobrassicin			4-methoxy-3-indolylmethyl	4-OMeGBS
Neo-glucobrassicin	Indole type	*Isatistinctoria*	1-methoxy-3-indolylmethyl	neo-GBS

1.5.2.1 Degradation of GSLs.

Glucosinolates are found in the vacuoles of S-cells in plants. Adjacent to them are the myrosinase cells which contain thioglucosidases known as myrosinases. Upon tissue disruption of brassicas, GSLs and myrosinases come into contact, where in a process of hydrolysis, GSLs are hydrolysed to produce an array of compounds (Brown et al., 2003). The hydrolysis process results in conversion of the compounds to corresponding aglycons (Figure 1.8), which then decompose to release of bioactive compounds such as nitriles, thiocyanates, and ITCs depending on the R-group and prevailing chemical conditions in a process known as GSL-MYR system (Dutta et al., 2019; Ngala et al., 2014; Wathelet et al., 2004). Brassicas additionally produce other toxic sulphur containing hydrolysis products such as dimethyl sulphide, methyl sulphide, dimethyl disulphide, carbon disulphide, methaneiol etc., which may contribute to the biofumigation process (Dutta et al., 2019).

Glucosinolates (GSLs), are also occasionally hydrolysed from myrosinase produced *in situ* by soil microbes. Isothiocyanates (ITCs) are toxic GSLs catabolites and are attributed to the biocidal activity of brassica green manures (Dutta et al., 2019). Some studies have suggested the possible reactions that occur between the nematode pest and the ITCs, one of them is reaction of the active sites of the ITC with the nucleophiles of the nematode, mainly thiols and amine groups of certain enzymes making them alkylated. In other cases, the ITC have been shown to induce oxidative DNA damage and affect the motility of the nematode by impairing its host finding ability (Murata et al., 2000). In an isolated study, it was observed that dorsal pharyngeal gland nucleus in *G. rostochiensis* reduced upon exposure to ITCs hence ultimately reducing the nematode parasitism (Dutta et al., 2019). The non-volatile residues produced by biofumigant crops also improve the soil organic matter, recycling nutrients hence contributing to good soil quality that gradually build management of soil borne pathogens. Figure 1.8 below shows the release of ITC upon hydrolysis of glucosinolates.

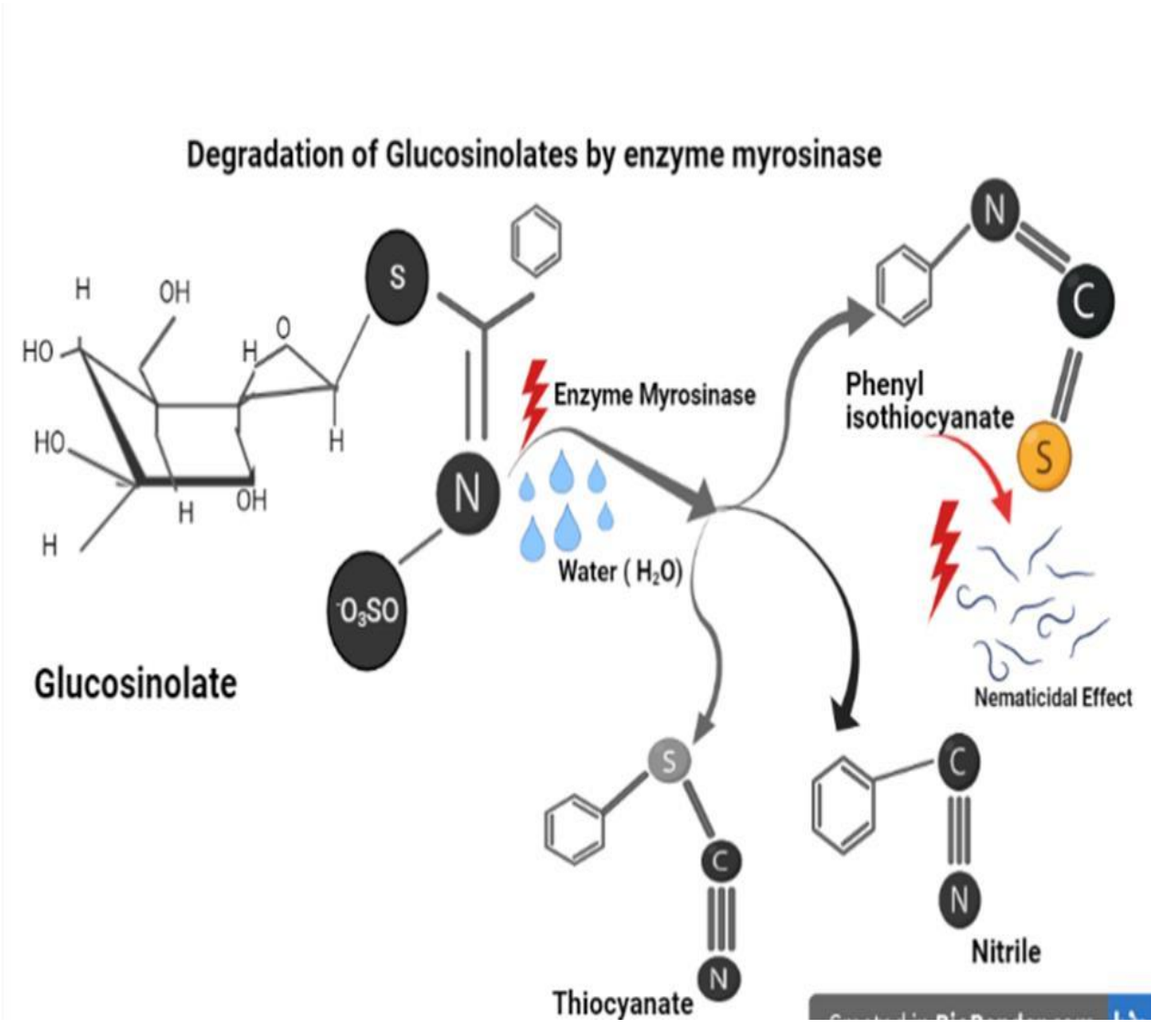

Figure 1.8: Hydrolysis of Glucosinolates into Isothiocyanates, thiocyanate and nitriles by the enzyme myrosinase (Created with BioRender.com).

1.5.2.2 Toxicity of isothiocyanates to plant parasitic nematodes.

Isothiocyanates (ITCs) derivatives differ in their level of toxicity among and within the different brassica species (Zasada & Ferris, 2003). Their effectiveness differs depending on the type and structure of ITC. This difference in toxicity can also be partly explained by their different biosynthetic pathways that are influenced by both genetic and environmental factors (Mithen, 2001; Li & Quiros, 2003; 4 Windsor et al., 2005).Commercially available ITCs as well as natural extracts from leaf, shoot and root extracts and macerates of different brassica crops have been evaluated under glasshouse and laboratory conditions to determine their nematicidal activities. The effects reported vary from hatching inhibition, reduced motility and death of target species and are influenced by a variety of factors.

For instance, the toxicity of aromatic ITC ‘s *to Tetrahymena pyriformis* has been correlated to reaction with cysteine residues of glutathione which has a role in respiration (Schultz et al., 2005). Toxicity of ITCs to nematodes is also known to be influenced by ITC-lipid solubility, ITC volatility and ITC hydrophobicity. Volatile ITC e.g., 2-propenyl are in gaseous form and are capable of dispersing evenly under suitable conditions and effectively interact with the target organism. Lipid soluble ITC’s e.g. 2-phenethyl can penetrate the nematode cuticle and permeate phospholipid membranes hence interacting with intercellular functions that kill the organism (Sarwar et al., 1998). However, the toxicity of the ITCs under field conditions are influenced by many other factors related to agronomic practices, soil factors and prevailing climatic conditions. Therefore, efficacy in this case involves manipulation of these factors for a pest and disease management (Fourie et al., 2016).

For instance, high organic matter content was found to lead to sorption of methyl ITC, leading to reduced pathogen suppression (Smelt & Leistra 1974). Elevated levels of organic carbon in the soil have also been associated with a decrease in 2-propenyl ITC (Borek et al., 1995). A similar observation was made with methyl ITC, which was seen to decrease in soils with high organic carbon. An explanation for the decrease may have been the reaction of the ITCs with nucleophilic groups such as phenols, amines, alcohols, carboxylic acids, and thiols contained in organic soil matter.(Gimsing et al., 2009).

The toxicity of the ITCs has also been shown in a study by Buskov et.al (2002), to be a related to type of GSLs in the brassica species used, where out of 13 GSLs tested, a high mortality of PCN juveniles was recorded after exposure to brassica extracts phenethyl- and benzyl GSLs which are both capable of producing ITCs upon hydrolysis. Similarly, another study evaluating efficacy of ITC produced by different plants recorded that *B. vulgaris* and *Moricandia moricandioides* lacked efficacy against *G. pallida,* because they contain indole-GSLs which are unable to produce stable ITCs (Halkier & Gershenzon, 2006).Nematicidal activity has been shown to only be achievable upon exposure to hydrolysis products, where intact GSLs have no effect due to the absence of myrosinase. Buskov *et al*. (2002) conducted an experiment to evaluate the toxicity of prop-2-enyl-, but-3-enyl-, (R)-4-methylsulfinylbut-3-enyl-, benzyl-, phenethyl-,4-hydroxybenzyl-, (2S)-2-hydroxybut-3-enyl-, and (2R)-2-hydroxy-2-phenylethyl GSLs and their hydrolysis products on the juveniles of *G. rostochiensis.* The GSLs were tested at three concentrations, 0.05, 0.3, and 1.0 mg/mL, in the presence or absence of the enzyme myrosinase. Results showed that intact GSLs had no effect in the absence of the myrosinase enzyme.

However, 100% mortality of *G. rostochiensis* juveniles was observed after 16h exposure to 1

mg/mL phenethyl glucosinolate and myrosinase at pH 6.5. Isothiocyanates derived from different GSLs differ in their potency. For example, 2- propenyl ITC derived from sinigrin (prop-2-enyl GSL) was shown to be more potent to *H schactii* when compared to ITCs derived from gluconapin, glucotropeolin and dehydroerucin, where at a similar concentration of 0.5%, sinigrin caused mortality after 24h exposure time while mortality for the other ITCs was recorded after 48h exposure time (Lazzeri et al., 1993). Further tests to investigate the toxicity of sinigrin ITCs showed that even at concentration of 0.05%, of crude extracts obtained from whole seed of *Brassica carinata,* a mortality of 100% for *H. schactii* was achieved after 48h exposure time as compared to hydrolysis products of sinalbin and glucoraphanin where no nematicidal effects were recorded (Lazzeri et al., 1993). Similar results were obtained in pot experiments where *Brassica macrocarpa* leaf flour, which is known to contain high levels of sinigrin, recorded a 50% reduction in root galling index on tomato plant roots in pots treated with sinigrin when compared to untreated control (Argento et al., 2019).

Leaf extracts from 8 weeks old plants of *R. sativus* cv. Weed check, *N. officinale* cv. Cress*,* and *B. juncea* cv. Nemfix were shown to inhibition the movement of *G. pallida* juveniles, by 97, 93, and 89%, respectively, in a sand column assay (Lord et al., 2011). Pure commercially sourced 2-propenyl ITCs, at a concentration of 0.002%, were also shown to exhibit high toxicity on eggs of *G. pallida*, where the hatching ability was decreased by 50% after 2h exposure time (Brolsma et al., 2014). In another study, silica sand in polyvinyl tubes was used to determine the lethal concentration of commercially available ITCs i.e., Allyl, benzyl, butyl, ethyl, phenyl, 2-phenylethyl and 4-methyl-sulfinyl(butyl) against *Tylenchulus semipenetrans* and *Meloidogyne javanica.* The tubes were incubated at 25°C for 48 h. The findings indicated that benzyl and 2-phenylethyl ITCs, with the highest molecular weights, were the most toxic ITCs. The LC90 values were 0.01 and 0.03 µmol/ml for 2-phenylethyl ITCs and 0.01 and 0.06 µmol/ml for benzyl ITCs for *T. semipenetrans* and *M. javanica*, respectively (Zasada & Ferris, 2003).

Effect of commercially available pure ITCs, namely ethyl ITCs, propyl ITCs, isopropyl ITCs, butyl ITCs, isoamylene ITCs, acryloyl ITCs, isovaleryl ITCs, phenyl ITCs, benzyl thiocyanate, benzyl ITCs, 1-phenylethyl ITCs, 2-phenylethyl ITCs and allyl ITCs, were tested against *M. javanica and* compared to metam sodium in an *in vitro* experiment. When exposed for three days to allyl, acroloyl and ethyl ITCs, the juveniles became irreversibly immobile at LC_{50} values of 2.76, 2.53 and 3.05 mg mL^{-1}, respectively (Wu et al., 2011). Allyl and acryloyl ITCs were applied to the soil at rates of 1.0 ml and 1.1 ml respectively. Results showed that there were significantly fewer galls on cucumber roots and fewer juveniles in the soil in ITC and metam sodium treated pots compared to the untreated control. The two ITCs were equally effective at a lower rate of 0.5 ml per kg of soil compared to metam sodium at its recommended rate (Wu et al., 2011). Volatile compounds (VOCs) obtained from macerated tissue of broccoli shoots and sunflower seeds were shown to reduce infectivity, mobility, and reproduction of *M. incognita* juveniles. The mobility, infectivity and reproduction of juveniles was also reduced when they were placed in water exposed to broccoli. This is because sulphurated VOC were found in the water exposed to broccoli macerates and were attributed to the nematotoxic effects to the juveniles. The study also showed that sunflowers produce toxic volatile organic compounds that have potential use in biofumigation against *M. incognita* (Carlos et al., 2018). Volatile compounds obtained from seeds of *B. juncea*, *B. napus* and *Sinapis alba* similarly indicated that they had a nematicidal activity when exposed to *Paratylenchus* spp and populations of *Aphelenchoides compositola* parasitizing white button mushroom (Kowalska & Smolinska, 2001).

Table 1.8: Effects of green manure amendments from different Brassicaceae species on plant parasitic nematode population densities.

Cover crop	Follow-up crop	Nematode spp	Reduction %	Country	Reference
Brassica campestris	*Solanum tuberosum*	*Meloidogyne chitwoodi, Pratylenchus neglectus*	48% reduction in *M. chitwoodi* juveniles and 54% reduction *P. neglectus*	USA	(Al-Rehiayani & Hafez, 1998)
Brassica campestris	*Vitis vinifera*	*M.javanica*	61-73% reduction in egg production	Australia	McLeod & Steel, 1999
Brassica carinata	N/A	*Pratylenchus neglectus*	0-65% reduction	Australia	Potter et al., 1998
B. juncea	*Vitis vinifera*	*M. javanica, Criconemoides xenoplax*	51% reduction in *M. javanica* and no effect on *C. xenoplax*	South Africa	Kruger et al.,2015,
B. juncea	*Solanum tuberosum*	*G. pallida*	Significant reduction in viable encysted eggs	United Kingdom	Ngala et al., 2014
B. juncea	*Solanum lycopersicum*	*Meloidogyne incognita*	14% reduction in galling	India	Randhawa & Sharma, 2008

Cover crop	Follow-up crop	Nematode spp	Reduction %	Country	Reference
B. juncea	N/A	*Pratylenchus neglectus*	6-68% reduction in population levels	Australia	Potter et al., 1998
B. napus	*Vitis vinifera*	*M. javanica, Criconemoides xenoplax*	Reduced by 14 and 8% for *M. javanica* and *C. xenoplax* respectively.	South Africa	Kruger et al., 2015
B. napus	*Vitis vinifera*	*Pratylenchus neglectus*	0-57% reduction in population levels	Australia	Potter et al.,1998
Raphanus. sativus	N/A	*G. pallida*	50% reduction in population levels	USA	Riga et al., 2010
R. sativus	N/A	*P. teres*	72% reduction	Netherlands	Hartsema et al., 2005

1.5.2.3 Potential of biofumigation under field conditions.

The potential of biofumigant brassica crops has also been extensively studied on different target nematodes under field conditions. The effects have been inconsistent with some studies recording high suppression of PPN from biofumigant cover crops (Lord et al., 2011) while others recording no effect to the target species (Vervoort et al., 2014). The selection of biofumigant cover crops needs to consider the host status of species or cultivars to PPNs to ensure that populations decline during the growth of the biofumigant as well as after residue incorporation (Matthiessen & Kirkegaard, 2006). To be effective, brassica green manures need to effectively release ITCs for soil fumigation under suitable conditions. A blend of high soil moisture content, thorough pulverization in the incorporation process and high plant biomass are vital in release of ITCs for biofumigation (Dutta et al., 2019; Kirkegaard et al., 1993; Kruger et al., 2013).

In the incorporation process, the strategy of incorporation and proper timing is essential. A study by Matthiessen, Warton and Shackleton (2004), showed that approximately 100 nmol ITC g^{-1} soil was achieved following thorough pulverization and irrigation after incorporation of mustard. On the contrary, a study by Gardiner *et al.*, (1999), recorded a concentration of 1 nmol g^{-1} Methyl ITC in the soil following plough down of winter rapeseed which is below the recommended rate of 291 kg ha^{-1} of Methyl ITC required for effective suppression of pests and pathogens.

Nevertheless, release of low concentrations over a long period of time can ultimately lead to suppression of pests and pathogens (Mattner et al., 2008). This is especially in the case of partial biofumigation, where brassica crops are grown but not incorporated. Instead, GSLs released from the plant roots during its growth period are slowly hydrolysed by enzymes secreted by the growing plants or soil microbes (Ngala et al., 2015). This strategy is beneficial when using winter hardy biofumigants such as *R. sativus,* which have large root biomass and have been shown to effectively suppress the encysted eggs of *G. pallida* (Ngala et al., 2015).

Timely incorporation of brassica materials is another key factor in maximising efficacy of biofumigants. Studies by Mattner *et al*., (2008) indicated that higher efficacy was obtained from maceration and incorporation of mature plants as compared to immature plants. Similarly, the suppression of *Rhizoctonia fragarie* using *B. rapa* and *B. napus* green manures was greatest when maceration and incorporation was done at anthesis compared to maceration at establishment stage (Mattner *et al.*, 2008). This is because the GSL concentration is highest/at peak during mid-flowering and this timing should be closely monitored (Ngala *et al*., 2014). High glucosinolate brassica crops can achieve more than 40 µmol GSL g^{-1} dry-weight tissue (Kirkegaard & Sarwar, 1998; Lord *et al*., 2011).

Plant biomass is another contributor to efficacy and can vary greatly between and within various brassicas species. For instance, 70t ha^{-1} fresh weight biomass was recorded in *B. juncea,* which was two times higher than *E. sativa* grown under similar field conditions during the mid-flowering stage (Ngala *et al.*, 2014; Watts *et al.*, 2014.). Low quantities of brassicaceous residues incorporated (20 kg ha^{-1}) were not effective in the suppression of *M. incognita* whereas 60 kg ha^{-1} effectively reduced infection and damage of *M incognita* in *Vigna subterranean* (Fourie et al., 2016). High amount of root biomass produced by *R. sativus* (oilseed radish) was linked to its effective partial biofumigation in reducing the viability of encysted eggs of *G. pallida, the* high root biomass which was linked to production of high concentration of GSLs hence toxic ITCs (Ngala et al., 2014).

It is also important to consider the time of planting as seasonal variations affect biomass and glucosinolate concentration (Booth et al., 1991; Price et al., 2005; Sarwar et al., 1998). In the UK, the glucosinolate concentration in *Brassica juncea* cv. ISCI 99 was found to reach up to 100 µmol GSL g^{-1} dry-weight of tissue, at the mid-flowering stage under field conditions (Ngala *et al*., 2014). The high concentration in summer, has been attributed to light intensity, long day length and temperatures which are higher in summer (Engelen-Eigles et al., 2006). Longer day length and higher light intensity ensures greater photosynthesis and accumulation of glucose which is an integral component of GSL and contributes to plant biomass (Agerbirk & Olsen, 2012). For that reason, summer grown brassicas have been shown to be more effective in pest and disease suppression as compared to winter grown brassica (Ngala et al., 2014).

Nutrients such as Sulphur and nitrogen are also very paramount in field grown biofumigants and are essential elements in the biosynthesis process of GSL which greatly influences the GSL concentration. Nitrogen also plays a vital role in protein biosynthesis, cation which influences the biomass produced by the biofumigant. Recommended field application rate for Nitrogen is 60-100 kg ha^{-1} (Lazzeri et al., 2004) while sulphur is applied as sulphate at a ratio of 5:1 (Pers. Comm. Dr Matthew Back: Reader in Nematology at Harper Adams University investigating nutrient applications to biofumigant crops for AHDB Potatoes).

During growth and development of brassicas crop, GSLs have also been shown to be released from young growing roots in the process of partial biofumigation. Soil microbes have been shown to play a crucial role in the degradation of glucosinolate, where they produce myrosinases which convert these GSLs into biocidal compounds such as ITCs. A study investigating the role of microbial activity in degradation of GSLs i.e., sinigrin, found that there was a strong correlation between *G. pallida* suppression by brassicas and microbial activity, which was attributed to sinigrin degradation from myrosinase produced by incorporated brassica material and myrosinase produced by soil microbes that were seen to build up post incorporation of the brassicas. The egg viability of *G. pallida* encysted eggs was significantly lower in pots where *R. sativus* was grown in unautoclaved field soil compared to pots with autoclaved soil, indicating the role of microbes in the partial biofumigation system (Ngala et al., 2015).

The content and profile of GSLs in the plant tissue is also key as it has been linked to effective suppression of pests and pathogens, this because the ITCs produced are dependent on the GSL content and profile (Dutta et al., 2019; Fourie et al., 2017). Production of high levels of aliphatic ITCs by some brassicas has been shown to give greatest suppression to soil borne pathogens. Other compounds released during decomposition of brassica materials have also been shown to contribute to the process of biofumigation. This includes compounds such as methyl sulphide, dimethyl sulphide, dimethyl disulphide, carbon disulphide and methanethiol (Lewis & Papavizas, 1971). These compounds have low toxicity compared to allyl ITCs but are released over a longer period, hence their suggested contribution in biofumigation (Lewis & Papavizas, 1971; Virtanen & Wahlross, 1965; Walker et al., 1937).

1.6 Non-brassica allelopathic plant species.

Crops producing allelochemicals have been investigated for their use in nematode management. They can be exploited either through crop rotations, intercropping or use as green manures. Allelopathy refers to the ability of plant species to produce allelochemicals, which are secondary metabolites or their products into the environment, which have negative effects on other plants and microorganisms (Wang et al., 2002). These allelochemicals are released either through

volatilization, exudation, leaching from plant roots or through decomposition of plant residues (Barnes & Putnam, 1987; Dutta et al., 2019; Halbrendt, 1996).

1.6.1 Sorghum -Sudan grass.

Sorghum-sudan grass is known for its allelopathic effects to nematodes is attributed to hydrogen cyanide, a hydrolysis product of dhurrin, which is a cyanoglycoside compound contained in all parts of Sudan grass (Viaene & Abawi, 1998). As a pre-planting green manure, *Sorghum vulgare*, was reported to suppress the ring nematode, *Criconemoides xenoplax,* in a field experiment. The densities of *C. xenoplax*/100 cm^3 on plots with *S. vulgare* green manure were comparable to the chemical fumigant methyl bromide (Nyczepir & Rodriguez-Kabana, 2007).

However, nematode suppression associated with Sudan grass should not be generalised, as variations have been reported between varieties and with different nematode species. For instance, *S. hybrida* was shown to be a poor host to *M. incognita* when grown in pots under glasshouse conditions *(*Curto et al., 2012). On the other hand, the sudan grass varieties Piper and Trudan and sorghum-sudan grass (Sordan 79, P855F, and P877F), had no effect on populations of *Pratylenchus*, *Xiphinema*, and *Paratrichodorus* spp. However, sorghum-Sudan grass was found to significantly lower populations of *Longidorus* spp. in the first year of the field experiment when used as green manures (MacGuidwin & Layne, 1995).

Additionally, nematode races within a species have been shown to vary in their rate of multiplication of the different sudan-grass varieties. For instance, *M. incognita* races 1 and 2 were able to reproduce in all sudan-grass varieties except Trudan 8 and Sordan 79, on the other hand, *M. hapla* was effectively suppressed by all cultivars tested (Mojtahedi et al., 1993). A follow-up glasshouse experiment aimed at evaluating the cause of suppression of *M. hapla*, showed that the juveniles were sensitive to hydrogen cyanide, which is degradation product of dhurrin found in *S. vulgare* and could be responsible for the observed suppression when used as a green manure (Mojtahedi et al., 1993). Dhurrin obtained from *S. hybrida* cv. Super dolce was further shown to have negative effects on juvenile mobility and egg hatching of *M. incognita.* A concentration of 0.58 mM of dhurrin was reported to cause 50% mortality of *M. incognita* juveniles and cause 50% egg inhibition at 0.38mM (Curto et al., 2012).

1.6.2 Rye.

Winter rye (*Secale cereale*) has been frequently used as a cover crop in many rotation systems due to its allelopathic effects on soil borne pests and pathogens. It is also recognised for its beneficial agronomic properties, such as reducing soil erosion, increasing nutrient sequestration as well as being suppressive to weeds, and PPNs (Zasada et al., 2005). Rye is associated with the production of allelochemicals, which are detrimental to soil borne pathogens including PPNs; it produces secondary metabolites known as benzoxazinoids, which are also found in other plants belonging to the Poaceae, Acanthaceae, Lamiaceae, Ranunculaceae and Scrophulariaceae (Chitwood, 2002).

This compound is released when crops are macerated/incorporated in the soil. The compound 2,4-dihydroxy-(2H)-1,4-benzoxazin- 3(4H)- one (DIBOA) and its breakdown product benzoxazolin2(3H)-one (BOA) has been linked to the allelopathy with rye (Barnes & Putnam, 1987; Sicker et al., 2000; Sicker & Schulz, 2002). The benzoxazinoids DIBOA and DIMBOA occur as glucosides in intact rye. Rye has been reported to reduce gall formation by *Meloidogyne hapla* in a rye-tomato crop rotation (Halbrendt, 1996b). Production of hydroxamic acids has also been linked with the suppression recorded with rye; hydroxamic acids have been

shown to exhibit acute toxicity on *M. incognita* and *X. americanum* populations. In addition to these allelopathic properties, rye has been shown to exhibit antagonistic mechanisms to plants, bacteria, insects and fungi due to production of the secondary metabolites (Zasada et al., 2005). Host status studies of rye cultivars to *M. incognita* demonstrated that nematode reproduction is mostly influenced by cultivar selection and less by concentration of the benzoxazinoids, where some cultivars of rye had significantly lower nematode reproduction than others, despite having similar concentration of the compound (Zasada et al., 2005).

1.6.3 Alfalfa.

Medicago spp. belongs to the family Fabaceae (Faboideae) and is composed of 83 species, with *Medicago sativa* (lucerne, alfalfa) being the most well-known species. This genus produces diverse secondary metabolites such as alkaloids, isoflavones, naphthoquinone, coumarins and saponins. The secondary metabolite saponin is known to be nematicidal.

Saponins are a wide group of phytochemicals containing triterpene or steroid aglycone and are particularly abundant in members of the Fabaceae. They are made up of different glycosylated triterpenic sapogenins (aglycone moieties) such as soyasapogenols A, B and E, zahnic acid, hederagenin, bayogenin and medicagenic acid, which is the dominant sapogenin typically accounting for 40-70% of total aglycones, although this varies in different plant tissues (D'Addabbo et al., 2011). Medicagenic and zahnic acid are the two main aglycones found in *M. sativa* (50% and 15%, respectively) and *M. arborea* (30% and 15%, respectively). Medicagenic acid is abundant in the roots of *Medicago* spp., while zahnic acid is only found in negligible amounts. Bayogenin has been mostly isolated from the shoots of *Medicago arborea*. In contrast, hederagenin (35%) and bayogenin (30%) are the dominant sapogenins in both shoots and roots of *M. arabica* (Avato et al., 2006).

The biological activity of the saponins have also been related to their structural differences where monodesmosides have been shown to be more active. The aglycone and position of the sugar molecule were also said to be important determining factors in the activity and efficacy of the saponins. In studies investigating antimicrobial activity of saponins, it was concluded that medicagenic acid and hederagenin most likely contribute to the activity detected in *M. sativa*, *M. arborea* and *M. arabica,* respectively against medically important yeasts and Gram-positive and Gram-negative bacteria (Avato et al., 2006).

Structurally, saponins may have one (monodesmosidic) or more sugar chains (bi-, tridesmosidic), linear or branched, linked to the aglycone moiety (sapogenin) on an ether or ester bond. Their occurrence in different plants is correlated to the structural type, where steroidal saponins are found in monocots and triterpenoid saponins are found in dicots (D'Addabbo et al., 2011). The specific mode of action of saponins in *Medicago* spp. has not been widely studied but they have been regarded as resistance factors in defense mechanism against pathogens (D'Addabbo et al., 2020).

In other studies, the biological effects of saponins have been attributed to the interference with the cell permeability of organisms as they have specific interactions with the cell membrane (Tava & Avato, 2006). Other studies have shown that exposure of *Meloidogyne* spp. eggs to saponins at different concentrations from *Medicago sativa*, reduced the cholesterol levels in the eggs and increased the general crop performance and growth as compared to untreated plants. Saponins were also shown to with reduce juvenile motility and egg viability of *M. incognita* (Ibrahim & Srour, 2013).

In an experiment conducted to determine activity of saponins obtained from different *Medicago* spp., no statistical difference was observed in mortality caused by the highest concentration of saponins and the nematicide oxamyl for *M. incognita*, *G. rostochiensis* and *Xiphinema index.* Tests conducted using extracts from different varieties of *Medicago sativa* were shown to be strongly active against *Xiphinema index* (dagger nematode) and *M. incognita.* Mortality of *M. incognita* juveniles was above 90% after 8 h exposure to an extract from *M. murex* or 16 h exposure to extracts from *M. hybrida*, and *M. truncatula* at a concentration of 500 μgmL^{-1}. *Xiphinema index* mortality was strongly affected by extracts from *M. lupulina* where a mortality of 93.3 % and 100% was achieved after 8 h and 4h exposure time at concentrations of 500 µg mL and 1000 µg $mL^{-}1$ extract, respectively. Up to 76% mortality of G. *rostochiensis* was achieved after 24h exposure time to 125 μgmL^{-1} concentration of the extracts from *M. hybrida* and *M. lupulina.* Moreover, the egg hatch of *G. rostochiensis* was reduced to 10-21% compared to untreated control after two weeks exposure to 1000µg mL of extracts from *M. lupulina* compared to untreated control (D'Addabbo et al., 2020).

The nematicidal effects of saponins from *M. arabica, M. arborea* and *M. sativa,* were studied in laboratory experiments on *Xiphinema index.* Related prosapogenins, obtained by basic hydrolysis of saponins, and sapogenins produced by acid hydrolysis of saponins were also included. As a comparison, soyasaponin and purified aglycones from *Medicago* spp. (medicagenic acid, hederagenin and bayogenin) and a commercial mixture of saponins from *Q. saponaria,* were also included in the study. All the saponins from *Medicago* spp. induced 100% mortality of *X. index* at the highest concentration (500 µg ml^{-1}) at 8 and 48 h exposure. Crude saponins from *M. sativa roots* and *M. arabica* tops and roots resulted in significantly greater mortality after 48 h than *M. sativa* and *M. arborea* tops at the same concentration. Medicagenic acid appeared slightly more active than bayogenin, causing 52% mortality at 62.5 µg ml^{-1} after 48 h of treatment. Prosapogenins were more nematicidal than the related saponins and sapogenins at the same dose, except for *M. sativa* tops at the maximal concentration (Argentieri et al., 2008).

Results obtained from a pot experiment investigating the nematicidal activity of dry foliage and root of *Medicago sativa* and *Medicago arborea* in pot mixes to suppress *M. incognita* and *G. rostochiensis* showed that both amendments were able to reduce the densities of both nematode species. In the field experiments, soil amendment with pelleted *Medicago sativa* at 20 or 40 t ha^{-1} increased the yield of tomato and reduced soil densities and root galling caused by *M. incognita* and densities of *G. rostochiensis* compared to the non-treated control (D'Addabbo et al., 2009).

1.6.4 Marigolds.

Marigolds (*Tagetes* spp.) are another group of plants that have been widely studied for their ability to suppress nematodes by producing compounds that are potentially allelopathic to PPNs (Chitwood, 2002). Alpha-terthienyl has been the major compound associated with the observed nematicidal activity. This compound contains sulphur and is concentrated in *Tagetes* spp. tissues. The activity of the compound is photoactivated or is released in response to root penetration by nematodes (Hooks et al., 2010).

The nematicidal effect of marigolds has also been attributed to other biologically active compounds such as essential oils which are believed to be working in combination with α-terthienyl (Dutta et al., 2019). These mechanisms may work separately on in combination either

as non-host, poor-host, allelopathy, trap crop or facilitation of other antagonistic flora and fauna (Wang et al., 2001). Marigolds have been mostly exploited against endoparasitic nematodes, such as root lesion nematodes (*Pratylenchus* spp.) as either an intercrop or in rotations schemes. This is because root peroxidases are produced in response to nematode penetration in the absence of light, which is the main activation factor. Therefore, nematodes that do not penetrate the root system i.e., ectoparasites may not be affected by the α- terthienyl biocidal compound (Bakker et al., 2006; Hooks et al., 2010).

Marigolds also act as trap crops where they cause arrested development of juveniles e.g. juveniles of *Meloidogyne* spp (Daulton & Curtis, 1963; Ploeg & Maris, 1999). Under field conditions, *T. patula* was shown to reduce the densities of Hawaiian *R. reniformis* as compared to the fallow control, evaluation of root penetration also showed that penetration was inhibited hence this effect by *T. patula* was attributed to its poor host status (Caswell et al., 1991). Marigolds can suppress a wide array of endoparasitic nematodes, though results can be inconsistent and there is little evidence of suppression for ectoparasitic nematodes (Hooks et al., 2010).

In field studies, evaluating the efficacy of summer cover crops on *Tylenchorynchus claytoni*, *P. minor, Pratylenchus brachyurus*, *Helicotylenchus dihystera,* and *X. americanum*, marigolds were planted in rotation with tomato transplants, with tomatoes being grown every third year. Marigold suppressed all nematode species except for *X. americanum* which increased during the 5th year of the study (Brodie et al., 1970). Similar results were obtained for the ectoparasitic nematodes *Belanolaimus longicaudatus* (sting nematode), *Dolichodorus heterocephalus* (awl nematode) and *Paratrichodorus allius* (SRN) where the marigolds used in the study were excellent hosts (Rhoades, 1980). Table 1.9 gives a summary of some of the allelochemicals from plants that have been shown to have nematicidal effects and inhibit hatching of plant parasitic nematodes.

Table 1.9: Alkaloids/secondary metabolites from diverse crop species with negative effects to plant parasitic nematodes.

Crop species	Family	Secondary metabolite	Nematode species	Effect	Reference
Chromolaena odorata *Crotalaria* spp.	Fabaceae	1,2 dehydropyrrolizidine	*Meloidogyne* spp *Rhabditis* spp.	Repulsion	Thoden et al., 2009
Sorghum sudan grass	Poaceae	Dhurrin – Hydrogen cyanide	*Meloidogyne* spp		Mojtahedi et al., 1993
Sorghum sudan grass	Poaceae	Dhurrin – Hydrogen cyanide	*Criconemoides xenoplax*	Nematicidal	Nyczepir & Rodriguez-Kabana, 2007
Secale cereale Rye	Poaceae	Hydroxamic acids	*M incognita Xiphinema americanum*	Nematicidal	Zasada et al., 2005
Medicago spp. (Alfalfa)	Fabaceae	saponins	*Xiphinema index* and *Pratylenchus thornei*	Nematicidal	Martín & Magunacelaya, 2005
Medicago spp.	Fabaceae	saponins	*Meloidogyne* spp	Hatching inhibition	Ibrahim & Srour, 2013)
Medicago spp	Fabaceae	saponins	*G. rostochiensis*	Hatching inhibition	D'Addabbo et al., 2020

Crop species	Family	Secondary metabolite	Nematode species	Effect	Reference
Quillaja saponaria	Quillajaceae	saponins	*Xiphinema index* *Pratylenchus thornei*	Nematicidal	Martín & Magunacelaya, 2005
*Macleaya cordata (*plume poppy)	Papaveracea	Sanguinarine, chelerytherine and allocryptopine	*Bursaphelenchus xylophilus*, *Caenorhabditis elegans* and *M. incognita*	Nematicidal	K. Wang et al., 2012
Chestnut (*Castanea sativa* L.)	Fagaceae	Tannins	*M. javanica*	Nematicidal	Maistrello et al., 2010
Chestnut (*Castanea sativa* L.)		Tannins	*G. rostochiensis*	Hatching inhibition	Renčo et al., 2012
Bean (*Phaseolus vulgaris* L.)	Fabaceae	Saponins	*Hoplolaimus* spp. and *Tylenchorynchus dubius*	Nematicidal	Miller et al., 1973

1.6.5 Hybrid grass (*Festulolium* spp.).

Section modified from: Mwangi, N. G., Stevens, M., Wright, A. J. D., Edwards, S. G., Hare, M. C., & Back, M. A. (2024a). Grass – Endophyte Interactions and Their Associated Alkaloids as a Potential Management Strategy for Plant Parasitic Nematodes. *Toxins*, *16*, 1–21.

Festulolium hybrids are cool season grasses which are an intergeneric cross between *F. pratensis* (Huds.) and *Lolium perenne* (L.) and/or *L. multiflorum* (Lam.). *Epichloë uncinata*, a fungal endophyte, which colonises these hybrids, produces bioprotective loline alkaloids, which can accumulate to 2% of the host plant dry weight (Zhang et al., 2009). The loline alkaloids are water soluble and able to translocate around host tissues to areas such as the roots, where the endophyte itself is not found actively growing (Patchett et al., 2008). The type of loline alkaloids produced by colonised grasses include norloline (NL); loline (L), N-methylloline (NML), N-formylnorloline (NFNL), N-acetylnorloline (NANL), N-formylloline (NFL) and N-acetylloline (NAL) (Yates et al., 1990). The alksloids NFL and NAL are the most isolated loline alkaloids from *Lolium-Festuca* hybrids. The endophyte's presence is required for high loline alkaloid expression and the fungus genotype determines whether lolines are produced or not (Blankenship et al., 2001). Importantly, loline alkaloids do not cause the animal health disorders (fescue toxicosis and ryegrass staggers) in grazing livestock associated with some of the other endophyte produced alkaloids, such as ergovaline and lolitrem B (Fletcher et al., 2017; Gooneratne et al., 2012).

Secondary metabolites produced from grass-endophyte interactions can have both direct and indirect effects on PPNs. Directly, they can interact with the nematodes motile stages causing paralysis (nematostatic) or death (nematicidal) (Clay & Schardl, 2015). Direct interaction of the secondary metabolites with soil inhabiting PPNs involves translocation of the compounds to the root systems and subsequent exudation, which then has a negative effect on development and reproduction of the nematode (Jia et al., 2013; Mwangi et al., 2024a).

Metabolites can also interact with immobile stages such as nematode eggs causing hatching inhibition (Meyer et al., 2013). The metabolites may lack nematistatic/nematicidal effects but may possess repellent activities which interfere with nematode chemoreceptors, hence impairing nematode host finding abilities, and cause mortality due to starvation (Clay & Schardl, 2015); nematode host finding can be evaluated in a chemotaxis assay, where the movement of the nematode from a centre of inoculation, usually in an agar plate, is monitored and the metabolite rated either as strong/weak repellent or an attractant (Bacetty et al., 2009). In other assays, seedlings of the plant are used to evaluate nematode attraction and repulsion, to evaluate compounds being exuded by the roots (Jia et al., 2013).

Factors such as the class of the alkaloid, concentration of the alkaloid, exposure time, part of the plant the extract is obtained i.e., shoots/roots, and age of the plant have been shown to cause variations in mortality, motility and attraction and repulsion activity to the nematodes. To evaluate the direct effects of these metabolites, nematodes are exposed to biologically relevant concentrations for a specific duration of exposure; the movement of the nematodes is then evaluated by stimulating their motion, with a lack of movement indicating that the compound is nematostatic (Desmedt *et al.*, 2020; Mwangi *et al.*, 2024a).

To discern whether the effect is nematostatic or nematicidal, the nematodes are placed in distilled water for a recovery assessment; the failure to recover qualifies the compound as

nematicidal. The effect of a compound may be either nematicidal or nematostatic depending on the dosage of the compound and the duration of exposure, as nematodes may recover at lower doses or die at higher doses (Desmedt et al., 2020). The metabolites may not possess nematostatic/nematicidal effects but might exhibit repellent activity, which disrupts nematode chemoreceptors, consequently impeding the nematodes' ability to locate a host and leading to mortality due to starvation. The ability of nematodes to locate a host is evaluated in a chemotaxis assay, where the movement of the nematode from a central point of inoculation, typically on an agar plate, is observed, and the metabolite is categorized as either a strong/weak repellent or an attractant (Bacetty *et al.*, 2009).

In other assays, seedlings of the plant are utilized to assess the attraction and repulsion of the compounds being exuded by the roots (Jia et al., 2013). The outcomes obtained in controlled laboratory conditions are sometimes incongruent with those observed in plants. Some of the factors contributing to this disparity include (i) the exclusive production of certain alkaloids from *Epichloë* spp. in the plant, and (ii) the significant influence of the host plant on the concentration levels of the metabolites from these interactions, as the environment in which it grows can modify the biosynthetic pathways involved in the production of the metabolites (Card et al., 2021; Spanu, 2012). Table 1.10 gives a summary of *in-vitro* studies conducted on different nematode species.

Table 1. 10: Summary of in vitro tests evaluating the direct effects of alkaloids from grass–endophyte interactions on different nematode species (Mwangi et al., 2024a).

Nematode Species	Grass Genotype	Endophyte Species/Alkaloids Tested	Exposure Material	Assay	Nematode Stage	Dose	Exposure Time	Effect	%Efficacy	Reference
Meloidogyne incognita	*Schedonorus. arundinacea*	*Epichloë coenophialum*	Seedlings	Chemotaxis	Juveniles		2 h	Repulsion	Chemotaxis factor = 0	Jia et al., 2013
M.incognita	*S. arundinacea*	*E. coenophialum*	Fungal filtrate	Mortality	Juveniles	100%	72 h	Nematicidal	72%	Jia et al., 2013
	Leymus chiniensis	*Epichloë* sp.	Fungal filtrate		Juveniles	100% fungal filtrate	72 h		91.7%	
	Achnatherum sibiricum	*E. sibiricum*	Fungal filtrate		Juveniles	100% fungal filtrate	72 h		66.8%	
Pratylenchus scribinieri		Ergovaline	Purified alkaloids	Mortality	Juveniles	5 µg mL^{-1}	72 h	Nematicidal	100%	Bacetty et al., 2009a
		Lolines				50 µg mL^{-1}	72 h	Nematostatic	100%	
		Ergocryptine				50 µg mL^{-1}	72 h	Nematostatic	100%	
P. scribinieri	*Festuca arundinacea*	*E. coenophialum*	Root extracts	Mortality	Juveniles	2400 µg mL^{-}	72 h	Nematostatic	80%	Bacetty, et al., 2009a

Nematode Species	Grass Genotype	Endophyte Species/Alkaloids Tested	Exposure Material	Assay	Nematode Stage	Dose	Exposure Time	Effect	%Efficacy	Reference
M. incognita	*F. arundinacea*	Agroclavine Setoclavine + Agroclavine *E. coenophialum* c	Root exudates	Mortality	Juveniles Eggs	21 µM 7 µM + 34 µM 1.4 *w/w* 1.4 *w/w*	24 h 24 h 7 days 7 days	No effect No effect Nematostatic Hatching inhibition	39.5% 97.6%	Meyer et al., 2013

Grass endophytes can induce the plant host immunity to PPNs, which is a mechanism commonly utilized by other antagonistic endophytes (Kuldau & Bacon, 2008; Bultman & Ganey, 1995). This induction involves activating genes responsible for producing various phytohormones, phytoalexins, volatile organic compounds, pathogenesis-related proteins, and initiating the salicylic acid, jasmonic acid, and ethylene pathways, which help protect plants from stressors (Desmedt et al., 2020). Some of these defense mechanisms counteract stressors such as PPNs, while others, like phytohormones, promote plant growth and mitigate stressor-induced damage (Kumar & Dara, 2021). Apart from induced host resistance, metabolic resistance is another scenario where the nematode may attempt to penetrate the host but encounters constitutively formed toxic metabolites that deter it from infecting the host (Desmedt et al., 2020). Additionally, endophytes can influence the composition and production of root exudates, further inhibiting PPNs. This has been demonstrated in *M. incognita* repulsion to root exudates extracted from roots colonized by *F. oxysporum* and preference for exudates from tomato (Dababat & Sikora, 2007).

Endophytes also deter PPNs by outcompeting them for resources (Kumar & Dara, 2021; Sikora et al., 2008). For example, *F. oxysporum* isolated from banana disabled and eliminated *Pratylenchus goodeyi* (Mwaura et al., 2010), while *Chaetomium globosum* produced secondary metabolites such as chaetoglobosin A, chaetoglobosin B, flavipin, 3- methoxyepicoccone, and 4,5,6-trihydroxy-7-methylphthalide, which directly affected *M. incognita* (Khan et al., 2019). Various grass–endophyte associations have been studied for their ability to suppress PPNs. Endophyte-colonized tall fescues have been shown to reduce the numbers of PPNs such as *Pratylenchus* spp. (Bacetty, et al., 2009a; Bacetty et al., 2009b) and *Meloidogyne* spp. (Meyer et al., 2020; Nyczepir & Meyer, 2010). Although the endophyte hyphae in grass– endophyte interactions do not occur in the root system, it has been suggested that the metabolites responsible for interacting with the nematodes in the roots are translocated from the leaves and stems, which are the points of synthesis (Cook & Lewis, 2001). This was confirmed as *Epichloë* spp. strains deficient in ergot alkaloid production were unable to reduce numbers of *Pratylenchus* spp. compared to ergot-producing strains, which had a negative effect (Timper et al., 2005).

However, other studies showed that the concentrations of the translocated ergot alkaloids in the roots were very low. Knockout mutants with silenced ergot alkaloid biosynthesis pathways were still able to effectively suppress nematodes, indicating that the ergot alkaloids were not solely responsible for nematode suppression (Panaccione et al., 2006). Nevertheless, in other groups of endophytes associated with antinematode activity, such as non-pathogenic strains of *F. oxysporum*, the culture filtrates have been shown to negatively affect *M. incognita*, suggesting that the direct interaction of endophyte toxins and nematodes can be a mechanism used by endophytes to suppress PPNs (Zabalgogeazcoa, 2008). In certain cases, the presence of endophytes has a notable impact on reducing PPN, while in other instances, disparities between E+ and E- have not been observed. Although endophytes can influence the susceptibility of grasses to nematodes, it has been noted that the host status also plays a significant role, as certain plant cultivars are non-hosts regardless of the presence or absence of endophytes (Meyer et al., 2020). Research on different grass-endophyte combinations shows variations in nematode suppression, depending on the grass genotype, the interaction between grass and endophytes and the species of nematode in question (Meyer et al., 2020; Nyczepir, 2011). Variations across various grass endophyte combinations are summarized in Table1.11 below.

Table 1.11: Summary of pot experiments on the multiplication of different nematode species on colonised and non-colonised grass genotypes (Mwangi et al., 2024a).

Nematode species	Endophyte		Grass		Reproduction on Colonised (E+) or Non-Colonised (E−)						
	Species	Strain	Genotype	Cultivar/Variety	Initial Densities (Pi)/Pot	Final Densities (Pf)		Assessment	Trial Duration (Days)	Country	References
						E+	E−				
Meloidogyne incognita	*Epichloë uncinata*	U6 U8 U10	*Festulolium* hybrids	FHCDO802 BUS 10–12 FHAB0802 ABA 10–22 FHCD0802 BUS 10–13	5000	285.5 71.2 803.2 75	500 NS 63.1 NS 95.3 NS 600 *	Eggs/gram roots Nematodes/100 cm^3 soil	49	USA	Meyer et al., 2020
Pratylenchus scribinieri	*Epichloë coenophialum*		*F. arundinacea*	Jesup.	1500	1	1734 *	Nematodes/gram roots	40–45	USA	Bacetty et al., 2009
Pratylenchus spp.	*E. coenophialum*	Endemic		Georgia	984	20–30	150–190 *	Nematodes in roots/pot	56	USA	Timper et al., 2005

Nematode species	Endophyte		Grass		Reproduction on Colonised (E+) or Non-Colonised (E−)					Country	References
	Species	Strain	Genotype	Cultivar/Variety	Initial Densities (Pi)/Pot	Final Densities (Pf) E+	Final Densities (Pf) E−	Assessment	Trial Duration (Days)		
M.incognita	*N. uncinatum*			Bishanon	500	50.5	42.5 NS	Egg mass/root system	42	Japan	Uesugi et al., 2014
				JFIR-18		37	44 NS				
M.arenaria				Bishanon	500	41	39 NS				
				JFIR-18		66.2	57.4 NS	Nematodes/root system			
P. coffeae				Bishanon	300	721.50	515 NS		48		
				JFIR-18		288.2	291.4				
P. penetrans				Bishanon	300	412.40	NS				
				JFIR-18		367.10					
							501.6				
							NS 370.1				
							NS				
							15 NS				

Nematode species	Endophyte		Grass		Reproduction on Colonised (E+) or Non-Colonised (E−)					Country	References
	Species	Strain	Genotype	Cultivar/Variety	Initial Densities (Pi)/Pot	Final Densities (Pf)		Assessment	Trial Duration (Days)		
						E+	E−				
Tylenchorynchus spp.				Kentucky 31	270	20	40 NS	Nematodes in	180	USA	Rogers et al., 2016
				Texoma MaxQII		35	32 NS	100 cm^3 soil			
				Flecha MaxQ		91	40 *				
Criconemella spp.				Kentucky 31	High rate	6	246 NS				
				Texoma MaxQII	(800)	159	236 NS				
				Flecha MaxQ		606	311 NS				
				Kentucky 31	Low rate (250)	14	64 NS				
				Texoma MaxQII		34	291 NS				
				Flecha MaxQ		1026	162 *				
Helicotylenchus spp.				Kentucky 31	225	55	174 NS				

Nematode species	Endophyte		Grass		Reproduction on Colonised (E+) or Non-Colonised (E−)					Country	References
M.incognita	*E sibiricum*		*Achnatherum sibiricum* *F. arundinacea*	Texoma MaxQII Flecha MaxQ Wild type Kentucky 31	1000	84 330 10 0–5	80 NS 147 * 20–25 * 10–20 *	Nematodes/root system	15	China	Jia et al., 2013
P. scribneri	*Epichloë spp.*	Wild-type Isolate Lp1	*Lolium perenne*	Isolate Lp1 IpsA knockout dmaW knockout	1000	80–100 100–150 100-110	400–410 * 400–410 * 400–410	Nematodes/pot	48	USA	Panaccione et al., 2006
P. vulnus	*E. coenophialum*		*F. pratensis*	Wild-type Jesup Jesup (Max-Q)	3000	2 0 6	12 * 12 * 130–140	Nematodes in 100 cm3 soil	153	USA	Nyczepir, 2011

There are limited field experiments that have been documented comparing efficacy of colonised and non-colonised grass genotypes on PPN suppression. In some field experiments certain varieties have demonstrated the ability to suppress PPNs. The tall fescue variety Jesup-Max-Q was shown to prevent the resurgence of *M. incognita* and *M. hapla*, but not *M. javanica* and *M. arenaria* when tomato plants were sown as a follow-up crop, suggesting its potential use as a pre-plant strategy for managing *M. incognita* and *M. hapla* (Nyczepir & Meyer, 2010).

The same variety (Jesup- Max-Q) was evaluated under field conditions as a pre-plant alternative to chemical nematicides for controlling *M. incognita* populations before establishing a peach orchard over a 7-year period. The study compared (i) 1 year of peach followed by 1 year of Jesup (Max-Q), (ii) 2 years of continuous Jesup (Max-Q), (iii) 2 years of continuous peach, and (iv) 2 years of continuous peach followed by fumigation with 1,3-dichloropropene (1,3-D). Initially, pre-plant treatments did not significantly impact nematode population density, but later sampling (13 months after planting) revealed lower populations in grass and Jesup (Max-Q) plots compared to continuous peach plots.

Over a three-year period, non-fumigated plots had the highest nematode populations, while fumigated plots had the lowest. Tree growth was greatest in fumigated and Jesup (Max-Q) plots, moderate in grass-planted plots, and lowest in non-fumigated plots based on trunk diameter measurements. These findings indicated that pre-plant and post-planting treatments influenced nematode populations and tree growth in peach orchards (Nyczepir et al., 2014). Assessment of rate of endophyte colonization of the tillers showed that the rate of colonization was low which was concluded to have compromised the efficacy of the endophyte in nematode suppression under field conditions.

A comparison between ryegrass and tall fescue E+ and E- revealed seven times more *Pratylenchus* spp. in ryegrass than in tall fescue. The endophyte status did not significantly affect the densities of *Pratylenchu*s spp., *H. pseudorobustus*, and *Tylenchus* spp., but the total nematode numbers were 26% lower in endophyte-colonized grass compared to non-colonized grass, indicating that the endophyte strain was unstable, leading to lower suppression, which could have been higher if all tillers were colonized by the endophyte (Pedersen et al., 1988).

1.7 Research objectives.

The general aim of this research is to evaluate the potential of brassica and non-brassica cover crops and some of their associated phytochemicals in the management of SRN. The specific objectives were: -

I. To determine the sensitivity of SRN to commercial ITCs associated with brassicas in an *in-vitro* assay.

II. To evaluate the efficacy of brassica and non-brassica cover crops species in management of SRN under field conditions.

III. Identify the type and quantities of GSLs present in the roots and shoots of brassica species used in the study.

IV. Investigate the sensitivity of SRN to root and shoot extracts obtained from hybrid grass (*Festulolium loliceum*) grown under glass-house conditions.

V. Determine the alkaloids and antioxidants present in shoots and roots of *F. loliceum.*

Chapter 2: General materials and methods.

2.1 Introduction.

This chapter describes the general methodologies used in the experiments. Detailed methodologies for specific experiments are described in the relevant chapters. Activities described in this section were undertaken in the Nematology Laboratory, at the Centre for crop and Environmental Science (CCES), Harper Adams University, Newport, UK.

2.2 Nematode extraction methods.

Two extraction methods were compared at the beginning of this study to determine their efficacy in extracting SRN from soil. No significant differences were observed in the recovery of SRN. Therefore, the two methods were used interchangeably, depending on the objective of the experiment. Nematodes used for *in-vitro* assays were extracted using the Seinhorst two flasks as this method maintains the integrity of the nematodes because water is used as the extraction fluid therefore does not affect the nematode physiology which might be a confounding factor when conducting exposure studies with compounds and extracts. For SRN quantification from field samples, centrifugal floatation method was used as this method is faster given the number of samples being extracted. The physiological state of the nematode in this case was not important as the nematodes were only extracted for quantification purposes.

2.2.1 Seinhorst two flask method.

The principle behind the Seinhorst two flask method is the difference in size, shape, and sedimentation rate between nematodes and soil particles. Soil is first passed through a 2mm sieve to remove debris and large stones. The soil is then washed into a 2L flask filled with water and placed upside down in another 2L flask containing water for a period of 10 mins (Figure 2.1). Nematodes are collected in a clean suspension and placed in sample bottles for identification and quantification. On the other hand, the centrifugal floatation method uses differences in specific gravity between the nematodes and other particles in a soil sample. The extraction liquid usually has a higher specific gravity than the nematode, hence the nematodes stay afloat and are decanted into sieves at the end of the extraction (Bezooijen, 2006; Decraemer et al., 1979).

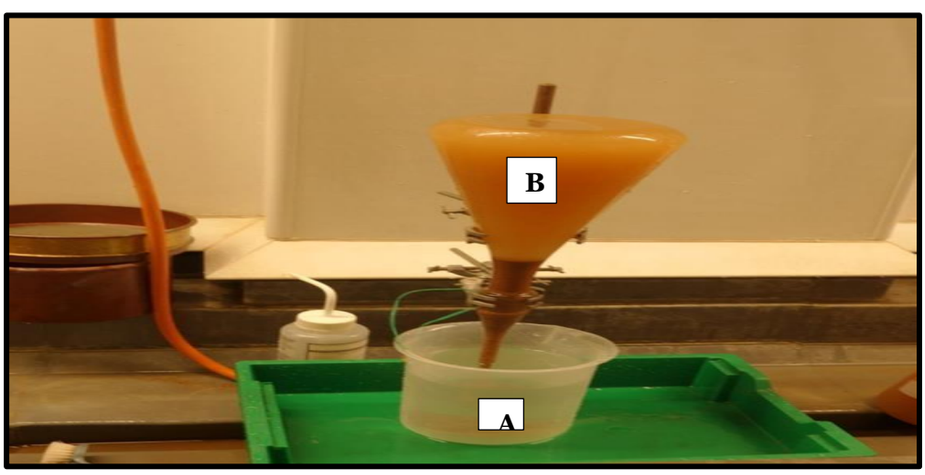

Figure 2.1: Seinhorst two-flask method showing the sedimentation process. Soil particles with a higher density than nematodes are collected in beaker (A) while nematodes remain in flask (B).

2.2.2 Centrifugal floatation method.

Stubby root nematodes were extracted using centrifugal flotation method using magnesium sulphate heptahydrate (MgS04.7H20) as the extraction solvent (Bezooijen, 2006). The bulk sample collected from field was gently mixed and passed through a 4mm sieve to remove large stones before taking a 200ml subsample for extraction. The soil was divided into four 50 mL centrifuge tubes and 20ml of MgS04.7H20 at 1.15 specific gravity added into each tube. The tubes were gently agitated to mix the extraction fluid and the soil and then centrifuged at 2680 RCF (1150 g) for 5 minutes (Figure 2.2). The supernatant was then decanted into 215 µm and 53µm sieves and gently rinsed in tap water before being washed into sample bottles. The suspension was concentrated into a smaller volume which was wholly quantified under a compound microscope at 20x magnification).

Figure 2.2: Nematode extraction using the centrifugal flotation technique using magnesium sulphate heptahydrate (MgSO4.7H20).

2.3 Nematode identification and quantification.

The whole suspension obtained from the extraction was quantified, where the suspension was concentrated and pipetted into a 2ml counting chamber under a compound microscope at 20x magnification. Morphological characteristics e.g., spicule shape in males, body cuticle and vaginal characteristics of the females was used to distinguish the genus and species of *Trichodorus* and *Paratrichodorus* spp.— (Figure 2.3) following the key as described by Decraemer (1995).

2.4 Molecular identification of stubby root nematodes.

Twenty stubby root nematodes were picked out with a needle under a microscope and then transferred into a PCR tube (200 µl) containing 18 µl of 1X PCR buffer (GoTaq/Promega). Four/five 1 mm glass beads were added into each tube and vortexed. for 30 seconds before adding 4 µl of proteinase K (100 µg ml−1) each tube. Tubes were incubated in a heating block at 60°C for 60 min, 95°C for 15 min to inactivate the Proteinase K and 10°C for 10 min. Tubes were then centrifuged at 16,000g and stored at −20°C. Real-time PCR amplifications were performed using a Step OnePlus Real-Time PCR System (Applied Biosystems) and optimised for the Fast-Run cycling approach. All probes were labelled at the 5'-end with FAM reporter dye and at the 3'-terminal with TAMRA quencher. Fragments were amplified using the primers D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-'3) and D3B (5'-TCG GAA GGA ACC AGC TAC TA'3) primers (De Ley et al., 1999) and cloned into pGEm-T vector (Promega) — (Table 2.1).

Each reaction contained: 10 *µ*l SensiFast Probe Hi-Rox Mix (Bioline Reagents), 0.3 *µ*M of probe, 0.6 *µ*M of primers, the volume made up to 18µL with sterile distilled water. A 2 *µ*L of template DNA was added to each reaction. The amplification conditions were 95˚C for 20s followed by forty cycles at 95°C for 1 sec with 60°C for 20s. Positive controls with plasmids and negative controls with sterilized water were included for each test performed. Positive controls/standards were received from James Hutton institute (JHI), where they were generated from the D2D3 expansion fragment of the 28srDNA fragment from representative examples of each of the 4 target nematode species. Standards were diluted in 10mM Tris (pH 8.0) to generate stocks containing from 10^8 to 10^2 copies µl-1 in 10mM Tris (pH 8.0). These standards were included in every PCR to allow quantification of the number of target-nematode-28S copies per sample, as well as negative (No Template) controls.

Table 2.1: Forward (F) and reverse (R) Primers and probes used in molecular identification of stubby root nematodes (SRN).

Target	Name	Sequence (5' - 3')	% G-C
Trichodorus primitivus	*T. primitivus* F	GCTTTCTCGTCGCGTGC	65
	T. primitivus R	CCTCGCAACACGTACAATCAA	48
	T. primitivus Probe	[FAM]CACGACCAGACAGTTCATTCAGCCAA[TAM]	50
Trichodorus Similis	*T. similis* F	GGCTTTCTCATGCTGTGCT T	50
	T. similis R	AGTGCCACCTCAAAGCTGT A	50
	T. similis Probe	[FAM]CCTTTGGCCGAATGCACTGTCTGACC[TAM]	58
Paratrichodorus Pachydermus	*P. pachydermus* F	GCTGATCGCAAGACATCGT G	55
	P. pachydermus R	CTGTCGGTCATGATGCTTT CTG	55
	P. pachydermus Probe	[FAM]CGAAAATGCACTAATCAAAAGCAGAATCAACCC [TA M]	39
Paratrichodorus anemones	*P. anemones* F	GTCCTGACCGAGTTGTGG	61
	P. anemones R	AACGCAGCCAAGAGAATCG	53
	P. anemones probe	[FAM]TCAACCCAATGGCTACGCACTACGAG[TAM]	54

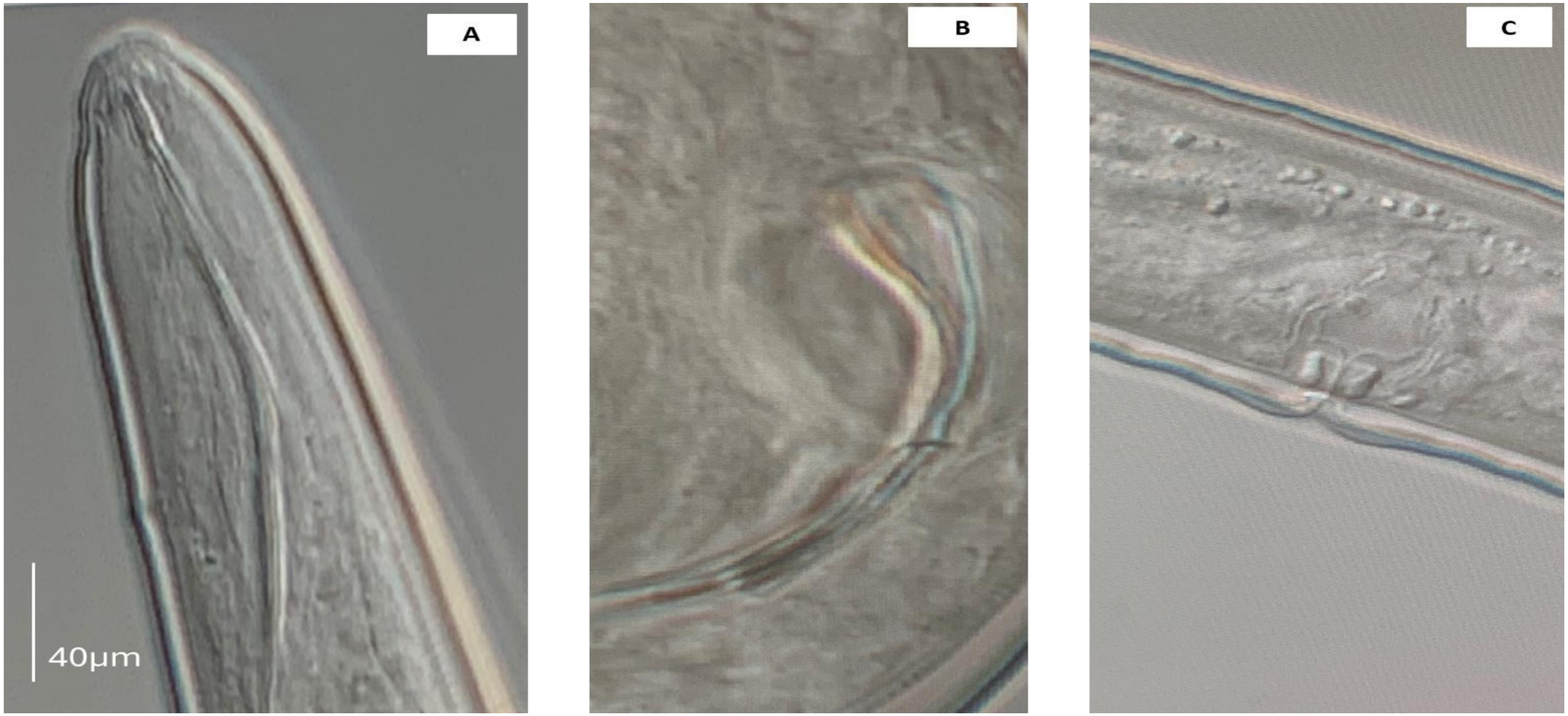

Figure 2.3: *Trichodorus primitivus* anterior (A), male spicule (B), and sclerotized vaginal pieces of the female (C) at x100 magnification (Photo taken by Nyambura Mwangi).

Chapter 3: Sensitivity of stubby root nematodes (*Trichodorus* and *Paratrichodorus* spp.) to ITCs associated with Brassicaceae in an In-vitro assay.

Chapter modified from: Nyambura, G.M., Stevens., M., Wright, A.J.D., Edwards, S.G., Hare, M.C., and Back, M.A. (2023). Sensitivity of stubby root nematodes (*Trichodorus* and *Paratrichodorus* spp.) to ITCs associated with Brassicaceae in an *in vitro* assay. *Nematology,* 26: 203-210.

3.1 Introduction.

Brassicas have been successfully used in management of PPNs in the process of biofumigation (Kirkegaard et al., 1993; Kruger et al., 2013; Kwerepe & Labuschagne, 2003; Ngala et al., 2014; Waisen et al., 2020). They contain secondary metabolites known as glucosinolates (GSLs) which protect them from pests and pathogens. Glucosinolates are sulphur containing metabolites, which chemically exist as β-thioglucoside from amino acids and are categorized based on the structure of their side chain (R). Glucosinolates have different profiles both quantitatively and qualitatively within the family brassica and occur in different quantities and in different cultivars and even species grown in the same environment (Bellostas et al., 2004).

The most predominant GSL in brassicas is sinigrin which is commonly found in brassicas such as *B. juncea* and *B. carinata*, glucoraphanin has been isolated from *B. rapa* and *R. sativus*, while gluconasturtiin has been found in *B. juncea* and *B. campestris.* Tissue damage/wounding in brassicas, results in a hydrolysis reaction of enzyme myrosinase and the GSLs which are in nearby cells within the cytoplasm (Brown et al., 2003). Soil microbes also hydrolyse glucosinolates by myrosinase produced *in situ* by soil microbes. The hydrolysis results to release of bioactive compounds such as nitriles, thiocyanates, and isothiocyanates depending on the R-group and prevailing chemical conditions in a process known as GLS-MYR system (Dutta et al., 2019; Ngala et al., 2015; Wathelet et al., 2004).

The most toxic of these bioactive compounds are the isothiocyanates and are attributed to the biocidal activity of brassica green manures (Dutta et al., 2019). The process of biofumigation takes advantage of this system where maceration and incorporation of brassica residues leads to production of bioactive compounds including isothiocyanates (ITCs) within the soil (Lord et al., 2011; Ntalli & Caboni, 2017). In addition, Brassicas also produce other toxic sulphur containing hydrolysis products such as dimethyl sulphide, methyl sulphide, dimethyl disulphide, carbon disulphide, methaneiol etc., which may contribute to the biofumigation process as they are released for a longer period unlike ITCs which have a shorter half-life (Bellostas et al., 2004).

The interaction of ITCs and nematodes has been explained by various studies that attempted

to determine the possible reactions. One study suggested that the active sites of the ITCs react with the nucleophiles of the nematode namely the thiols and amine groups making them alkylated (Avato et al., 2013). Isothiocyanates have also been shown to induce oxidative DNA damage hence impairing host finding abilities of the nematode which affects the motility (Murata et al., 2000). In *G. rostochiensis*, exposure to ITCs lead to a reduction of the nucleus of the dorsal pharyngeal gland hence ultimately reducing the nematode parasitism (Wu et al., 2011). Exposure of *Tetrahymena pyriformis* to ITCs was correlated to the reaction of glutathione cysteine residues, which are involved in respiration (Schultz et al., 2005).

Isothiocyanates differ in their toxicity among and within the Brassica species (Zasada & Ferris, 2003). The type and structure of the parent glucosinolate from which the ITCs are derived is known to influence their efficacy (Matthiessen & Kirkegaard, 2006; Pinto et al., 1998). The biosynthetic pathway for the different ITCs also explains their differences in level of toxicity as the pathways are influenced by both genetic and environmental factors (Fahey et al., 2001). Other factors that influence toxicity of ITCs include ITC lipid-solubility, volatility and hydrophobicity. For instance, ITCs which are in gaseous form such as 2-propenyl can disperse evenly under ideal conditions such as where the soil is properly sealed to mimimize loss through volatilization and effectively interact with target organisms.

On the other hand, ITCs such as 2-phenethyl are lipid soluble and can penetrate and permeate the phospholipid membrane of the nematode cuticle, hence interacting with the intercellular functions of the organism leading to death (Sarwar et al., 1998). The suppression of *G. pallida* was shown to be influenced by brassica used and the type of glucosinolate present, where mortality of *G. pallida* juveniles was recorded only after exposure to brassica extracts phenethyl and benzyl glucosinolates out of 13 glucosinolates tested (Buskov *et al.*, 2002). Similarly, ITCS obtained from *Barbarea vulgaris* and *Moricandia moricandioides* were unable to suppress *G. pallida* as the ITCs were derived from indole-glucosinolates which are not capable of producing stable ITCs (Halkier & Gershenzon, 2006).

Isothiocyanate products of the glucosinolates sinigrin, gluconapin, glucotropeolin and glucodehydroerucin were suppressive against *H. schactii* whereas those from glucoraphanin and sinalbin were not suppressive, indicating that toxicity of the ITCs is influenced by the type of glucosinolates and variations may also be linked to target nematode (Zasada & Ferris, 2003). Isothiocyanates are very volatile in nature and have a short half-life, therefore a lot of research has focused on how to prolong them in the soil to ensure they are not easily lost before encountering the target pest. The concentration present and the exposure time influences the efficacy of ITCs on target organisms.

Isothiocyanate concentration in the soil reduces soon after and during incorporation process. A study monitoring ITC concentration from *Brassica napus* during biofumigation found that

the highest concentration was released within 2h and 90% of the production was lost after 24h (Brown et al., 1991). Similar results were found when using *B. napus* and *B. juncea* where highest concentrations were released within 30mins of incorporation and no ITCs were recovered 12 days later (Gimsing & Kirkegaard, 2006; Morra & Kirkegaard, 2002). Research should therefore focus on screening brassicas with high concentration of glucosinolates to maximize the suppression of target pests in biofumigation process. The use of commercially available pure ITC makes it possible to evaluate the toxicity of the ITC by eliminating the conversion process from GSLs (Zasada & Ferris, 2003).

The objectives of this study were to: -

I. Compare the effect of commercially available ITCs on SRN mobility and mortality.

II. Determine the lethal dose (LD_{50}) and effective dose (EC50) values of the different ITCs.

III. Determine the effect of exposure time on the mortality of SRN.

Null hypotheses:

I. Isothiocyanates have no effect on mobility and mortality of SRN.

II. Exposure time to ITCs has no effect on the mortality of SRN.

3.2 Materials and methods.

3.2.1 Assay chemicals.

Commercially available ITCs were obtained from Sigma-Aldrich, UK. The ITCs used in this study included allyl (AITC), sulforaphane (SITC) and 2-phenylethyl (PEITC). AITC and SITC are aliphatic while PEITC is aromatic; these ITC are derived from the parent GSLs sinigrin, glucoraphanin and gluconasturtiin respectively. The criteria used in selecting ITCs used in this study was guided by previous studies that have reported their toxicity on a wide range of plant parasitic nematodes (Ntalli & Caboni, 2017; Wood et al., 2017; Wu et al., 2011; Zasada et al., 2009) as well as their association with brassica plants used in biofumigation (Aydınlı & Mennan, 2018; Lord et al., 2011; Ngala et al., 2015; Waisen et al., 2020)— (Table 3.1).

Table 3.1: Properties and characteristics of commercial isothiocyanates (ITCs) used in the study.

Isothiocyanate	Category	Parent glucosinolates	Molecular mass (g/mol)	Plant species
Allyl	Aliphatic	Sinigrin	99.15	*Brassica. juncea*, *B. carinata*
Sulforaphane	Aliphatic	Glucoraphanin	177.29	*Raphanus. sativus, B. rapa*
2-Phenylethyl	Aromatic	Gluconasturtiin	163.23	*Brassica juncea*, *Brassica campestris*

3.2.1 Source of SRN.

Mixed stages of SRN (*Trichodorus* and *Paratrichodorus* spp.) were obtained from infested soil collected from Docking, Norfolk site, UK, 52°54'01.7"N 0°36'32.4"E, which has a history of SRN infestation. Nematodes were extracted using Seinhorst two flask method (Bezooijen, 2006). Soil was gently mixed and washed through a 1 mm sieve to remove large stones and debris that would otherwise block the flasks. The extract was then washed through 215 µm and 53 µm sieves to collect a clean suspension, which was then transferred into sample bottles. Nematodes were used to set up the assay immediately after extraction to prevent any deterioration at storage. The composition of SRN used in this study, were identified as *T. primitivus (80%)*, T. *cylindricus 15%,* and *P. pachydermus* (5%) (Table 3.2), using morphological features key as described by Decraemer (1995)

.

Table 3.2: Composition of average males, females and juveniles of stubby root nematodes (SRN) ± SE (standard error), extracted from 200ml soil sample, n=10.

Stubby root nematode species	Males	Females
Trichodorus. primitivus	15.5±2.20	48.4±5.11
Trichodorus. cylindricus	4±0.68	7.6±1.03
Paratrichodorus. pachydermus	1.4±0.28	2.5±0.40
Juveniles		7.10±0.87

3.2.2 Assay protocol.

An *in vitro* assay was carried out by pipetting 1 ml of nematode suspension containing 20 mixed stages of SRN into a 25 ml bottle. Stock solutions of each ITC were prepared using 1% dimethyl sulfoxide (DMSO). Dilutions were made to make six concentrations; 1.625, 3.125, 6.25, 12.5, 25 and 50 µg ml^{-1} for each of the ITC and then 2 ml of test ITC added. Two controls included, i.e., distilled water and 1% dimethyl sulfoxide (DMSO). The experiment was incubated at room temperature (20 ± 1°C) in the dark, and each treatment was replicated four times, and the experiment repeated once. The effect of the ITC on SRN mobility was assessed after 24h, 48h and 72h exposure period in a repeated measures design.

Nematodes were subjected to mechanical stimulation using a fine eyelash needle, and their locomotory response was observed and categorized as either mobile or immobile. Immobility in each treatment was expressed as number of immobile/total number of nematodes assessed. After the last assessment at 72h, the nematodes were washed using distilled water in a 38 µm sieve to remove the ITC treatment and then transferred into distilled water and incubated for 48h for recovery assessment. The nematode stimulation procedure was repeated to determine whether they were dead or alive, which would indicate that the immobility effect observed was reversible or irreversible. Mortality was expressed as dead/total for each ITC.

3.3 Data Analysis.

All data were analysed using R-studio software (R Core Team, 2022). A Levene test was conducted to compare the variances between the two experiments. Data from two experiments was combined as the variances were homogeneous (P=0.24). Data on nematode mobility was analysed using generalised linear mixed effects model with

concentration and ITC as fixed effects and time as a random effect (package lme4). Data on mortality were analysed by fitting a binomial generalised linear model to predict the response of mortality to the variable's concentration and ITC. The package Emmeans was then used to extract the contrast and mean estimates and for pairwise comparisons with significant differences at $P<0.05$. The package drc (dose response curve) on R-studio software was used to generate a dose response regression model for Lethal dose (LD_{50}) and effective dose (ED_{50}) values and for pairwise comparisons among the different ITCs.

3.4 Results

3.4.1 Effect of ITC on SRN immobility.

The effect of ITCs on SRN mobility was compared using effective dose (ED_{50}) values to determine which concentration causes 50% reduction in mobility at different exposure times, using log logistic models to generate dose response curves (Figure 3.1). The type of ITC and concentration had a significant effect ($P< 0.05$), on the immobility of SRN, where immobility increased in a dose dependent manner. Increase in exposure time had no significant effect on nematode mobility. Immobility was significantly higher ($P<0.05$) after 24h exposure and this did not significantly increase after 48 and 72h (Figure 3.1). The ED_{50} values were 7. 5 and 44 µg ml $^{-1}$ after 24h, 6.5 and 30 µg ml $^{-1}$ after 48h and 5.98, 4.91 and 25 µg ml $^{-1}$ after 72h for AITC, PEITC and SITC respectively. Increase in exposure time caused higher immobilisation of SRN for SITC, however the increase was not significant. A pairwise comparison of the ED_{50} values showed that AITC and PEITC were not significantly different ($P>0.05$), while both were significantly lower compared to SITC for all the exposure times.

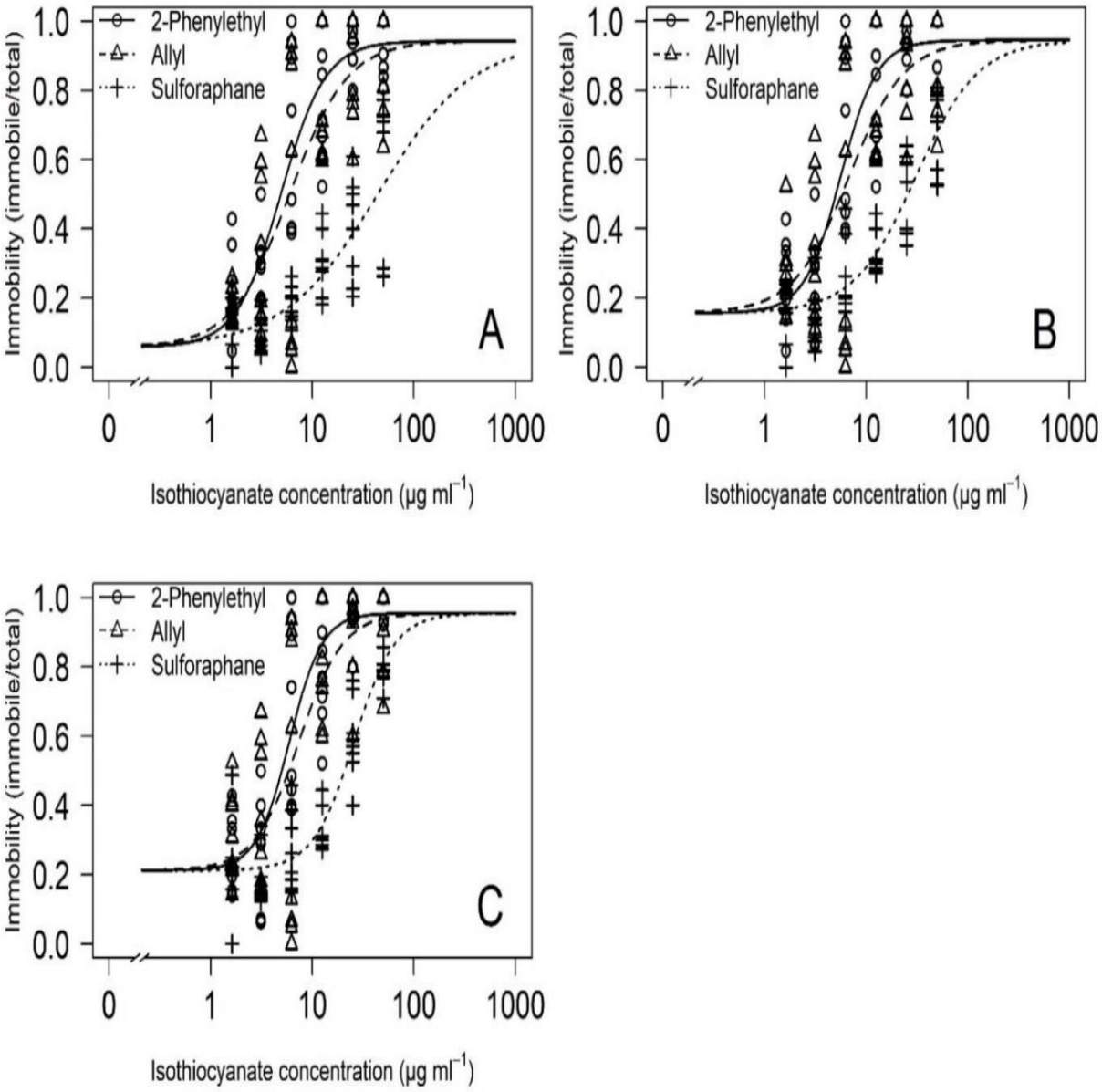

Figure 3.1 Dose response curves for stubby root nematodes (SRN) immobility (n=8) after exposure to 2-Phenethyl (PEITC), Allyl (AITC) and sulforaphane (SITC) for A: 24h; B: 48h and C: 72h at different concentrations.

3.4.2 Effect of ITC on SRN mortality.

All the ITC tested induced a significantly higher mortality in SRN compared to the controls distilled water and DMSO. In general, mortality significantly increased with increase in ITC concentration, and the type of ITC also had a significant effect on the mortality of SRN (P<0. 05), (Table 3.3).

Table 3.3: Analysis of Deviance Table (Type II tests). On effect of the type of isothiocyanate, concentration and their interaction on mortality of stubby root nematodes. Response variable: Mortality.

	LR Chisq	Df	Pr(>Chisq)
Isothiocyanate	34.65	3	1.446e-07 ***
Concentration	251.42	5	< 2.2e-16 ***
Isothiocyanate X Concentration	19.19	10	0.0379 *

LR Chisq – Likelihood Ratio Chi-squared; Df – Degrees of freedom; Pr(>Chisq) – Probability (p-value) of observing a Chi-squared value greater than the test statistic.

There was also a significant, linear positive correlation (R=0.73; P<0.001) between ITC concentration and SRN mortality Nematode mortality was recorded after 48h recovery assessment in distilled water. Mortality for all ITC at all tested concentrations was significantly higher (P<0.05) than the controls distilled water and 1% DMSO and no differences were recorded between the two controls. Steep dose response curves were obtained for each ITC where small increases in ITC concentration led to a significant increase in SRN mortality. The first three doses of AITC and SITC induced a similar degree of mortality effect whereas a dose of 6.25 µg ml^{-1} of PEITC caused seven times more mortality than the 1.625 and 3.125 µg ml^{-1} concentrations (Figure 2). The LD_{50} values for AITC, PEITC and SITC were 10.67, 6.91 and 24.31 respectively. PEITC had the lowest LD_{50} value, which was twice and four times lower than AITC and SITC, respectively. Pairwise comparisons of LD_{50} values generated from the ITCs tested indicated that PEITC was significantly more potent than both AITC and SITC. The LD_{50} of AITC was also significantly lower than SITC. At the highest concentration, 50 µg $ml^{-}1$, overall SRN mortality was 100, 92 and 83% for PEITC, AITC and SITC, respectively (Table 3.4).

Table 3.4: Lethal dose (LD_{50}) estimates values (ug/ml) for SRN (SRN) for Allyl (AITC), 2-Phenethyl (PEITC) and Sulforaphane (SITC) ITCs with standard error (Std.Error), upper limit, lower limit and P-value.

	LD_{50} estimate*	Std.Error	Lower limit	Upper limit	P-value
Allyl (AITC)	10.67[a]	1.95	6.80	14.53	2.20e-07
2-Phenethyl (PEITC)	6.91[b]	0.95	5.04	8.78	2.15e-11
Sulforaphane (SITC)	24.31[c]	4.10	16.20	32.42	2.40e-08

*LD_{50} estimates with different letters within the column are significantly different ($P < 0.05$); data are from two experiments, n = 8.

3.5 Discussion.

Brassicas contain various glucosinolate profiles, which translate to distinct ITCs with different toxicities (Bellostas et al., 2004). The values obtained from the LD_{50} calculations indicated a wide range in toxicity of the ITCs tested in this study. Differences in the structure of the ITCs i.e., the chemical properties of the R side chain can confer differences in their biological activity (Lazzeri et al., 1993). Aliphatic ITCs are known to be more toxic than aromatic ITCs (Lewis & Papavizas, 1971). This has been shown in a study investigating the sensitivity of *Fusarium graminearum* to different ITCs where aliphatic ITCs i.e., allyl (AITC), methyl ITCs (MITC) and ethyl ITCs (EITC) were found to be more toxic compared to aromatic ITCs i.e., 2-phenethyl (PEITC) and benzyl (BITC) (Ashiq *et al*., 2021). Our study showed contrary results where the aromatic ITC (PEITC) was more toxic than aliphatic ITCs.

Similarly, another study reported aromatic ITCs to be more toxic to *M. javanica* and *Tylenchulus semipenetrans* compared to aliphatic ITCs and found no relationship between ITC structure and toxicity to the nematodes (Zasada & Ferris, 2003). This shows that variations exist depending on the target nematode species and pathogens. For instance, non-toxic effects have even been reported when *Caenorhabditis elegans* were exposed to SITC at doses of up to 70 ppm where the exposure instead increased the longevity of *C. elegans*(Qi et al., 2021). On the other hand, the study by Wood et al. (2017) found contrary results where they reported 100% juvenile mortality of *G. pallida* when exposed to 50 µg/ml SITC. Our study agrees with the latter, where at the highest dose of 50µg/ml, SITC caused a

mortality of 83% and had a low LD_{50} value showing its potency to SRN. Despite the mortality variations based on concentration and type of ITC, SRN proved to be sensitive to the ITCs tested. SRN are known to be more sensitive to chemical compounds than other PPNs as shown in an experiment where several PPN species were exposed to CuSO4 or MnSO4.

Stubby root nematodes were more sensitive than other plant parasitic nematodes as none of them were mobile after 36 h exposure time indicating their vulnerability to toxic compounds compared to other PPN (Cooper, 1971). Toxicity of ITCs to nematodes is also known to be influenced by ITC-lipid solubility, ITC volatility and ITC hydrophobicity (Sarwar et al., 1998). Volatile ITCs e.g., 2-propenyl are in gaseous in form and are capable of dispersing evenly under suitable conditions and effectively interacting with the target organism. Lipid soluble ITCs e.g., 2-phenylethyl can penetrate the nematode cuticle and permeate phospholipid membranes, interacting with intercellular functions that kill the organism (Sarwar et al., 1998).

The lipid solubility of PEITC might best explain its toxicity in our study when compared to AITC and SITC. The parent glucosinolate associated with the different ITCs might also have contributed to the variations recorded, as the three different ITCs are associated with different GSLs i.e., sinigrin, gluconasturtiin and glucoraphanin for AITC, PEITC and SITC respectively. In a study investigating different glucosinolates, ITC products of sinigrin, gluconapin, glucotropeolin and glucodehydroerucin were suppressive against *H. schactii* at a concentration of 0.5% after 48 hours exposure time, whereas those from glucoraphanin and sinalbin were not suppressive, indicating that toxicity of the ITC is influenced by the type of GSLs (Lazzeri et al., 1993; Ntalli & Caboni, 2017).

A similar study also showed that the toxicity of ITC to potato cyst nematodes (PCN), (*G.pallida*), was related to the type of GSLs in the brassica species used (Buskov *et al.*, 2002); out of 13 GSLs tested, high mortality of PCN juveniles was recorded after exposure to brassica extracts phenylethyl and benzyl glucosinolates, which are both capable of producing ITCs upon hydrolysis. *Barbarea vulgaris* and *Moricandia moricandioides,* containing indole-glucosinolates, which are unable to produce stable ITCs, lacked efficacy against *G. pallida* (Halkier & Gershenzon, 2006).

The high potency of AITC and PEITC in our study is consistent with studies where AITC and PEITC were recommended as potential candidates in suppression of *T. semipenetrans* and *M. incognita* (Zasada & Ferris, 2003). The potency of AITC has also been reported in increased mortality of *G. pallida* juveniles when exposed to high concentrations of 50 µg/ml compared to benzyl ITC (BITC) (Wood et al., 2017). High immobility was recorded after 24h exposure time for all ITC and concentration tested and increasing the exposure did not cause a significant increase in mortality.

The short time to cause immobility by PEITC and AITC also highlights their potency. Under field situations, a shorter time to induce mortality/paralysis is desirable especially because ITC are very volatile in nature and have a short half-life of 20-60h in the soil environment (Borek et al., 1995). The volatile nature of ITCs has led to research focusing on how to prolong them in the soil to ensure they are not easily lost before encountering the target pest. The concentration present and the exposure time influences the efficacy of ITC on target organism variations in efficacy may also be linked to target nematode species (Zasada & Ferris, 2003). Concentration of ITC in the soil reduces during and soon after the incorporation process. A study monitoring ITC concentration from *B. napus* during biofumigation found that the highest concentration was released within 2 h and 90% of the production was lost after 24 h (Brown et al., 1991). Similar results were found when using *B. napus* and *B. juncea* where highest concentrations were released within 30 mins of incorporation and no ITC were recovered 12 days later (Gimsing & Kirkegaard, 2006; Morra & Kirkegaard, 2002).

All the ITC tested showed very low LD_{50} values showing their potency towards SRN. These concentrations have been reported as achievable under field conditions when certain brassica varieties are used in the biofumigation process. For instance, concentration of 100 μmol g^{-1} dry weight of 2-propenyl which is a glucosinolate that produces AITC, has been recorded in *Brassica nigra* (Bellostas et al., 2007), 93 μmol g^{-1} dry weight in *B. carinata* (Zasada et al., 2009) and 90 µmol g^{-1} dry weight in *B. juncea* leaves (Ngala et al., 2015). Ideally, 2-propenyl GSL concentrations above 13 µmol g^{-1} dry weight can produce at least 50 ppm of AITC. In *R. sativus*, gluconasturtiin GSL, whose derivative is PEITC, has also been detected at concentrations of 53.6 μmol g^{-1} dry weight (Ngala et al., 2015).

Additionally, 14-25 µmol gluconasturtiin g dry $weight^{-1}$ has been recorded in the leaves of Indian mustard (*B. juncea*), black mustard (*B. nigra*) and Ethiopian mustard (*B. carinata*) (Bellostas et al., 2007), while 15.8 µmol g dry $weight^{-1}$ was reported in the root tissue of *B. napus* (Gimsing & Kirkegaard, 2006). As for sulforaphane (SITC), parent GSL glucoraphanin, studies have shown that concentrations achieved are dependent on plant species in question. For instance, Lord et al. (2011) found concentration of less than 3 μmol g dry $weight^{-1}$ in *E. sativa* while Ngala (2015) found higher amounts of 25.4 μmol g dry $weight^{-1}$ in the leaf tissue of *R. sativus*.

The low-level production by some plant species can therefore be mitigated by careful selection of the highest producing brassica species in a biofumigation system to achieve desirable nematode suppression. In conclusion, this study demonstrates the potential of ITCs in suppression of SRN. It also confirms the nematicidal activity of the ITCs tested at concentrations that are achievable under field conditions i.e., 1.625 to 50 µg/ml.

In conclusion, Brassicas such as *B. juncea* and *R. sativus* contain significant levels of sinigrin

and gluconasturtiin respectively, which are the parent GSLs of PEITC, AITC and SITC, and hence these species could be explored more in management of SRN. This study focused on field population of as they occur naturally in sugar beet fields. Therefore, there was a mixture of SRN genera and species, although the most dominant was *T. primitivus*. Other specialised tests can be conducted for *Paratrichodorus* spp which were in negligible numbers in this study to verify whether these compounds are equally toxic and establish an LD_{50} for this species.

Chapter 4: Field evaluation of efficacy of cover crops in suppression of SRN (*Trichodorus* and *Paratrichodorus* spp.).

4.1 Introduction.

Sugar beet (*Beta vulgaris* L.) is the second most important sugar crop in the world after sugar cane (Ahmad et al., 2017). The primary product is sugar and the biproduct, pulp, is mostly utilised for livestock feed. In East Anglia and the East Midlands regions of England, Sugar beet is an economically important crop, occupying in the region of 105,000 hectares, covering 3.7% of the total arable cropping area and supplies 55% of sugar consumed in the United Kingdom (Tzilivakis et al., 2005).

Plant parasitic nematodes, (PPNs) are economically Important pests globally resulting in crop losses which equate to US $80 billion (Nicol et al., 2011). Sugar beet is also subject to infection by a variety of different PPN species such as beet cyst nematode *(Heterodera schactii)* (Wright et al., 2019), root knot nematodes— *Meloidogyne hapla* and *M. chitwoodi* (Griffin et al., 1982) and SRN —*Paratrichodorus* and *Trichodorus* spp. (Hafez, 1998; Whitehead & Hooper, 1970). Stubby root nematodes are polyphagous ectoparasites and are widely distributed in light sandy soils (Cooke, 1973; Whitehead & Hooper, 1970; Winfield & Cooke, 1975).

In a survey conducted in the UK, in 50% of sites positive for SRN, they were found to occur in soils with a sand fraction greater than 80% and a less than 10% silt. *Trichodorus primitivus* was reported as the most prevalent and cosmopolitan species, followed by *P. pachydermus* (Alphey & Boag, 1976). The prevalence of these two species was also recorded in sugar beet fields, in Eastern England, where out of seven species of SRN reported, 35% were *P. pachydermus* and 30% were *T. primitivus* (Whitehead & Hooper, 1970).

In sugar beet grown in East England, SRN are known to attack young seedlings leading to a condition known as Docking disorder, named after the parish “Docking”, where it was first recognised and described (Gibbs, 1959). *Trichodorus* and *Paratrichodorus* genera have been isolated in 75% of samples collected in fields with Docking disorder symptoms (Cooke, 1973). Foliage of sugar beet suffering from Docking disorder appears to be deficient in nitrogen or magnesium (Cooke, 1989; Whitehead & Hooper, 1970).

Sugar beet infected by SRN, have stubby lateral roots, which turn grey- brown and later black as they die and decay. In fields where the symptoms persist, root yield has been found to be up to 17.5 t/ha less and more fangy than those from unaffected fields (Cooke, 1973). Yield losses of up to 50 % have also been recorded because of the fangy root symptoms (Cooke, 1989). The prevalence of Docking disorder has been correlated with environmental and agronomic factors such as rainfall, physical conditions of the soil, previous cropping, rate and

timing of fertiliser application/herbicides. Damage symptoms on the roots are mostly visible at the end of May, which is characterised by high rainfall while damage on the foliage is mostly visible in June (Cooke, 1973).

For many years, management of SRN relied upon the prophylactic use of pesticides including soil fumigants such as 1, 3 dichloropropene. Application was undertaken either shortly after drilling or before sowing sugarbeet (Cooke & Draycott, 1971). In the UK, granular aldicarb was also used at drilling to sugar beet crops at risk from Docking disorder to prevent root damage by SRN, but the expense and inconvenience limited its use (Cooke, 1989). In the recent past, Vydate (Oxamyl), was the nematicide applied by the sugar beet growers but was banned in December 2020, leaving growers with only NEMguard as the chemistry available for the management of SRN. Other crop management strategies therefore need to be evaluated for future recommendations to sugar beet growers.

Use of cover crops (CC), may provide a potential nematode management option as certain CC can reduce nematode populations by either i) non-hosts, poor hosts or resistant host ii) release of secondary metabolites that can have nematicidal, nematistatic or repellent effects iii) Promote the diversity of antagonistic microbial communities by providing an ecological niche and iv) acting as trap crops for nematodes (Wang et al., 2002)— (Figure 4.1). Phytochemicals such as polythienyls and polyacetylenes from widely used CC such from family Asteraceae, 2- dehydropyrrolizidine (PAs) from the families Asteraceae, Boraginaceae and Fabaceae, ITCs from Brassicaceae, saponins from Leguminosae and glucosides from Poaceae have also been shown to suppress nematodes (Thoden et al., 2009). These nematicidal phytochemicals can be exploited through crop rotations, intercropping or use as green manures (Zhou et al., 2012), where they can be released either through volatilization, exudation, leaching from plant roots or through decomposition of plant residues (Dutta et al., 2019; Halbrendt, 1996).

As a green manure Sorghum sudan grass has been shown to effectively suppress *Meloidogyne* spp. (Mojtahedi et al., 1993) and *Criconema. xenoplax* (Nyczepir & Rodriguez-Kabana, 2007) and these effects have been attributed to hydrolysis of dhurrin to hydrogen cyanide which has nematicidal properties (Viaene & Abawi, 1998). Utilisation of rye in a rye-tomato crop rotation, was shown to reduce gall-formation of *M. hapla* (Halbrendt, 1996). *In vitro* assays with hydroxamic acids from rye were later shown to possess nematicidal activity towards *M incognita* and *Xiphinema americanum* (Zasada et al., 2005). Brassicas such as radishes and mustards have also been effectively used in suppression of plant parasitic nematodes in the process of biofumigation under field conditions (Ngala et al., 2014; Lord et al., 2011; Kirkegaard et al., 1993; Kruger et al., 2013).

Additionally, different species in the genus *Medicago* are also known to produce saponins

which have been shown to have different modes of action to different PPNs in *in-vitro* assays e.g., nematicidal effects to *X. index* and *Pratylenchus thornei* (Martín & Magunacelaya, 2005), reduced cholesterol levels in the eggs of *Meloidogyne* spp. (Ibrahim & Srour, 2013) and reduced hatching *G. rostochiensis* (D'Addabbo et al., 2020). These compounds have been further exploited for development of biopesticides for nematode management (Renco et al., 2014). *Endophytic fungus in the genus Epichloë mostly* form a symbiotic relationship with cool season grasses, and these interactions result in production of bioactive secondary metabolites such as peramines, lolines, ergot alkaloids and indole-diterpenes. Tall fescue colonised by the endophyte have been shown to effectively suppress densities of *P. scribineri* (Bacetty et al., 2009) and was considered a poor host to *P. vulnus* (Nyczepir, 2011). In other cases, the grass-endophyte combinations selectively suppress some nematode species of the same genera. For instance, tall fescue variety Jesup-Max-Q, colonised with an endophyte, was also shown to suppress *M. incognita* and *M. hapla* but not *M. javanica* and *M. arenaria* (Nyczepir & Meyer, 2010).

Alkaloids produced because of these interactions have also been shown to possess nematicidal effects where purified alkaloids i.e., ergovaline demonstrated nematicidal effects towards *P. scribineri* under in-vitro conditions Root exudates, shoot and root extracts obtained from endophyte infected tall fescue Jesup (Max Q) infected influenced egg hatch and juvenile activity of *M incognita* (Meyer et.al., 2013). Under field conditions, incorporation of biomass from this cover crop material provides numerous benefits such as promoting proliferation of bacteria during decomposition of the organic material, hence becoming a source of food for microbiovorous nematodes and in turn becoming a food base for nematophagous fungi which are suppressive to PPNs (Carrascosa et al., 2014; Van Den Boogert & Deacon, 1994).

The aim of the study was to investigate the effect of growing cover crops from diverse plant families on the suppression of SRN under field conditions.

4.2 Null hypotheses:

I. Biofumigation using brassica cover crops does not affect densities of SRN.

II. Cover crops from diverse plant families and soil disturbance have no effect on population densities of SRN and subsequent sugar beet yield and quality parameters.

III. Cover crop mixtures have no effect on population densities of SRN.

4.3 Materials and Methods.

4.3.1 Field experiment 1: Effect of growing and incorporating brassicas in suppression of SRN.

4.3.1.1 Site description.

The first field trial was conducted in a site located in Bury St Edmunds, Suffolk, 52°17'03.0"N 0°43'39.8"E and had a sandy loam soil with 87.7% sand, 7.1% silt, 5.2% clay, pH= 8.07 and 10.12% organic matter—Site 1. This site was selected as it had a previous history of SRN infestation. The experiment was conducted from 29th June 2021 to 15 December 2021. The CC used in these experiments were selected based on their potential as suppressive CC and commercial availability. Moreover, the brassica CC were selected because of their known high production of bioactive compounds i.e., ITCs that have shown to be suppressive to other nematode species.

4.3.1.2 Experiment design and treatments.

The experiment was laid out in a randomised complete block design (RCBD), comprising plots measuring 22 by 4m, with a 6m buffer between blocks. Four treatments (Table 4.1) were assigned across five blocks. Blocks were spaced 6m apart to allow movement of machinery without damage to nearby plots.

4.3.1.3 Soil sampling.

Soil samples were collected i) prior to cover crop drilling, to establish the initial nematode densities (Pi), ii) four weeks after cover crop drilling (4WAP), iii) before cover crop incorporation (Bi), iv) post-incorporation of cover crop residues (Ai). Sampling was performed on a 15m by 3m area within the plot, targeting the middle of the plot in a W sampling pattern. A total of 28 cores were randomly sampled at a depth of 30cm to obtain a composite sample of 1-1.5kg from each plot. Figure 4.1 shows the sampling pattern used. Monthly rainfall and soil temperature data for the different sampling dates was obtained from the nearest meteorological station, i.e., Brooms Barn Research Station which is 5.3 kilometers away. Nematodes were extracted using centrifugal floatation method and quantified as described in section (2.2.2).

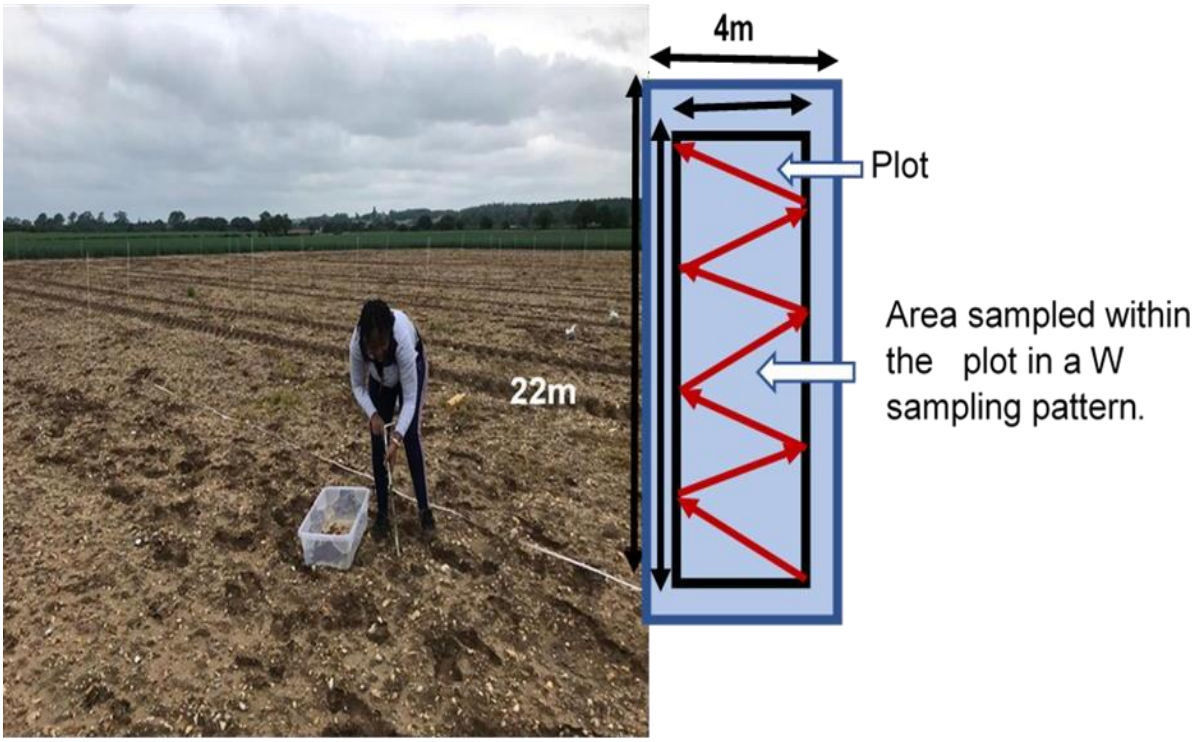

Figure 4.1: Soil sampling of experimental plots at the Bury St. Edmunds site, Suffolk**.**

4.3.1.4 Field operations.

Brassica CC in site 1 were drilled on 29th June 2021 following an onion crop. The seed rate recommended by the seed supplier was used (Table 4.1). Plots were uniformly treated with sulphur and nitrogen fertilizer (Yara Bela AXAN) at a rate of 100kg N and 34kg S ha^{-1} at planting as is the recommended practice (Cite AHDB Report by Matt Back) when growing brassica CC for biofumigation. Cover crops were flailed and incorporated on 9th September 2021, where the CC grew for 71 days.

Prior to incorporation of the brassicas, two subplots measuring 0.33 m^2 were selected for biomass samples. The sub plots were randomly selected at distinct locations within the plot and assessed for plant density; average plant counts were used to represent the number of plants of the whole plot. Ten plants were collected per plot, and fresh weight was measured. Dry weight measurements were also taken by first drying the plants at 60ºC for 72h h. At incorporation, the green tissue was flailed using a flail topper and incorporated within the top 30 cm of soil using a rotary tiller. The soil surface was immediately rolled using a Cambridge roll to trap volatile compounds produced by the brassica CC.

Table 4.1: Treatments used in Suffolk (site 1) to assess the effect of cover crops (CC) on field populations of Stubby root nematodes (SRN).

Species name	Common name	Variety	Seed rate kg ha^{-1}	Seed supplier
Brassica juncea	Indian mustard	Brons	10	Joordens Zaden BV
Raphanus sativus oleiferus	Oil seed radish	Terranova	20	Joordens Zaden BV
Raphanus sativus longipinattus	Daikon radish	Daikon	20	RAGT seeds UK
Fallow	Control	Control	------	

4.4 Field Experiment 2: Effect of cover crops from diverse families on the suppression of SRN and subsequent effect on sugar beet yield and quality.

4.4.1 Experiment design and treatments.

Field experiment was conducted at a site located in Docking, Norfolk Eastern England 52°54'01.7"N 0°36'32.4"E. This site had sandy loam soil with 88.2% sand, 5.9% silt, 5.9% clay, pH= 7.15 and 9.22% organic matter. The site was selected for its history of Docking disorder in sugar beet caused by SRN. The CC used in this experiment were selected based on their potential to suppress SRN and their commercial availability as indicated in Table 4.2. The field experiment was conducted from July 2021 to January 2023. The experiment was laid out in a randomised complete block design (RCBD). Seven cover crop species from four different plant families i.e., Papaveraceae, Brassicaceae, Boraginaceae and Poaceae, were evaluated as highlighted in (Table 4.2).

Treatments were assigned to plots measuring 9 by 6m and were arranged in four blocks with a 6m buffer between the blocks. Three fallow controls were included at this site: i) Sterile fallow – in this fallow, glyphosate (SHRAPNEL®) was applied as a post-emergence herbicide to ensure that it was weed free; ii) Disturbed fallow – This fallow was disturbed by rotavating the soil in a similar way to plots containing CC during the flailing and incorporation period; iii) Undisturbed fallow – this fallow was left undisturbed, and no weed management was undertaken.

Table 4.2: Treatments used in Suffolk (site 1) and Docking (site 2) to assess the effect of cover crops (CC) on field populations of SRN.

Species name	Common name	Family	Variety	Seed rate kg ha-1	Seed supplier
Brassica. juncea	Brown/Indian Mustard	Brassicaceae	Brons	10	Joordens Zaden BV
Raphanus sativus oleiferus	Oilseed radish	Brassicaceae	Terranova	20	Joordens Zaden BV
Raphanus sativus Longipinattus	Daikon radish	Brassicaceae	Daikon	20	RAGT seeds UK
Festulolium loliceum	Hybrid Grass with endophyte (E+)	Poaceae	Green solutions	25	Cropmark Seeds NZ
Festulolium loliceum	Hybrid grass without endophyte (E-)	Poaceae	Green solutions	25	Cropmark Seeds NZ
Papaver. somniferum	Opium poppy	Papaveraceae	Marianne	1.5	Joordens Zaden BV
Phacelia tanacefolia	*Phacelia*	Boraginaceae	Factotum	8	Joordens Zaden BV
Lolium multiflorum	Ryegrass - susceptible control	Poaceae	Syntilla	25	RAGT seeds UK
Fallow disturbed	Control		---------	----------	
Fallow undisturbed	Control		---------	----------	
Sterile fallow	Control		---------	---------	

4.4.2 Field operations.

Cover crops were drilled on 28th July 2021 following a crop of spring barley (Figure 4.3). Glyphosate was applied in the sterile fallow treatments four weeks after CC had been drilled to manage weeds. Plots drilled with brassica CC were uniformly treated with sulphur and nitrogen fertilizer at the same rate as site 1.

Figure 4.2: Field operations during the field experiment at Docking, Norfolk: Cover crop drilling (A), Flailing (b), incorporation of cover crop material into the soil (C) and rolling to seal the soil incorporated with brassica material using a roller (D).

The rest of the CC were not fertilised to mimic farmer practices, where no fertiliser is applied. The various field operations and timings for each field experiment are summarised in Table 4.3. The CC grew for 113-days post drilling, where they were flailed and incorporated on the 19th of November 2021; prior to incorporation two subplots measuring 0.5 m^2 were selected for biomass samples. Incorporation at this site was done later due to slow brassica crop emergence due to the lower soil temperatures. The biomass was measured as described for site 1. At incorporation, the green tissue was flailed using a flail topper followed by incorporation within the top 30 cm of soil with a rotary tiller.

The soil surface was immediately rolled using a Cambridge roll to trap volatile compounds produced by the brassica CC. The same process was applied for the disturbed fallow control, where soil was rotavated and rolled to create a disturbance effect. Brassica samples for glucosinolate analysis were randomly taken from each plot. Three plants per plot were collected and taken to the lab for processing. The samples were flash frozen using liquid nitrogen and stored -80ºC. The samples were later frozen (GVD6/13 MKI freeze dryer,

GIROVAC Ltd, North Walsham, UK) for a week and milled into a fine powder. The samples were sent to NIAB lab test, Cambridge, UK, for GSL analysis, performed following ISO 9167 “Rapeseed and rapeseed meals- Determination of GSLs content – Method using HPLC.

Table 4.3: Timings of management practices used during the field experiments conducted at Bury St. Edmunds, Suffolk (site 1) and docking, Norfolk (site 2)

Field operations	Site 1	Site 2
Sampling for initial SRN densities (Pi)	29th June 2021	28th July 2021
Cover crop drilling	1st July 2021	30th July 2021
Sampling after cover crop drilling (4 WAP)	28th July 2021	25th August 2021
Sampling before cover crop incorporation	7th September 2021	16th November 2021
Biomass assessment of Brassica species	7th September 2021	16th November 2021
Cover crop incorporation	9th August 2021	19th November 2021
Sampling after incorporation of cover crop material	15th December 2021	4th February 2022
Sugar beet drilling	---------	3rd March 2022
Sugar beet harvest and Sampling for final densities of SRN (Pf)	---------	5th January 2023

4.4.3 Soil sampling and nematode assessments.

Soil samples were collected at different dates, before and during cover crop growth and at harvest of the main crop (sugar beet) as summarised in Table.4.3. A W shape sampling pattern was used with random sampling points along the pattern. Soil samples were collected at 30 cm depth using a 2 cm diameter corer (Figure 4.3). At each sampling point, detritus on the soil surface, such as dead plant material, were removed before sampling (Boag et al., 1989). At least 28 cores were taken from each plot to make a 1-1.5kg composite sample. Soil samples were collected before cover crop drilling (Pi), four weeks post drilling cover crops (4WAP), before cover crop incorporation (Bi) and at sugar beet harvest (Pf). Soil was carefully placed in labelled plastic bags and stored in the cold-room at 4˚C to await extraction. Monthly rainfall and soil temperature data for the different sampling dates was obtained from the nearest meteorological station i.e., Denver station.

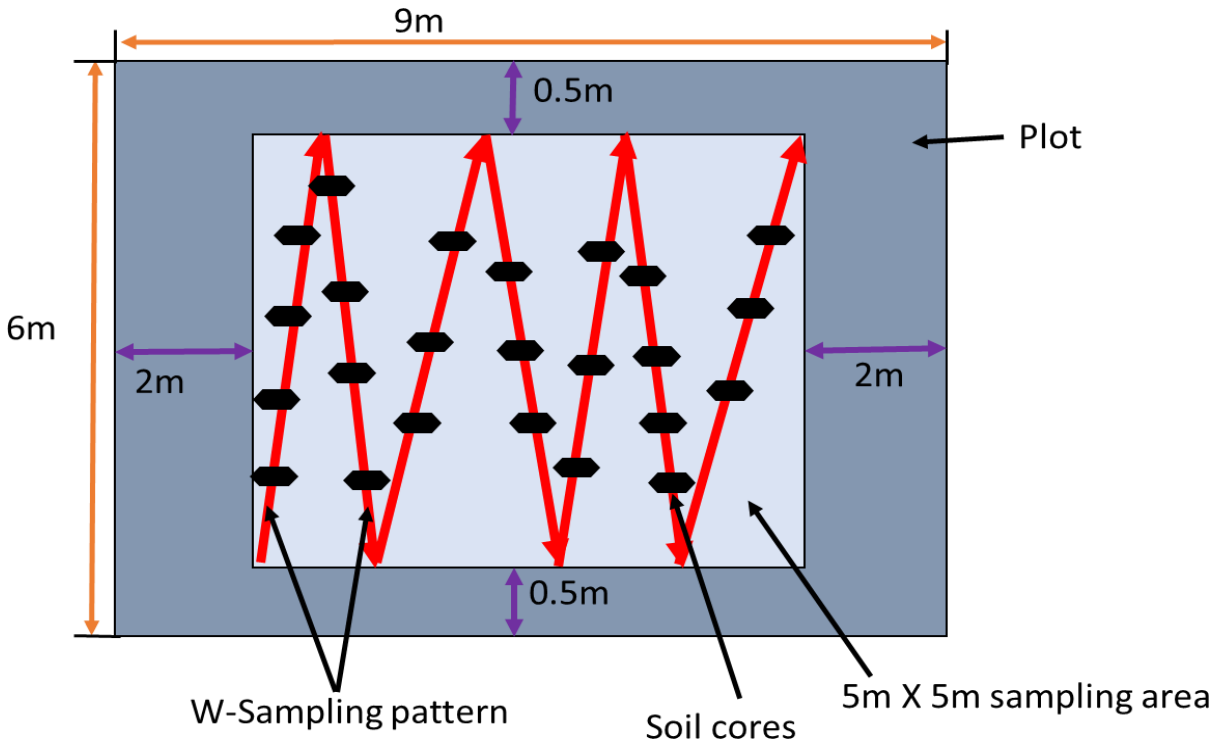

Figure 4.3: Soil sampling pattern and sampling points on a 5 x 5m^2 sampling area for each plot (Site 2).

4.4.4 Nematode extraction, Identification, and quantification.

Stubby root nematodes (SRN) were extracted using centrifugal flotation method using magnesium sulphate heptahydrate ($MgS04.7H20$) as the extraction solvent (Bezooijen, 2006). The bulk sample collected from the field was gently mixed and passed through a 4mm sieve to remove large stones before taking a 200ml subsample for extraction. The soil was divided into four 50 ml centrifuge tubes and 80ml of $MgS04.7H20$ at 1.15 specific gravity added into each tube. The tubes were gently agitated to mix the extraction fluid and the soil and then centrifuged at 2680 RCF (1150 g) for 5 minutes.

The supernatant was then decanted into 215 µm and 53µm sieves and gently rinsed in tap water before being washed into sample bottles. The suspension was concentrated into a smaller volume which was wholly quantified under a compound microscope at 20x magnification. Morphological characteristics e.g., spicule shape in males, body cuticle and vaginal characteristics of the females was used to distinguish the genus and species *of Trichodorus* and *Paratrichodorus* spp. following the key as described by Decraemer (1995).

4.4.5 Sugar beet yield and quality assessments.

The main crop— sugar beet was drilled on 3rd March 2022, three months after cover crop incorporation. Sugar beet crops were grown for 10 months and harvested on the 5th of January 2023. At harvest, two rows of sugar beet per plot were lifted by hand and a total of 25-30 roots were scored for fanging using a fanging score scale (Figure 4.5). Fanging percentage for each plot was calculated using the formula: Fanging percentage % = $(\frac{2b+2c+d+e}{a+b+c+d+e})$ ×100 (Cooke, 1973). The whole plot was then harvested, bagged and sent for sugar and impurity analysis at the BBRO tare house at the Wissington sugar beet factory (Norfolk, UK). Soil tare (the amount of soil which adheres to the storage root at harvest) was determined by weighing each sample while dirty and then washed and reweighed to obtain clean sample weight; Soil tare % was calculated to express the proportion of dirt as follows: Soil tare % = $(\frac{Dirty\ sample\ weight - clean\ sample\ weight}{Dirty\ sample\ weight})$ ×100 (.Wright et al., 2022).Polarimetry was used to calculate the sugar content while the concentration of the impurities was determined using flame photometry (sodium and potassium impurities) and colorimetry (amino nitrogen) according to standard methods. The yield of sucrose was calculated as sugar % multiplied by clean weight of the samples (Whalley, 2013).

Fanging Score scale:

a— No evidence of fanging; b— Roots moderately fangy but main tap root evident; c— Moderately fangy with bearding evident ; d— Roots exhibiting severe fanging; e— Very severe fanging with tap root absent.

Figure 4.4: Root fanging scores for sugar beet roots at harvest a— no evidence of fanging, b— moderately fangy, with main taproot evident, c— moderately fangy with bearding evident, e— very severely fanged and possessed no main tap root.

4.5 Field experiment 3: Effect of cover crop mixtures and single cover crops on population densities of SRN.

4.5.1 Site description and experiment design.

The trial was carried out at Harper Adams field site in Tibberton Grange 52°46'00.8"N 2°28'15.1"W, Grid Reference SJ683188, which had a clay loam soil with 38% sand, 22% silt, 30% clay, pH= 6.4 and 7.5% organic matter. The experiment was laid out in a randomised complete block design with six cover crop treatments in four blocks. The field experiment was conducted from September 2022 to April 2023. The CC used in these experiments were selected based on their suitability as forage for sheep. Five cover crop species and a cover crop mix were tested as highlighted in Table 4.4. Cover crops were assigned to plots measuring 6 by 6m in four blocks with 3m buffer strips separating the blocks. Samples were taken before cover crop drilling (i) before sheep were let to graze on the plots (ii) and after sheep grazing (iii). The change in SRN densities were then monitored. Soil sampling was carried out as described in experiment 2.

Table 4.4: Treatments used in Tibberton Grange (site 3) to assess the effect of cover crops (CC) on field populations of stubby root nematodes (SRN).

Species name	**Common name**	**Variety**	**Seed rate Kg. ha^{-1}**
Brassica. rapa	Stubble turnips	Samson	7.5
Avena strigosa	Black Oats	Strigosa	75
Trifolium alexandrinum	Clover	Berseem	30
Raphanus sativus oleiferus	Radish	Siletta nova	20
Vicia sativa	Vetch	Common vetch	100
Mixture	Vitality mix	K56 Soil	20-25

4.6 Data analysis.

All data was analysed using R-studio software (R Core Team, 2022). Data from each sampling date was also analysed separately because there were sampling dates by treatment interactions. Data was analysed using Poisson generalised linear mixed effects model (GLMMs) with block as a random effect and cover crop treatments as fixed effect. The nematode initial densities were included as a covariate in the model for site 1 and site 2 when analysing differences between treatments at each sampling point to account for the baseline differences of the initial SRN densities between the plots prior to cover crop drilling. The package Emmeans was used to generate contrasts and significance level at ($P<0.05$). Linear mixed effects models were used to analyse cover crop biomass, sugar beet root yield and other sugar beet quality parameters, with block as a random effect and treatment as the fixed effect. Data on the effect of environmental variables on SRN densities was also analysed using Poisson GLMMs with block and treatment as random effects and environmental variables as fixed effects. Spearman rank correlations coefficients were generated to determine the relationship between the different yield parameters using ggscatter in library ggpubrr on R studio.

4.7 Results

4.7.1 Species composition.

At Bury St. Edmunds, Suffolk (site 1), the study evaluated the effect of growing and incorporating brassica CC in the process of biofumigation (Table 4.2). SRN (SRN) densities were monitored at different time points during plant growth and post termination of the CC (Table 4.3). The SRN species present in this site were identified as *T. primitivus* and *P. pachydermus* and the composition ratio was 1:1 for the two species with more juveniles present than males and females (Table 4.5). Diagnosis of SRN species using molecular methods was not very conclusive due to challenges of cross-reactivity of the primers. Resulting in the species-specific primers binding to and amplifying DNA from other, unintended species which resulted in false positive results.

Table 4.5: Composition of SRN species (SRN) (means) ± SE, extracted from 200ml soil samples (n=10) at Docking, Norfolk (Site 2) and Suffolk (Site 1).

	Site 1			Site 2		
Stubby root nematode - species	Males	Females		Males	Females	
T. primitivus	14± 5.09	10± 5.24		15.53±2.20	48.40±5.11	
T. cylindricus	--	--		4±0.68	7.6±1.03	
P. pachydermus	8± 4.06	16± 5.33		1.35±0.28	2.5±0.40	
Juveniles			43± 8			7.10±0.87

4.7.2 Cover crop effects on SRN densities in Suffolk.

The general trend was a decline of SRN densities from initial densities (Pi) after cover crop incorporation (Ai), with variations observed at different sampling points. At four weeks post cover crop drilling (4 WAP), plots drilled with brassica CC had significantly lower $P<0.05$, SRN compared to the fallow control (Table 4.6).

Table 4.6: Change in SRN densities in 1L soil at different sampling times during cover crop growth and post incorporation of cover crop residues at Bury St. Edmunds, Suffolk (Site 1). Values are means ±standard errors (n = 5). Different letters in the same column indicate significant effect of cover crop treatment (P≤0.05).

Treatment	Pi	4 WAP	Bi	Ai	Rf (Ai/Pi)
Daikon radish	34 (±7.95) ^{ab}	8 (±1.20) ^{b}	8(±3.65) ^{ab}	21 (±11.10) ^{ab}	0.73 (±0.37) ^{c}
Indian mustard	58 (±26) ^{bc}	11 (±1.85) ^{b}	1 (±0.50) ^{b}	8 (±1.95) ^{a}	0.29 (±0.15) ^{a}
Oilseed radish	88 (±19) ^{c}	15 (±4.15) ^{b}	1 (±0.50) ^{b}	23 (±5.80) ^{b}	0.29 (±0.07) ^{a}
Fallow	26 (±6.95) ^{a}	58 (±8.45) ^{a}	14 (±2.90) ^{a}	12 (±3.35) ^{ab}	0.47 (±0.07) ^{b}

Pi; Initial densities before drilling cover crops, 4 WAP; Four weeks after drilling cover crops, Bi; Before cover crop incorporation, Ai; Six weeks after cover crop incorporation, Rf; Reproduction factor ratio.

However, briefly before incorporation of the cover crop material (Bi), the soil was ploughed to loosen it before sampling, which created a disturbance effect in all plots, therefore no differences in SRN densities were recorded among all plots at this sampling point. A resurgence in SRN densities was then observed six weeks after cover crop incorporation (Ai), due to cultivation of a susceptible host crop (winter wheat) two weeks before sampling. Plots following Indian mustard had significantly lower SRN densities compared to daikon radish at this sampling point. Results from calculation of the reproduction factor (Rf), calculated as Ai/Pi, showed that SRN significantly reproduced in plots following daikon radish, followed by fallow control while plots following oilseed radish and Indian mustard had significantly lower Rf compared to the fallow control and daikon radish (Table 4.6).

4.7.3 Brassicas biomass and Glucosinolate profile.

The brassica CC at site 1 established well achieving high fresh shoot biomass of 42, 46 and 49 t ha^{-1} for daikon, oilseed radish and Indian mustard respectively, and no significant differences were recorded in both root and shoot biomass among the three brassica CC. At site 2, the fresh shoot biomass for both Indian mustard and oilseed radish were significantly lower when compared to the biomass achieved in site 1, which was more than double, and no significant differences were recorded between them. However, for the fresh and dry root weight at site 2, oilseed radish was higher as compared to Indian mustard (Table 4.7).

Table 4.7: Shoot and root fresh and dry weights (t. ha^{-1}) of Indian mustard, Daikon radish and oilseed radish at Suffolk (Site 1) and Norfolk (Site 2).

Cover crop	Site 1				Site 2			
	Fresh weight		Dry weight		Fresh weight		Dry weight	
	Shoots [b]	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
Indian mustard	48.53± (5.43)	4.25± (0.47)	5.75± (0.66)	0.77± (0.80)	17.67± (9.17)	1.2± (0.71) [a]	3.97± (2.47)	0.33± (0.19) [a]
Daikon radish	45.65± (6.95)	6.73± (1.29)	4.81± (0.73)	1.03± (0.16)	---------	---------	---------	---------
Oilseed radish	42.42± 4.48	5.72± (0.95)	4.16± (0.37)	1.19± (0.14)	12.18± (4.45)	8.86± (4.65) [b]	0.89± (0.23)	1.92± (1.31) [b]

[a] Values are the average of 5 blocks for site 1 and 4 blocks for site 2 [b] Means in same column followed by different letter are significantly different according to Tukey HSD (P≤0.05)

A total of nine GSLs were detected in the shoots of both *R. sativus* and *B. juncea* while ten were detected in the roots of both brassicas. The glucosinolate progoitrin was only present in roots and absent in shoots for both brassicas (Table 4.8). The most abundant GSL in shoots of *R. sativus* was sinigrin, followed by glucobrassicin and glucoraphasatin, which were significantly high compared to the other GSLs. In the roots of *R. sativus,* the most predominant GSL was glucoraphasatin followed by sinigrin, where they were significantly higher compared to the other GSLs. Glucoraphasatin was in a significantly high concentration in roots and shoots of *R. sativus* as compared to shoots and roots of *B. juncea.*

The most predominant GSL in shoots of *B. juncea* was sinigrin, which was significantly higher compared to all other GSL s recorded in the shoots. In the roots, sinigrin was also predominant, followed by gluconasturtiin and both were significantly higher compared to all other GSLs recorded. All the other GSLs recorded were not significantly different in the two brassicas. The total GSLs in the shoots were not significantly different between the two brassicas, however in the roots, the total GSLs were significantly lower in *B. juncea* when compared to *R. sativus*, which was three times higher.

Table 4.8: The average (µmol/g dry weight) GSL concentrations ± standard error of the mean (SE) found in *R. sativus* and *B. juncea*, used in field experiment 2 (Docking, Norfolk). For each GSL, the means that have the same letter are not statistically different according to Tukey's multiple range test (P < 0.05). Standard error of the means is shown in parentheses.

Glucosinolate	Brassica species			
	R sativus (µmol/g)		*B. juncea* (µmol/g)	
	Shoots	Roots	Shoots	Roots
Glucoberin	0.8± (0.37) ^{abc}	0.3± (0.07) ^{abc}	0±0.0 ^{a}	0± (0.0) ^{a}
Progoitrin	ND	0.225± (0.03) ^{abcdef}	ND	0.2± (0.0) ^{abc}
Sinigrin	13.4± (1.84) ^{hi}	7.75± (1.70) ^{ghi}	20.68± (2.28) ^{i}	6.575± (0.36) ^{ghi}
Glucoraphanin	0.425± (0.06) ^{abcd}	0.525± (0.09) ^{abc}	0±0.0 ^{a}	0.875± (0.875) ^{a}
Gluconapin	0.025± (0.025) ^{abc}	0± (0.0) ^{abc}	0.175± (0.025) ^{a}	0± (0) ^{a}
4 hydroxy glucobrassicin	0.05± (0.03) ^{abc}	0.075± (0.05) ^{abc}	0± (0.0) ^{a}	0± (0.0) ^{a}
Glucoraphasatin	5.075± (1.19) ^{efghi}	23.2± (2.93) ^{i}	0± (0.0) ^{a}	0± (0.0) ^{a}
Glucobrassicin	7.45± (1.25) ^{ghi}	0.725± (0.09) ^{abcdef}	0.225± (0.05) ^{abcd}	0.1± (0.0) ^{abce}
Gluconasturtiin	1.45± (0.18) ^{abcdef}	1.2± (0.04) ^{abcdef}	0.925± (0.13) ^{abcd}	3.175± (0.125) ^{efghi}
Neooglucobrassicin	0.25± (0.03) ^{abc}	0.125± (0.03) ^{abc}	0.3± (0.08) ^{a}	0.375± (0.09) ^{a}
Total GSLs	28.925± (4.16) ^{a}	34.125± (3.10) ^{a}	22.31± (2.47) ^{ab}	11.3± (1.06) ^{b}

4.7.4 Cover crop effects on SRN densities in Docking, Norfolk.

At Docking Norfolk (Site 2), CC from diverse plant families were evaluated (Table 4.2). At this site, SRN densities were monitored before and during cover crop growth and at harvest of the main crop sugar beet (Table 4.3). The SRN species identified in this site were *T. primitivus (80%)*, *T cylindricus 15%,* and *P pachydermus* (5%), where there were more females than males and juveniles (Table 4.5).

The brassica cover crops on this site did not establish well. The fresh shoot biomass of Indian mustard was two times lower while the fresh shoot biomass for oilseed radish was four times lower in site 2 compared to site 1 (Table 4.7). Prior to cover crop drilling, the initial densities (Pi) were not uniform, as shown in (Table 4.9, where some plots had significantly higher SRN densities than others. In that case Pi was used as a covariate in analysing to account for the baseline differences. Four weeks after cover crop drilling (4Wap), all treatments were not significantly different from the fallow undisturbed control, except for oilseed radish which was significantly lower and Nil- endophyte grass (E-), which was significantly higher.

During cover crop growth, before incorporation of biomass (Bi), there was a spike in SRN densities when compared to the previous sampling points. However, the rate at which the increase occurred, significantly differed among the different CC ($P<0.05$). All the fallow treatments at this sampling point were not significantly different but had significantly lower numbers compared to plots drilled with cover crops. Nil-endophyte grass (E-) had significantly higher SRN densities compared to all the treatments, whereas CC like opium poppy, *Phacelia* and Italian rye grass had significantly lower SRN densities when compared to Indian mustard, oilseed radish and endophyte grass (E+).

There was a reduction in SRN densities after flailing and incorporation of cover crop materials (Ai); SRN densities decreased in all plots except in the fallow undisturbed plots and in plots that previously had Italian ryegrass, where densities increased instead. A resurgence of SRN densities (Pf) was recorded at sugar beet harvest, where an increase was observed in all the plots. However, variations were observed in the plots following the different cover crops. Plots following Nil-endophyte grass (E-) had significantly higher SRN densities compared to all the treatments. Plots following fallow disturbed, and *Phacelia* had significantly lower nematodes compared to the fallow undisturbed, followed by opium poppy, sterile fallow plots, oilseed radish and Endophyte grass (E+). The densities recorded at sugar beet harvest were positively correlated with densities recorded before sugar beet drilling (Ai), $R = 0.45$, $p=0.0014$; plots that had highest densities post incorporation (Ai) also had the highest final nematode densities at sugar beet harvest (Pf).

The effect of soil disturbance during the cover crop incorporation was observed where the fallow disturbed plots had significantly lower nematodes compared to the fallow undisturbed. The sterile fallow which was weed free also had significantly lower nematodes than the undisturbed fallow. An overall reproduction factor (Rf) was calculated from cover crop drilling to sugar beet harvest (Table 4.9). The reproduction factor based on densities recorded at sugar beet harvest /densities prior to cover crop drilling, indicated that different CC multiplied nematodes at different rates. Differences were observed between and within different cover crop species. *Phacelia* and opium poppy had significantly lower multiplication rates when compared to CC like Indian mustard, Italian ryegrass, and non-infected grass, which multiplied the nematodes. Variations were also observed within plant families e.g., Indian mustard multiplied SRN three times more than the oilseed radish. Similarly, the Endophyte grass (E+), had seven times fewer SRN than the Nil-endophyte grass (E-).

Table 4.9: Change in average stubby root nematode densities in 1L soil ±SE (standard error) at different sampling times at Docking Norfolk (Site 2). Treatments with similar letters are not significantly different at each sampling point.

Treatment	Pi	4 WAD	Bi	Ai	Pf	Rf (Pf/Pi)
Opium poppy	152.5 (±20.87) [c]	123.75 (±34.90) [a]	352.5 (±87.57) [cd]	271.25(±107.03) [bc]	562.5 (±102.66) [f]	3.69 (±0.47) [ab]
Phacelia	152.5 (±31.26) [c]	146.25 (±54.37)[ab]	342.5 (±97.56) [cd]	306.25 (±95.97) [ac]	396.25 (±59.03) [d]	2.59 (±0.69) [a]
Indian mustard	101.25 (±52.69)[bc]	90 (±18.37) [a]	463.75 (±94.46) [b]	327.5 (±120.70) [ab]	1043.75(±140.60) [b]	10.31 (±3.10)[def]
Oilseed radish	126.25 (±27.32) [ac]	36.25 (±3.75) [c]	491.25 (±67.47) [b]	371.25 (±46.25) [ad]	688.75 (±131.82) [c]	5.44 (±0.67) [bd]
Endophyte grass	140 (±53.81) [ac]	125 (±43.54) [a]	438.75 (±42.93) [bc]	241.25 ±73.78) [bc]	726.25 (±81.43) [c]	5.19 (±1.63) [d]
Nil-endophyte grass	93.75 (±22.67) [ab]	190 (±34.09) [b]	618.75 (±128.97)[e]	263.75 (±68.78) [bc]	1221.25 (±230.45)[a]	13.02 (±4.68) [ef]
Italian ryegrass	98.75 (±11.97) [bc]	126.25 (±55.35) [a]	355 (±96.48)[cd]	375 (±112.53) [a]	987.5 (±188.91) [b]	10 (±3.11) [ef]
Fallow disturbed	62.5 (±17.85) [b]	103.75 (±38.48) [a]	260 (±88.34)[ad]	270 (±106.69) [bcd]	417.5 (±96.88) [de]	6.68 (±1.53) [de]
Sterile fallow	153.75 (±64.53) [c]	141.25 (±43.99) [ab]	246.25 (±78.69) [a]	232.5 (±61.59) [c]	537.5 (±101.75) [ef]	3.49 (±0.69) [abd]
Fallow undisturbed	90 (±22.73) [ab]	91.25 (±15.75) [a]	215 (±64.06) [a]	341.25 (±84.37) [ab]	1080 (±66.90) [ab]	12 (±5.30) [f]

Pi: Initial densities before drilling cover crops, 4 WAD; Four weeks after drilling cover crops, Bi; Before cover crop incorporation, Ai; Six weeks after cover crop incorporation, Pf; Final densities at sugar beet harvest, Rf; Reproduction factor.

4.7.5 Effect of environmental variables on SRN Densities.

The sampling dates for SRN densities were characterised with differences in the soil temperature and rainfall (Figure 4.5). At site 1, the sampling dates were in the same season in 2021. The soil temperatures were consistent in June, July and September with sampling points ranging from 18-21°C, except for the last sampling date in December when the soil temperatures reduced to an average of 6°C. The rainfall was high in July (4mm) and decreased in September (<1mm) and then increased to 2.8mm towards end of the year in December 2021. At this site, weak relationships were recorded between SRN numbers and soil temperatures (R=0.28, p=0.011) and rainfall (R=0.22, P=0.052) – Figure 4.5- C and D. At site 2, the average soil temperatures at sampling in 2021 were higher ranging from 10 to 20°C compared to 2022 and 2023 where soil temperatures ranging from 3 to 5°C at time of sampling. A negative relationship between the soil temperatures and SRN densities was recorded (R=-0.71, P<2.2e-16) (Figure 4.5-A). Rainfall at sampling was lowest in February 2022 and July 2021, and highest towards the end of the year in November 2021. The highest rainfall amount at sampling was recorded in January 2023 and a positive relationship was recorded between high rainfall amount and increasing nematode densities (R=0.48, P=5.6E-13) — Figure 4.5-B.

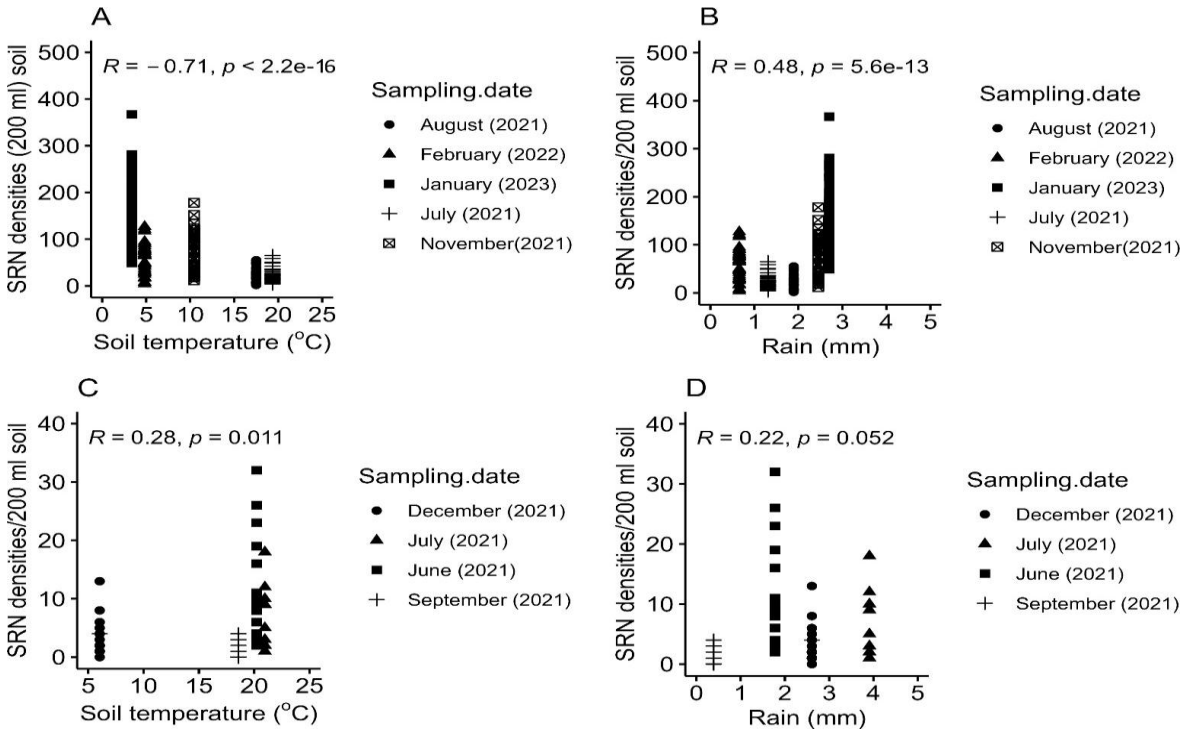

Figure 4.5: Relationship between average monthly soil temperature and rainfall on number of stubby root nematodes in 200ml soil, at different sampling dates in Docking, Norfolk—Site 2 (A and B) and in Bury St Edmunds, Suffolk— Site 1 (C and D).

4.7.6 Effect of cover cropping on sugar beet parameters.

Sugar beet root yield was not significantly between by the CC treatments (Table 4.10), however there was a negative correlation between root yield and SRN densities four weeks after cover crop planting, R=-0.61, P<0.001, where plots with high SRN densities at 4 weeks after cover crop planting ended up having lower root yield. High root yield was also positively correlated to sugar percentage; plots with lower root yield had lower sugar %, R=0.47, P=0.002. Cover cropping also had no effect on sucrose yield and the differences observed were mainly due to block effect rather than treatment effect. Sucrose yield was positively correlated to sugar %, R=0.46, P = 0.003. Sodium, potassium, and amino-nitrogen impurities were positively correlated with each other and with root yield.

Despite the root yield being unaffected by cover cropping, the root fanging was significantly affected by the treatments. SRN densities recorded at sugar beet planting (Ai), were positively correlated with the degree of fanging at sugar beet harvest R =0.45, P= 0.003. The root fanging percentage was significantly higher ($p<0.05$) in plots previously drilled with Indian mustard, Italian ryegrass and fallow undisturbed plots compared to plots following a sterile fallow, fallow disturbed and plots previously drilled with opium poppy, *Phacelia*, Endophyte grass (E+). Root fanging was positively correlated to soil tare %, R = 0.34, P = 0.02. where soil tare % was highest in plots following Indian mustard which also had the highest root fanging %.

Table 4.10: Effect of cover crops on sugar beet quality and quantity parameters measured at harvest Differences among treatments followed by the same upper-case letter within the same column are not significant (P> 0.05), according to Tukey HSD. No letters within columns indicate no significant difference (P>0.05).

Treatment	**Soil tare (%)**	**Fanging (%)**	**Root yield (t ha^{-1})**	**Sucrose yield (t ha^{-1})**
Fallow undisturbed	5.11± (0.33) ^{bc}	67.25± (2.75) ^{ab}	111.31± 4.79	17.90±1.51
Fallow disturbed	3.49± (0.40) ^{a}	56.81± (4.71) ^{a}	103.21± 11.98	18.09±1.55
Endophyte grass	4.79± (0.78) ^{abc}	58.82± (9.86) ^{ab}	*114.23± 12.19*	18.28± 1.95
Nil-endophyte grass	4.92± (0.47) ^{abc}	58.75± (8.93) ^{ab}	*93.08± 8.36*	14.89± 1.34
Indian mustard	5.61± (0.59) ^{c}	73.06± (4.31) ^{b}	110.28± 4.24	17.64± 0.68
Italian ryegrass	5.28± (0.27) ^{bc}	70.08± (4.85) ^{ab}	107.40± 7.18	17.18± 1.15
Oilseed radish	5.02± (0.69) ^{abc}	63.16± (2.65) ^{ab}	124.03± 11.17	19.84± 1.79
Opium poppy	4.63± (0.39) ^{abc}	57± (4.02) ^{a}	109.97± 18.49	17.59± 2.96
Phacelia	4.01± (0.25) ^{a}	57.25± (4.03) ^{a}	120.91± 10.91	19.34± 1.75
Sterile fallow	4.43± (0.92) ^{ab}	56.28± (5.52) ^{a}	99.66± 12.94	15.94± 2.07

Sugar content was also not significantly affected by CC treatments (P>0.05) but was negatively correlated to high SRN densities at harvest (R=0.39, P=0.01), where plots with lower densities (at sugar beet harvest) had a higher sugar %. Sodium, amino-nitrogen and potassium impurities were not affected by cover cropping. Potassium impurities were significantly higher compared to sodium and amino nitrogen (Figure 4.6).

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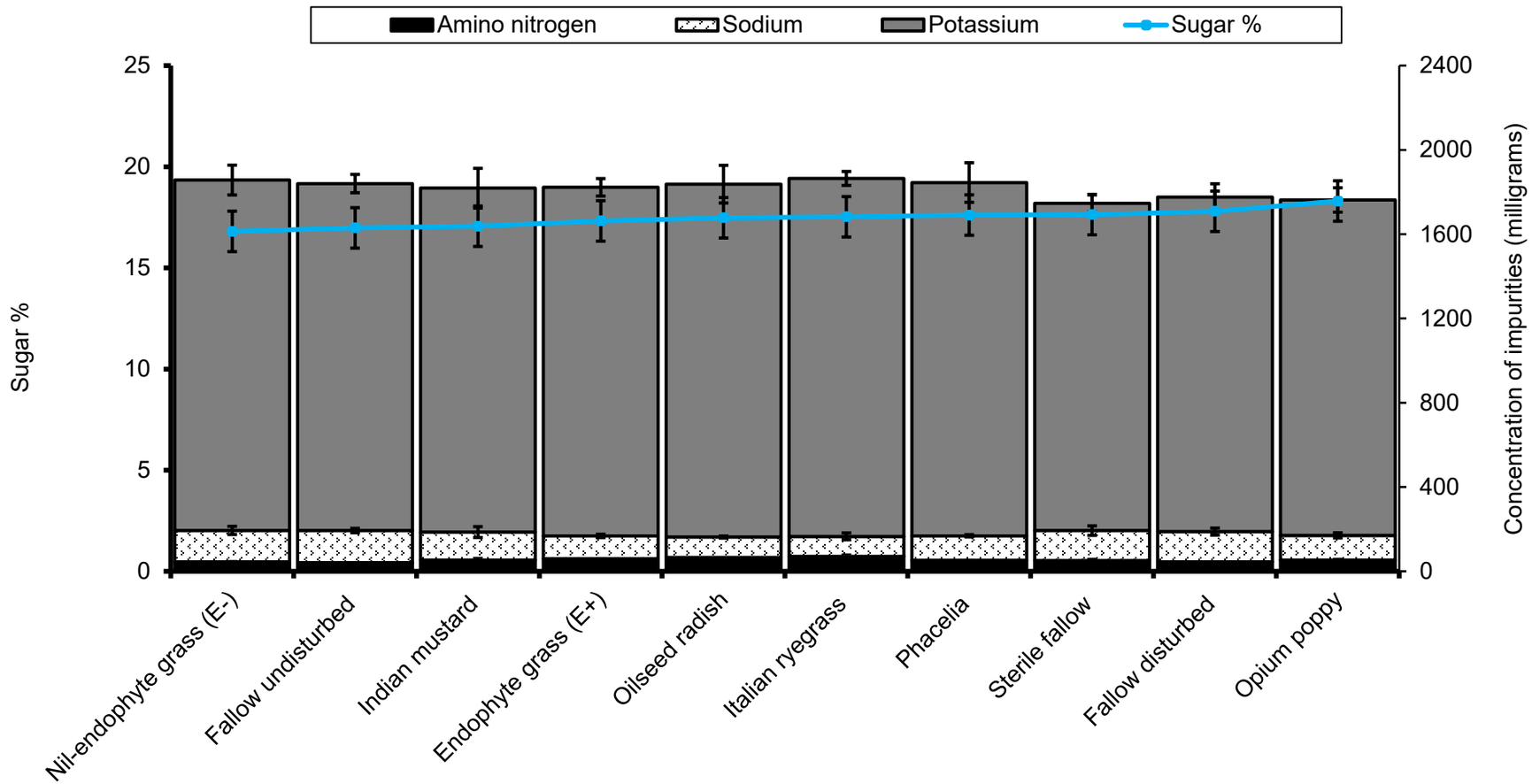

Figure 4.6: Graph of percentage average sugar % and average concentration of impurity components (sodium, potassium, and amino-nitrogen concentration (mg/100 g of sugar) ± standard error.

4.7.7 Effects of cover crops on SRN at the Tibberton Grange site.

Trichodorus primitivus was the only SRN species detected at the Tibberton Grange field site. The initial SRN densities (Pi) were not significantly different in the different plots. There was an increase in SRN densities during cover crop growth as SRN numbers significantly increased before sheep grazing in all the plots when compared to the previous densities at Pi (Table 4.11.). The increase occurred equally in all plots with the different cover crop treatments; there were no significant differences between the treatments ($P>0.05$). Nematode densities recorded three weeks post sheep grazing, declined sharply in all the plots. The reproduction factor of SRN after sheep grazing, was significantly lower in the vitality mix treatments as compared to the oat cover crop ($P<0.05$). When the overall reproduction factor was compared, it indicated that the different cover crops multiplied SRN at different rates, where clover had significantly higher multiplication rate of SRN compared to all the other cover crops. It was four times higher than the vitality mix, three times than radish and vetch and twice higher than oats and stubble turnips. Combination of the cover crops in a mix (vitality mix) had the greatest effect in reducing SRN multiplication compared to using individual cover crops like clover and stubble turnips. The reproduction factors of oats, radish and vetch were not significantly different from the vitality mix.

Table 4.11: Change in average SRN (SRN) densities in 1 Liter soil ±SE (standard error) at different sampling times at Grange Farm (site 3). Treatments with similar letters are not significantly different at each sampling point.

Cover crop	Pi	Pre-grazing	Post-grazing	Rf (Post-grazing /Pi)
Clover	103.75±23.93	828.75±112.83	637.5±110.69^{ab}	6.91±1.81^{c}
Oats	297.5±17.85	832.5±255.66	860±156.68^{b}	2.84±0.40^{ab}
Radish	285±110.08	546.25±127.06	365±167.01^{a}	2.25±0.83^{a}
Stubble turnips	217.5±75.62	877.5±187.31	650±232.84^{ab}	3.03±0.31^{b}
Vetch	316.25±106.88	903.75±293.22	402.5±185.40^{ab}	2.44±0.56^{ab}
Vitality mix	345±90.99	718.75±319.16	410±49.20^{a}	1.51±0.46^{a}

Pi: Initial densities before drilling cover crops, Rf; Reproduction factor.

4.8 Discussion.

I. Effect of growing and incorporating cover crops on SRN.

Trichodorus primitivus and *P. pachydermus* were found in both sites 1 and 2. These species were also reported to be prevalent in a previous UK survey (Alphey & Boag, 1976), and abundant in fields in East England with sugar beet crops exhibiting Docking disorder symptoms (Whitehead & Hooper, 1970). *T. cylindricus* was recorded in site 2 but was absent in site 1. The distribution and prevalence of SRN is influenced by agronomic practices, environmental conditions, and soil physical properties (Cooke, 1973; Winfield & Cooke, 1975).

In this study, the type of cover crops, environmental variables i.e., soil temperature and rainfall,

soil disturbance and presence of weeds, influenced the densities of SRN recorded. The sampling dates that took place during cover crop growth were characterised by significant differences in rainfall and soil temperature, and this had a significant effect on the SRN densities. This observation agrees with previous studies which reported that densities of SRN were positively correlated to high rainfall in May (Cooke, 1973). The strong negative correlation between soil temperature and SRN densities in our study can be explained by the behavior of SRN, which tend to move deeper in the soil profile during dry conditions, as they are highly susceptible to desiccation (Winfield & Cooke, 1975).

Despite SRN being able to feed and reproduce in many crop species due to their polyphagous nature (Ayala et al., 1970), it was clear in this study that the rate of reproduction was significantly different among the cover crop species tested. *Phacelia* and opium poppy were less suitable hosts compared to cover crops such as grass without endophyte (E-), Indian mustard and Italian ryegrass with endophyte (E+). One of the beneficial effects of *Phacelia* as a cover crop is its ability to suppress weed infestation (Wach, 2016), the allelochemicals contained in roots, stems, leaves, and flowers of *Phacelia* inhibit germination of seedlings (Kliszcz et al., 2023).

In previous host-status studies with *Meloidogyne hapla*, *Phacelia* was classified as a maintenance host which implies that nematode densities neither increased nor decreased during the cropping season (Viaene & Abawi, 1998). For *M. chitwoodi, Ditylenchus dipsaci* and *H. schactii, Phacelia* was classified as a poor host (Van Himbeeck et al., 2024), a fair host (Augustin & Sikora, 1989), and a non-host (Gardner & Caswell-Chen, 1993), respectively. *Phacelia* was also shown to successfully suppress densities of *M. hapla* when biologically enhanced with *Pochonia chlamydosporia* (Uthoff et al., 2024).

Like our study, opium poppy has been shown to be a poor host for several PPNs. For instance, under field conditions, twelve species of nematodes in the family tylechidae were recorded in low frequencies ranging from 1-41% in poppy in the Afyon region, Turkey (Akgül & Ökten, 2001). Poppy was also recorded as a non-host to *Pratylenchus thornei* and *Merlinius brevidens* in pot experiment studies (Tobar et al., 1995). During a field survey to investigate nematodes parasitizing poppies, only 10-12% of poppy infected with *M. incognita* showed stunting symptoms (Pandey et al., 1999).

In this study, the differences in SRN reproduction were not only recorded between crop species from diverse plant families, but also within families where the reproduction factor in grass containing endophyte (E+) was significantly lower compared to grass without endophyte (E-). The difference observed is likely to be due to the endophyte status of the grass. The symbiotic relationship between *Festulolium* spp. and the endophyte *Epichloë uncinata* results in the production of bioactive secondary metabolites known as lolines (Meyer et al., 2013, 2020).

The lolines are exuded from plant roots and are also abundant in stems and leaves of the grass, hence these compounds continue to be released when grass residues are decomposing (Blankenship et al., 2001; Bush et al., 1993; Roberts & Lindow, 2014). A similar study reported low densities of *Paratrichodorus minor*, in tall fescue colonised by *A. coenophialum* when compared to non-colonised fescue (Pedersen et al., 1988). The densities of *P. scribneri* recovered in 100 cm^{-3} soil from pots with tall fescue containing the endophyte *N. coenophialum* (E+) were 49-85 compared to 467 to 750 nematodes from pots with tall fescue without endophyte (E-), indicating that the presence of the endophyte had a suppressive effect on the nematode (Bacetty et al., 2009).

Brassica species performed differently within and between sites. In site 1, plots drilled with brassicas significantly suppressed SRN densities as early as four weeks after planting. The decrease in SRN densities in the drilled plots could be explained by the GSLs and myrosinase reaction in brassicas which leads to release of ITCs, which have been shown to possess nematicidal effect towards SRN in *in-vitro* assays using commercially sourced ITCs (Mwangi et al., 2024b). In this case, the GSLs may have been exuded through the young roots, which have been documented to possess high concentrations of GSLs during early growth. The roots of brassicas such as oilseed rape (Choesin & Boerner, 1991) and mustard (Paul Schreiner & Koide, 1993) are also known to release GSLs into the root rhizosphere.

Soil microbes in turn hydrolyse the GSLs into ITCs by releasing the enzyme myrosinase (Dutta et al., 2019). Exudation of ITCs from actively growing roots has also been reported (Elliott & Stowe, 1971) and is thought to be due to superficial cell damage during active root development when the plant is young (Ngala et al., 2015). Conversely, when the plant matures, the GSLs become more concentrated in the reproductive organs i.e., flowers and are incorporated in the soil in the biofumigation process (Bellostas et al., 2004). The biofumigation effect was observed at this site upon cutting and incorporation of Indian mustard and oilseed radish residue, but the effect was not observed for daikon radish where densities were higher than the fallow plots. The oilseed radish used in this study (Terranova) was bred for resistance to nematodes and explains why it performed better than the daikon radish.

At site 2, cultivation of oilseed radish resulted in greater SRN suppression than Indian mustard, where it had significantly lower SRN densities compared to the fallow undisturbed control, while Indian mustard was like the control. When the glucosinolate amounts and profiles were assessed between the two brassicas, oilseed radish had higher total GSLs (combined in shoots and roots) as compared to the Indian mustard, which could have contributed to these differences. The differences in the performance of the brassicas at the two sites might be due to several factors. At site 1, the initial nematode densities at drilling were not as high as in site 2. Initial nematode densities at planting play an important role in the rate of multiplication of nematodes (Mwangi et al., 2019)

The other factor is the establishment of the brassicas, whereby high biomass (40-48 t ha^{-1}) was achieved at site 1, close to the target of 50 t ha^{-1} (Lazzeri et al., 2004) whereas at site 2 the biomass was seven times lower than the recommended rate. Previous studies have reported that low levels of brassicaceous residues incorporated (20 kg ha^{-1}) were not effective in suppression of *M. incognita* as compared to 60 kg ha-1 which effectively reduced infection and damage of *M. incognita* in *Vigna subterranean* (Fourie et al., 2016; Kwerepe & Labuschagne, 2003). A similar observation was made where increasing the rate of *B. oleracea* residues increased the percentage reduction of *M. incognita* (Youssef & Lashein, 2013). High amounts of root biomass produced by *R. sativus* were also associated with high concentration of GSLs leading to release of toxic ITCs that enhanced efficacy in reducing the viability of encysted eggs of *G. pallida* in partial biofumigation (Ngala et al., 2014).

The disturbance effect created in the process of flailing and rotavating during cover crops incorporation was shown to influence SRN densities in this study. Fallow disturbed plots had significantly lower SRN densities compared to the fallow undisturbed plots, indicating the sensitivity of SRN to disturbance. Numerous studies have reported the susceptibility of SRN to mechanical damage. Manual handling and transportation of soil was attributed to reduced *P.*

teres densities, whereby soil carefully transported from field to laboratory yielded 2240 nematodes l^{-1} soil compared to soil transported via post in a cardboard box, yielding 628 nematodes l^{-1} soil (Bor & Kuiper, 1966). In The Netherlands, the effect of biofumigation on nematode communities was attributed to combined tillage and green manuring (Vervoort et al., 2014). The impact of weeds recorded at site 2 on SRN densities was also evident in this study; plots where weeds were managed using glyphosate (sterile fallow), had significantly lower SRN densities. SRN have been reported to feed and transmit viruses i.e., tobacco rattle virus (TRV) in many arable weed species such as field pansy, knotgrass, groundsel, shepherd's purse and chickweed (Cowgill, 2015) indicating that weed management serves as an especially important practice in keeping SRN densities low in fallow land.

II. Effect of using sole cover crops and a mixture on SRN densities.

At site 3, a comparison of the overall reproduction factor indicated that SRN multiplied at different rates on the different cover crop species, where clover had significantly higher multiplication rate of SRN compared to all the other cover crops. It was four times higher than the vitality mix, three times than radish and vetch and twice higher than oats and stubble turnips.

Combination of the cover crops in a mix (vitality mix) had the greatest effect in reducing SRN multiplication compared to using individual cover crops like clover and stubble turnips. Cover crop mixtures offer numerous advantages compared to monocultures (Ziech et al., 2015).

Incorporating species from diverse plant families such as Brassicaceae, Fabaceae, Poaceae, and Polygonaceae families, is advisable as their diverse characteristics complement each other, leading to system benefits (Silva et al., 2021). The selection of cover crop combinations should align with the objectives of crop rotation and the plants' ability to thrive in local conditions. This strategy aims to optimize the benefits for the soil microbial community, soil parameters, and the yield of subsequent crops (Silva et al., 2021). It is recognized that the interaction between cover crop cultivars and PPNs significantly influences nematode management success and rotation system performance. Particularly in the context of cover crop mixtures, it is crucial to choose mixture components that do not elevate pathogen densities. Use of antagonist or non-host plants in a crop rotation system is crucial for reduction of PPNs densities in agricultural fields. Cover crops such as black oat (*Avena strigosa* Schreb.) and white oat (*Avena sativa* L.) offer several benefits as rotational crops, including rapid growth, high biomass production, extensive root development (Silva et al., 2021), and the potential to lower *Meloidogyne* spp. (Marini et al., 2016; Riede et al., 2015) and *Pratylenchus brachyurus* (Gabriel et al., 2018), depending on the cultivar used (Machado et al., 2015).

In cover crop mixtures several mechanisms may occur depending on the mixtures in question, making them more effective compared to single cover crops. Firstly, the dilution effect of allelochemicals produced may occur as different species in the mix have a reduced density when compared to when grown singly and making the host-finding ability of pests that infest them difficult (Boudreau, 2013). Secondly the diversity in root architecture of the different crop species used enhances a physical and visual barrier that further complicates the host-finding process of the pest (Ratnadass et al., 2012) Thirdly the plant-plant interactions between the species used may change the morphological traits of the stand, further interfering with host-location by the pest (Ratnadass et al., 2012) and finally, depending on the species used in the mixture, there can be changes in the chemical composition of the exudates released making them either more attractive or repulsive to the pest (Ratnadass et al., 2012).

In this study the reproduction factor of SRN in oats, radish and vetch were not significantly different from the vitality mix, indicating their potential to be sown individually. Singular cover crops have also been shown to effectively reduce nematode populations as reported in the suppression of *Belanolaimus longicaudatus and M. incognita* by hairy indigo and joint vetch (Rodríguez-Kábana & Canullo, 1992). Clover was shown to be an excellent host when compared to all the other cover crops in the study. In studies with *M. javanica*, clover was shown to be an intermediate host (McLeod et al., 2001). Bhan et al. (2010) demonstrated that cover crops which were good hosts when planted as sole crops yielded similar results when planted in mixtures. For instance, combining a shrub that suppressed root-lesion nematodes with a susceptible host did not reduce the population of these nematodes (Desaeger & Rao, 2001).

Conversely, the densities of *Meloidogyne* spp. In the soil reduced when a host shrub was planted in a mixture with an antagonistic shrub of this nematode (Desaeger & Rao, 2001). Further clarification is needed regarding the host status of mixtures composed of host and non-host cover crops. Interestingly, (Cortois et al., 2017) demonstrated that the abundance of nematode plant feeders increased with the increasing C:N ratio of the aboveground biomass of the cover crops. As a result, crucifer- legume mixtures may suppress nematode densities compared to pure crucifer crops, as their C:N ratio is lower (Couëdel et al., 2018). Nematodes multiplied at the same rate in the vetch-radish mix as compared to vetch alone and multiplied at a lower rate in radish alone when compared to the mix (Barel et al., 2018; Summers et al., 2014). Nevertheless, no differences in the reduction of PCN were recorded when mixing Indian mustard, white mustard, and rocket compared to sole crops. Mixtures of white and Indian mustard are commonly used to suppress plant parasitic nematodes, but their efficiency compared to sole crops is unclear (Kokalis-Burelle et al., 2013; Kruger et al., 2013).

III. Effect of cover cropping on sugar beet parameters.

Cover cropping had a significant effect on the quality parameters of the roots of sugar beet at harvest. Root fanging and soil tare were significantly lower in plots that had lower SRN reproduction. Stubby root nematodes densities at sugar beet drilling were positively correlated with root fanging, hence plots with high nematode pressure recorded higher root fanging which was positively correlated to the soil tare as increased fanging leads to more accumulation of dirt in the roots. Direct feeding on roots of young sugar beet seedlings by SRN causes stubby lateral roots (fanging) which later turn roots grey- brown and then black as they die and decay (Christie & Perry, 1951; Winfield & Cooke, 1975). Young sugar beet have been shown to be more susceptible to SRN infestation and this was shown in a study where high densities of *T. cylindricus* or *P. pachydermus* were common mostly around young seedlings (1500 l^{-1}) than around large plants (600 l^{-1}) at Gayton, Thorpe, England (Whitehead & Hooper, 1970), hence this explains the positive correlation of root fanging and initial SRN densities at sugar beet drilling observed in our study.

At sugar beet drilling, high SRN densities were positively correlated to higher rainfall and lower soil temperature. The combination of these factors may have significantly contributed to the degree of root fanging. Similar observations were recorded where severe damage to young sugar beet seedlings was correlated with high total rainfall in the month of May (Cooke, 1973; Jones et al., 1969; Winfield & Cooke, 1975). The influence of soil moisture on SRN densities can be explained by the fact that SRN are most active when soils are at or near field capacity (Cooke, 1973).

Stubby root nematodes SRN have also been shown to be more susceptible to desiccation than other nematodes species i.e., *Rotylenchus* and *Pratylenchus* spp. (Rössner, 1971). The type of soil inhabited by SRN is mostly sandy, and this means that there is high drainage where the topsoil dries out as water percolates deeper through the soil profile (Cooke, 1973). This scenario causes the SRN to move up and down the soil profile following the soil moisture, and as such, densities of SRN are likely to vary depending on time of sampling (Cooke, 1973). Higher rainfall also leads to leaching soil nutrients such as nitrogen and manganese, which have been associated with high incidence of Docking disorder symptoms (Whitehead & Hooper, 1970). Potassium impurity was significantly higher in sugar beet grown in plots following *Phacelia* and Italian ryegrass compared to fallow undisturbed control. One of the factors known to influence the impurity levels in sugar beet is the amount of soil mineralizable nitrogen in the soil; increasing levels of N up to 285 kg N ha^{-1} was reported to significantly increase impurity levels and sugar loss percentage (Abdel-Motagally & Attia, 2009).

The amount of nitrogen post incorporation of the cover crops was not measured in this study. However, a study investigating the effect of cover crops on yield and N requirements of sugar beet attributed increased level of amino nitrogen impurities by fodder radish to increased availability of nitrogen to the beet crop (Allison et al., 1998). In this study, cover cropping did not significantly affect the root yield of sugar beet. Previous studies have shown that sugar beet suffering from docking disorder may recover later in the season (Cooke, 1973). This might explain why significant differences in yield were not recorded even though root fanging symptoms were still visible during scoring at harvest.

In conclusion, the studies carried out in the three experimental sites show that the population dynamics of SRN under field conditions are influenced by many factors. The host-status of the cover crop grown, the prevailing environmental conditions, the susceptibility of the follow-up crop, the presence of weeds and field operations that involve soil disturbance, all play a significant role in the population dynamics. It was clear that brassicas can be optimised to effectively manage SRN as seen in site 1 where brassicas established well and had high biomass hence suppressed the SRN, compared to site 2 where the biomass was lower. Cover crops such as *Phacelia* and opium poppy were also seen to have lower multiplication compared to the fallow, and other cover crops in the study can be utilised for management of SRN and further work should be conducted to optimise their efficacy.

The use of cover crop mixes should be further explored to enable selection of diverse mechanisms in cover crops that are compatible for use in suppression of SRN as some cover crops may produce repelling phytochemicals while others may act as poor host, and this combined leads to effective nematode suppression. The vertical distribution of SRN needs further investigation to establish the depth of sampling for these nematodes as there maybe be more factors at play influencing how deep the nematodes can be found. These include the type of crops, farming practices and the soil properties such as pH., moisture and particle size distribution of the soil.

Chapter 5: Nematotoxic effects of Endophyte infected hybrid grass *Festulolium loliaceum* shoot and root extracts on *Trichodorus primitivus.*

5.1 Introduction.

The symbiotic relationship between cool season grasses and claviceptaceous endophytic fungi in the genus *Epichloë,* leads to production of secondary metabolites such as lolines, peramines, indole diterpenes and ergots (Kuldau & Bacon, 2008; Schouten, 2016). These alkaloids are variably distributed in plant tissues with the highest concentrations being in the seed, followed by the vegetative tissue mainly in the pseudostems. In the roots these alkaloids are also found, which is because of translocation from points of synthesis through the xylem, as the endophyte does not colonise the roots (Meyer et al., 2020). The interaction between the host and the endophyte is very intimate and leads to synthesis of different alkaloids that may not be produced by either of them independently (Card et al., 2021). For instance, *Festuca rubra* colonised with *E. festucae* had high concentration of the alkaloid ergovaline compared to other *Festuca* spp colonised by a different endophyte (Schardl et al., 2012).

The occurrence of alkaloids in the various grass-endophyte interactions is known to be influenced by several factors with the main drivers being the host genotype and the associated fungal strain (Kuldau & Bacon, 2008; Vázquez-De-Aldana et al., 2007). In Lolium-Festuca hybrids, which are used in this study, the fungal endophyte *E. uncinata* is required for high loline alkaloid production, and its absence means no alkaloid production (Blankenship et al., 2001; Meyer et al., 2020). The alkaloids produced by these hybrids include N-acetylloline (NAL), N-methylloline (NML), N- formylnorloline (NFNL), nor- loline (NL), loline (L), N-acetylnorloline (NANL), and N-formylloline (NFL). N-formylloline (NFL) and NAL being the most predominant loline alkaloids (Meyer et al., 2020).

Concentration of the alkaloids produced by these grass-endophyte combinations are not always stable and vary depending on biotic and abiotic stresses. Environmental factors such as temperature and humidity are known to greatly influence the concentration of alkaloids. For instance, the alkaloids ergovaline and lolitrem B increase as temperature increases in summer period and is lower in early spring. Concentrations also increase during reproductive development and as the plant ages and accumulates older leaves (Bush et al., 1993).

The nutrient levels during growth of tall fescues colonised by *E. coenophialum* was also shown to influence the alkaloid concentration recorded, where in the shoots, the concentration of ergot alkaloids increased when phosphorus availability ranged from 17 to 50 mg kg^{-1} and decreased at 96 mg kg^{-1}. However, the concentration in the roots increased linearly with increasing soil phosphorus (Malinowski & Belesky, 2008). Age of the plants is another factor known to influence some alkaloid concentration such as ergovaline and lolitrem B which increases with age, while the alkaloid peramine decreases as the plant senescence (Schardl et al., 2012).

Wounding/artificial damage of fescues is also known to influence alkaloid concentrations. In meadow fescue colonised with *Neotyphodium siegelii,* the loline alkaloids were almost twenty times higher from 0-11 days post-clipping (Bylin, 2014). The loline concentration in artificially damaged tall fescue colonised with *E. coenophialum* also increased by two-fold when compared to the undamaged plants (Bultman et al., 2004). Under natural conditions, many plants exhibit increased chemical/structural mechanisms following wounding/damage by herbivores. However, this is not a general reaction in all plants, as some plants lacking chemical defences tend to become more susceptible to the damage/attack. Upon damage/attack by herbivores, plant cell

surface localised pattern recognition receptors (PRRs), induce plant immunity by recognising damaged associated molecular patterns (DAMPs), microbe-associated molecular patterns (MAMPs) (Zhang et al., 2022), herbivore-associated molecular patterns (HAMPs) (Gandhi et al., 2020), and phytocytokines, thereby activating pathogen triggered immunity (PTI). Phenolic compounds such as lignin, coumarins, furanocoumarins, flavonoids, and tannins are notably increased when plants are wounded (Zhang et al., 2022). The interaction between grass and endophytes also leads to production of phenolic compounds and flavonoids, amino acids and sugars which are key in nutrient availability and osmoregulation. These compounds are exuded by grasses colonised by the endophyte rendering them allelopathic to some soil dwelling microbes (Lee et al., 2021). These compounds have also been isolated in absence of the endophyte, where chlorogenic acid, caffeoylquinic acid isomers, and 3,5-DICQA were detected in the roots of both E+ and E− grass (Bacetty, et al., 2009). The amount of carbohydrates and organic carbon phenolic compounds released by E+ grass has however been shown to be greater than E− grasses (Van Hecke et al., 2005).

Experiments using purified compounds of the grass metabolites, and the crude extracts have indicated the potential of these compounds to be used as repellents, nematicidal or agents that inhibit nematode egg hatch. The efficacy of these compounds and extracts on mortality, motility or repulsion/attraction of nematodes is influenced by several factors such as class and concentration of the alkaloid and exposure time (Mwangi et al., 2024a). For instance, pure form of the loline alkaloid N-formylloline has been demonstrated to repel the parasitic nematode *P. scribneri* in in vitro assays (Bacetty et al., 2009). The fescue grasses themselves have been shown to cause negative effects even in the absence of endophyte, for instance the mortality of juveniles of *M. incognita* were recorded in tall fescue root and shoot extracts from both grass with and without the endophyte.

Similar results were also obtained when using hybrid grass *Festulolium* spp. where the inactivity of *M. incognita* juveniles obtained from root and shoot extracts was recorded in the presence and absence of the endophyte (Meyer *et al*., 2020), concluding that other compounds might be associated with the negative effects observed. The roots of tall fescue grasses have been reported to exude phenolic compounds such as cinnamic, ferulic, gallic, gentistic, and syringic acids during an assessment in a hydroponic system (Malinowski et al., 1998). Nematicidal effects have also been associated with phenolic compounds (López-Martínez et al., 2011).

The increase in phenolic compound content when plants are challenged with PPNs is the reason why researchers have drawn conclusions on their effects on soil-dwelling nematodes (Chitwood, 2002). Indirectly phenolic compounds have been shown to contribute to plant resistance to nematodes. For instance, chlorogenic acid in rice resistance to *Ditylenchus angustus* (Plowright et al., 1996), caffeic and chlorogenic acid in alfalfa resistance to *P. penetrans* (Baldridge et.al.,1998), and induced resistance of tomato roots to *Rotylenchus. reniformis* when immersed in the pyrocatechol, hydroquinone, phloroglucinol, pyrogallol, and orcinol (Mahmood & Siddiqui, 1993).

Some flavonoids are also known to be phytoalexins and contribute to resistance to nematodes. For instance, the flavonoids coumestrol and psoralidin were isolated from lima beans in response to infection by *P. scribneri* (Rich et al., 1977), similarly the flavonoid medicarpin, is a major phytoalexin that is expressed in high concentrations in alfalfa resistant cultivars to *P. penetrans.* (Baldridge et al., 1998). The flavonoid phytoalexin glyceollin in soybean roots was

demonstrated to have nematostatic effects towards of *M. incognita* in vitro bioassays at a dose of 15 µg ml^{-1} (Kanagy & Kaya, 1996; Kaplan et al., 1980).

The aim of this study was to evaluate the nematotoxic effects of root and shoot extracts obtained from grass with endophyte and without endophyte and identify the present phytochemicals.

5.2 Null hypotheses:

I. Shoot and root extracts from E+ and E- grass have no nematotoxic effects on SRN.

II. Bruising and wounding E+ grass has no effect on subsequent nematotoxic effects to SRN.

III. The level of alkaloids in E- and E+ grass is not affected by age and wounding of the grass.

5.3: Materials and Methods.

5.3.1 Glasshouse experiment layout.

I. Experiment 1.

The seeds of hybrid grass (*Festulolium loliaceum*) with endophyte and without endophyte, used in this study were supplied by CropMark Seeds, New Zealand. Seeds were sown in John Innes No.2 growth medium in 20 cm-diameter pots at a depth of 2cm, following the seed rate as recommended by the seed supplier. The first glasshouse experiment was set up from December 2022 to May 2023, while the repeat experiment was set up from February 2023 to June 2023. Shoot and root material were harvested at different plant ages i.e. 8, 12, 16 and 20 weeks old. The growth conditions were a 12h photo period, the mean day and night temperatures were 26 and 8°C respectively and the average relative humidity during the experiment was 67%. In trial 2 the average day and night temperatures during the experiment were 28 and 5°C respectively and 62% relative humidity. Plants were fertilised monthly using Wuxal liquid fertiliser 8-3.5-5 NPK. The treatments were arranged in a randomised complete block design, with three replications for every grass and age combination.

II Experiment 2.

This experiment sought to determine whether artificially wounding grass with endophyte (E+), would enhance the nematotoxic effects observed in the first experiment. Grass with endophyte was grown in the glasshouse in John Innes No.1 growth medium in 20 cm-diameter pots at a depth of 2cm, following the seed rate as recommended by the seed supplier. The glass house conditions were 12h day length, 62% relative humidity and an average day and night temperature of 23 and 8°C. The grass was allowed to grow until it was 8 weeks old. The plants were then wounded by first cutting ca. 4 cm off every leaf tip with scissors and then bruising the remaining leaf blade eight times with a tailor's rolling wheel, control plants received no cutting or bruising (Figure 5.1). The bruised shoots were harvested after 3-, 7-, 11- and 30-days post bruising (dpb). The activity of the extracts obtained from the bruised shoots on *T. primitivus* were prepared and tested as described for Experiment 1. Mechanical wounding was implemented to mimic the damage caused by animal grazing under field conditions or grass mowing and rolling practices.

Figure 5.1: A-Bruised grass foliage; B- control-non bruised; C- Equipment used for clipping and wounding the plants.

5.3.2 Assessment of endophyte status.

Endophyte status of the individual plants was assessed using commercial endophyte tiller test kit (*Epichloë* Endophyte Tissue Print Immunoblot Tiller Kit; Cropmark Seeds Ltd., Christchurch, New Zealand). This was done by sampling 10 tillers of 8-week-old plants in grass with endophyte (E+) and without endophyte (E-) plants. The components of the kit included: a nitrocellulose membrane, 1st antibody (a monoclonal anti-endophyte antibody, 2nd antibody (alkaline phosphate conjugated), chromogen, two blocking solutions (tris/NaCl and Skim milk).

Grass tillers were cut low down the stem, ensuring a flat bottom and all dead/diseased parts removed. They were then pressed onto the nitrocellulose membrane (NCM) and held for 3-4 seconds. The blotted NCM was then placed into a Petri dish and 10ml of blocking solution added, this was then placed on a shaker at 60rpm for 30 minutes, after which it was poured out. The 1st antibody was then added to the NCM and put on a shaker again for 1hour after which it was poured. The NCM was rinsed twice with 1oml of blocking solution and placed on a shaker for 5mins.

The 2nd antibody was then added and placed on a shaker (HS 501 digital, IKA, Staufen, Germany) for 1 hour, after which it was poured and the NCM rinsed again twice with 10ml blocking solution on a shaker for 5minutes.

Chromogen solution was then added, and the Petri dish immediately covered with an aluminium foil and placed on a shaker for 30 minutes (Figure 5.2).

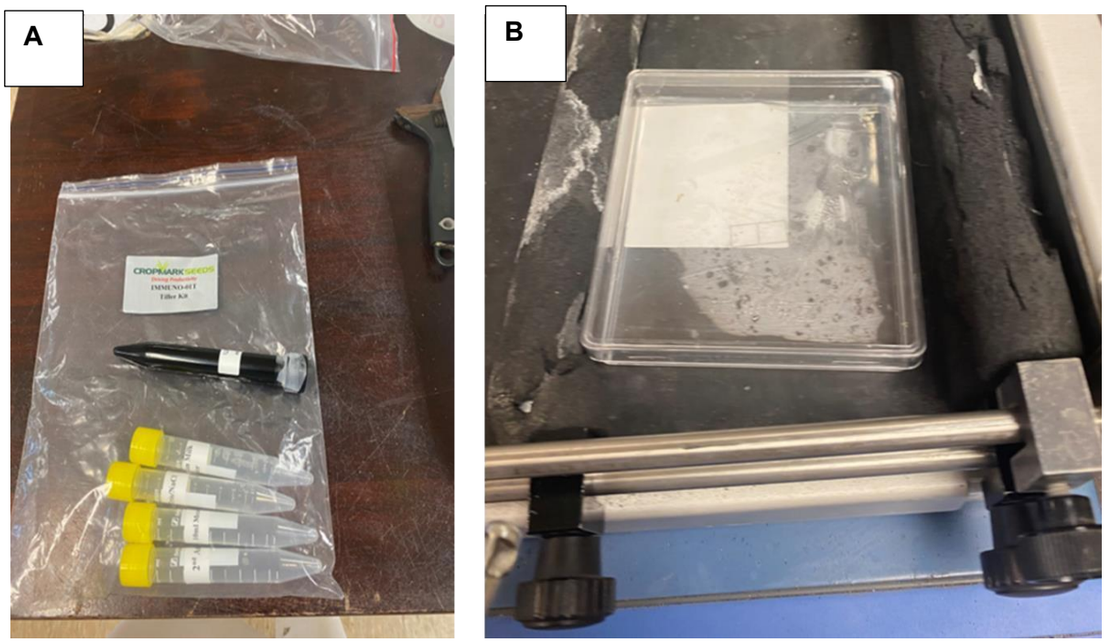

Figure 5.2: A- Endophyte kit with reagents and antibodies; B-Nitrocellulose membrane immersed in an antibody and placed on a shaker.

The blot development was assessed every 15 minutes for development of blots. Once the blots had developed, the chromogen was poured and rinsed thoroughly in cold tap water and the staining observed under the microscope at 20X magnification (Figure 5.3).

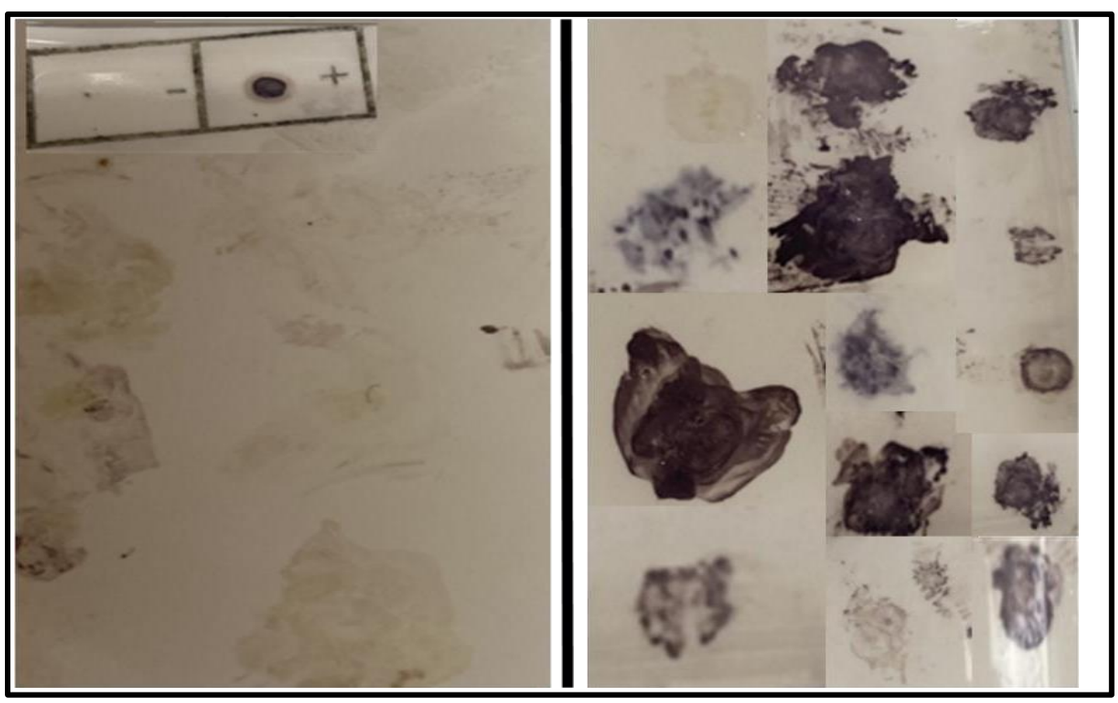

Figure 5.3: Blots of grass without endophyte (Left) and grass with endophyte (Right) on a nitrocellulose membrane.

5.3.3 Source of SRN.

Stubby root nematodes were obtained from infested soil collected from a site in Crabtree leasow. 52°46′15.73″N 2°25′35.51″W, Harper Adams University fields, which had a history of SRN infestation. Nematodes were extracted from soil using the Seinhorst two flask method as described in section 2.2. The SRN used in this study were identified as *T. primitivus.*

5.3.4 Preparation of plant extract and in-vitro assay set-up.

Shoot and root extracts were obtained from E+ and E- plants at different ages.i.e., 8 weeks,12 weeks,16 weeks and 20 weeks old plants. At each sampling point, three pots were harvested. The fresh shoot and root weight of the different treatments were weighed and were later freeze dried (GVD6/13 MKI freeze dryer, GIROVAC Ltd, North Walsham, UK) for a week and milled into a fine powder to pass a 1-mm-diametre sieve. Methanolic extracts were prepared by adding 4g of powder of E+ and E- to 40ml of 98 % methanol and placed in a shaker (HS 501 digital, IKA, Staufen, Germany) at 100rpm for 20 hours.

The extracts were then filtered using Whatman N0.1 filter paper, by folding the paper into a cone, placing it in a funnel, and allowing the extract to pass through, thereby separating the solid residues from the clear filtrate. The filtrate was then transferred to pre-weighed containers and dried using a rotary evaporator. The dried residue was then resuspended in sterile distilled water and stirred until dissolved. The solution was filtered again using 0.45 and 0.2µm syringe filters. Five dilutions i.e., 5000, 2500,1250,625 and 312.50µg/ml were made using sterile distilled water for each extract and replicated five times for each treatment and dose combination.

The dilutions were based on literature of similar studies targeting other nematode species (Bacetty et al., 2009) to reflect biologically relevant concentrations The experiment was repeated once. Stubby root nematodes were exposed to shoot and root extracts of grass with endophyte (E+) and grass without endophyte (E-), and effect on mobility was assessed after 24,48 and 72h. the nematodes were subsequently transferred in distilled water for 48h recovery assessment. SRN mobility was assessed after 24, 48 and 72 h exposure periods in a repeated measures design.

Nematodes were categorised as either mobile or immobile based on their response to mechanical stimulation using an eyelash needle. After the last assessment at 72 h, the nematodes were transferred to distilled water and incubated for 48 h for recovery assessment. The nematode stimulation procedure was repeated to determine whether they were dead or alive (mortality), which would indicate that the immobility effect observed was reversible or irreversible. Mortality was expressed as dead/total for each treatment. The preparation process is summarised in Figure 5.4.

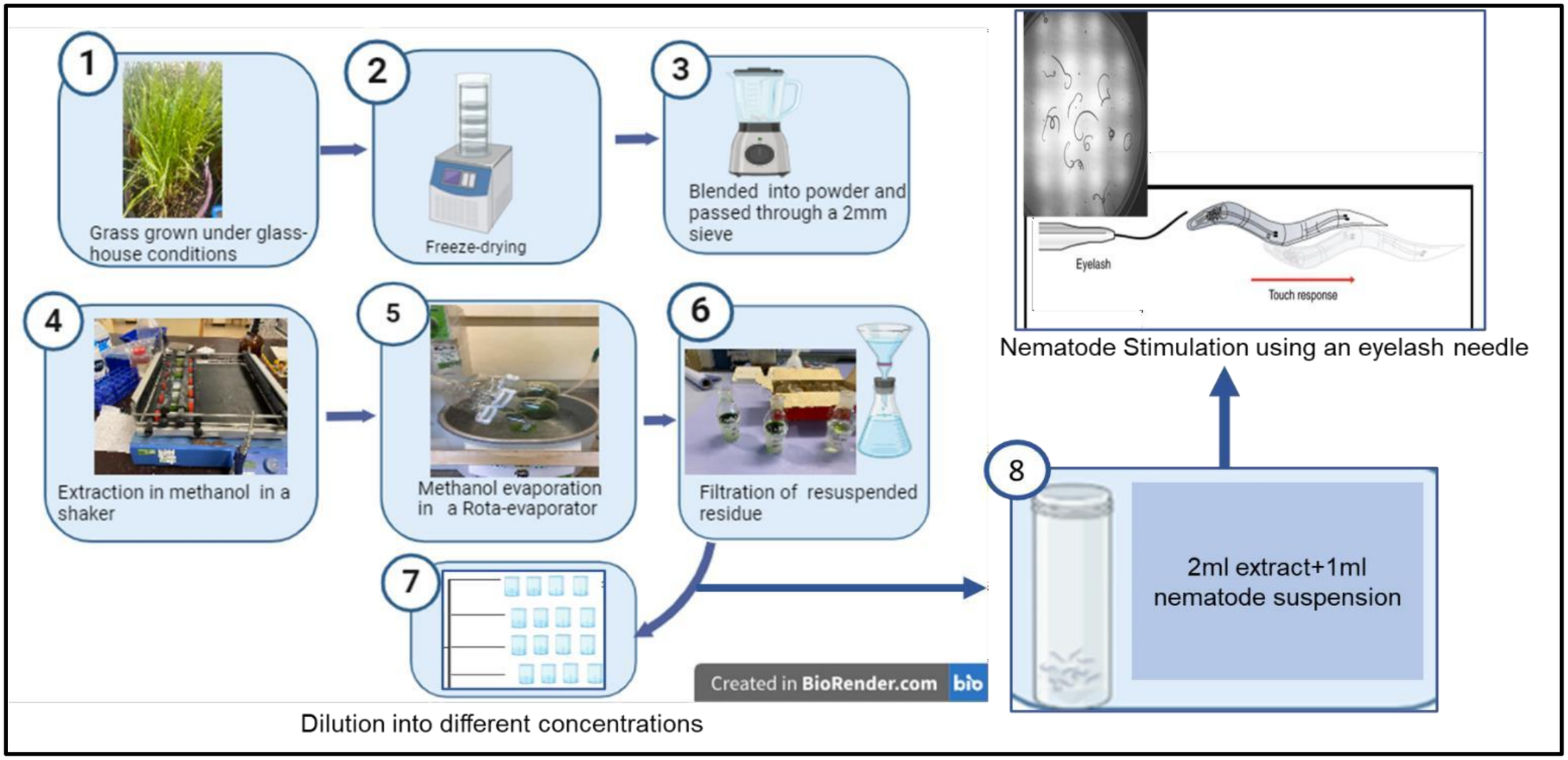

Figure 5.4: Steps followed in preparation of root and shoot extracts of *Festulolium loliceaum* and setting up of the in-vitro assay. .

5.3.5 Quantification of Phytochemicals.

Total flavonoids, total phenols and loline alkaloids were quantified in the E+ and E- samples. Methanolic crude extracts of E+ and E- samples were used to determine the phytochemicals and each analysis was done in triplicates.

5.3.5.1 Total flavonoid content (TFC).

Flavonoids in root and shoot samples we.re analysed using an aluminium chloride (AlCl3) assay (Woisky & Salatino, 1998). Quercetin was used as a standard at different concentrations (0-200 μg ml^{-1}). Methanolic solutions of E+ and E- samples (5000 μg^{-1}) were prepared and 500 μL aliquots of the solutions mixed in a test tube with 250 μL of AlCl3 (50 g/L in methanol) and 4.25 mL of methanol. The mixture was centrifuged at 1300 g for 2 mins. Pure methanol was used as a blank. Absorbance was measured at 510 nm after 30 min incubation using a spectrophotometer. A quercetin calibration curve (y = 0.007x-0.006, R2 = 0.97) was generated to determine the TFC, and this was expressed as μg quercetin equivalent per g crude extract (μg QEg^{-1}).

5.3.5.2 Total phenolic content (TPC).

The TPC in root and shoot of E+ and E- samples was determined using the Folin–Ciocalteu method (Singleton & Rossi, 1965). Gallic acid at different concentrations (0-250 μg ml^{-1}) was used as a standard. Methanol solutions E+ and E- samples (5000μg ml^{-1}) were prepared, 250μL aliquots of these solutions, were added to a test tube and 250 μL Folin–Ciocalteu reagent (diluted in water 1:1) was added, followed by 500 μL of saturated Na_2CO_3 solution and 4 mL of distilled water. The mixture was then incubated in the dark for 25 mins and later centrifuged at 3000g for 10 minutes. Absorbance was read at 725 nm using a spectrophotometer. A gallic acid calibration curve (y = 0.085x-0.066, R^2 = 0.98) was generated to determine the TPC. The TPC was expressed as μg gallic acid equivalent (GAE) equivalent per g crude extract (μg GAE g^{-1}).

5.3.5.3 Loline alkaloids.

Loline alkaloids in root and shoot of E+ and E- samples were analysed following a modified method from Yates et al. (1990) and Blankenship et al. (2001). A 250mg freeze dried and ground sample was mixed in 5ml 95:5 dichloromethane: ethanol, extraction solvent and 250 μl saturated sodium bicarbonate in a 6ml glass vial. The mixture was then placed in an orbital shaker for 1h at 200rpm. Samples were freeze dried and ground and a 250mg sample extracted in 5ml of 95:5 dichloromethane: ethanol, extraction solvent and 250 μl saturated sodium bicarbonate. A standard, 60 μg/ml 4-Phenomorpholine (Sigma Aldrich®, Sydney, Australia) was added in the extraction solvent. The samples were then filtered using a cotton -plunged pasteur pipette and 1ml of filtrated placed in a 2ml gas chromatography (GC) vial. GC analysis was conducted using a Shimadzu GC-2010 with a flame ionization detector and a ZB-5 (30 m × 0.25 mm × 0.25 μm) capillary column (Phenomenex®, Auckland, New Zealand). Hydrogen was used as a carrier gas at a flow rate of 6 ml per min. H2 and air flows at the detector were 40 and400 ml per min, respectively. The oven was heated from 40°C to 320°C at a rate of 20°C per min and held there for 5 min. Samples were introduced via 1 μl split-less injections. Retention times were as follows: N-methylloline (5.9 min), 4-phenomorpholine (6.9 min), N-acetylnorloline (8.2 min), N-formylloline (8.4 min), and N-acetylloline (8.7 min). Loline standards purified from Barrier U2TMseed (Cropmark Seeds Ltd., Christchurch, New Zealand) and a Festulolium cultivar infected with *E. uncinata*, were used to standardize the GC., using the methods of Briggs et al.

(2017). Limit of detection was 30 µg/g.

5.4 Statistical analysis.

All data were analysed using R-studio software (R Core Team, 2022). A Levene test was conducted to compare the variances between the two experiments. Data from two trials were analysed separately as they were significantly different (P <0.05). Data on immobility over 24,48

and 72h exposure time was analysed by fitting a mixed effects beta regression model, using (package glmmTMB), with age, treatment and concentration as fixed effects and time as random effect, the package emmeans was used to generate contrasts at p<0.05. Data on mortality was analysed by fitting dose response curves to generate the lethal dose concentrations causing 50% mortality (LD_{50}). The package drc (dose response curve) was used to generate log logistic regression models for lethal dose (LD_{50}) and used for comparisons of the LD_{50} values between the different treatments (Ritz & Streibig, 2012).

5.5 Results.

5.5.1 Effect of shoot and root extracts on SRN mobility.

I. **Shoot extracts.**

Results obtained from the first *in-vitro* bioassay indicated. that extracts obtained from both E+ and E- grass had the ability to significantly immobilise *T. primitivus* when compared to the distilled water control (DH_2O) (Figure 5.5). Concentration of extracts had a significant effect on SRN immobility (p=0.001). However, lower concentrations i.e., 312.50 and 625µg ml^{-1} were not significantly different from the control except at 8 weeks for both E+ and E- extracts. At lower concentrations i.e., 312.50 and 625ug/ml for 16- and 20-weeks old extracts, the endophyte status significantly affected the immobility observed, though the immobility was less than 10% for E+, compared to E- extracts where no immobility was recorded.

There was a significant concentration dependent response where immobility increased linearly with every increasing concentration, with highest values (80-100%) immobility observed at 500 µg $ml^{-}1$ and lowest (0- 4%) at 312.50 µg $ml^{-}1$ across all treatments. The immobility in most treatments increased cumulatively with increasing exposure time, however at highest concentration of 5000 µg ml^{-1} most SRN were immobilised after 24 h exposure period, for instance at 5000 µg /ml for both E- and E+ grass, more than 60% *T. primitivus* were immobilised after 24 h exposure time. In the case of lower doses more exposure time was needed to immobilise more SRN. The factors age also significantly affected immobility (p= 0.0002)

The rate of immobilisation generally declined with increasing age for both E+ and E-grass extracts. However, for E+ extracts, there was an increase in rate of immobilisation for the 20-week-old extracts. Significant interaction effects were also recorded for the factors age: treatment, treatment: concentration and age: concentration (p=0.01,0.003 and 0.04) respectively.

In the second repeat *in-vitro* assay, a similar trend was observed, however the endophyte status was not seen to affect immobility at any point. Both E- and E+ extracts significantly reduced immobility compared to the distilled water control (DH2O), except at lower doses (312.50 µg ml-^{1}) for 16-week-old plants in both E+ and E- treatments. A concentration dependent response was also recorded with high concentration (5000 µg ml^{-1}) achieving higher immobility (62-95%) compared to lower concentrations (312.50 µg ml-1), which had 0-9% immobility rate across all

treatments. No differences in immobility were recorded at the highest concentration of 5000 µg ml^{-1}, except for 16-weeks-old plants where there was significantly lower immobility in both E+ and E- extracts (Figure 5.6). At lower concentrations of 312.50 -1250 µg ml^{-1} in E- extracts, younger extracts significantly increased immobility as compared to older extracts. The effect of age also followed a similar trend with more immobility across different concentrations being observed in extracts obtained from younger grass as compared to older ones in E- extracts, this was similar in E+ extracts until 16-weeks-old after which at 20-weeks-old the immobility significantly increased. Significant interaction effects were also recorded for the factors age: treatment, treatment: concentration and age: concentration ($p=0.011$,0.003 and 0.03) respectively

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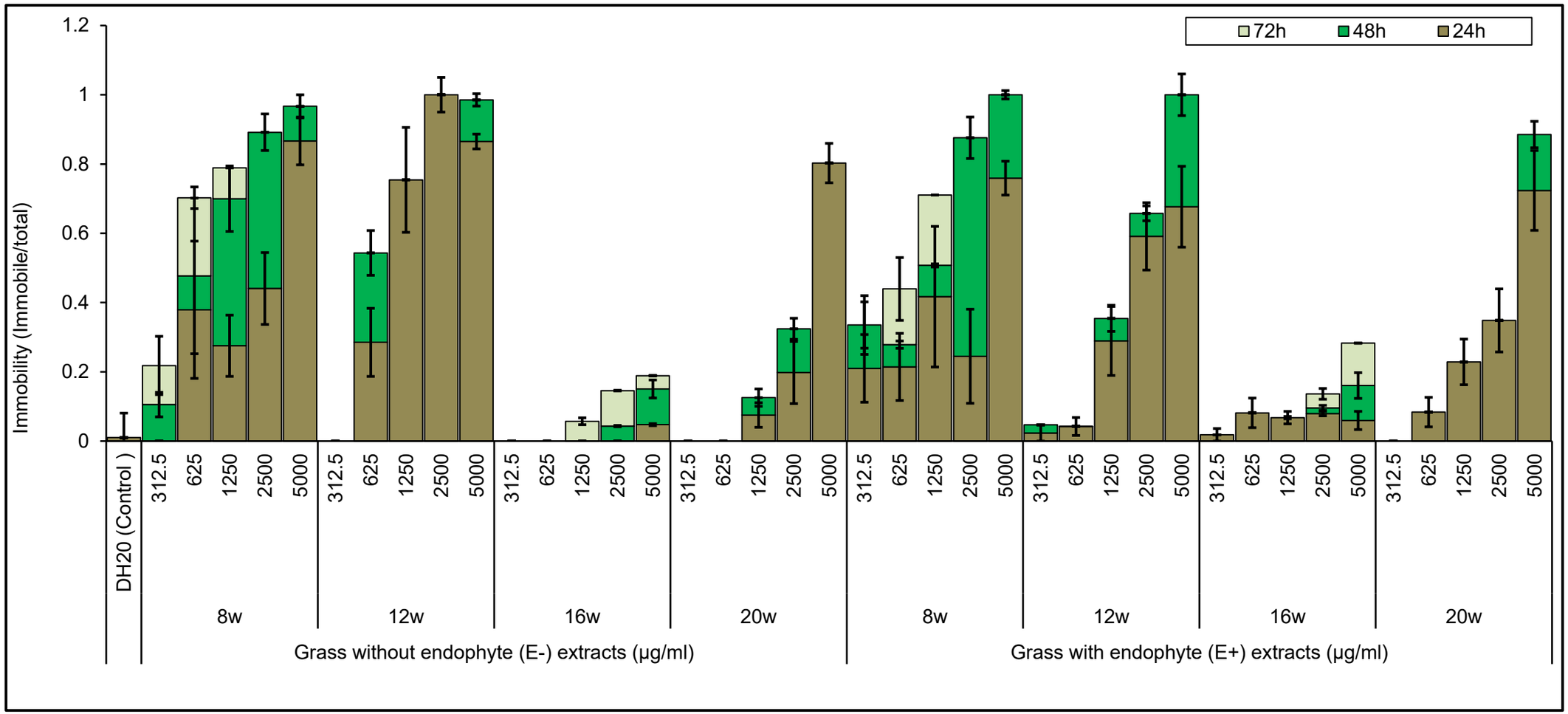

Figure 5.5: Experiment 1: The average immobility of stubby root nematodes (SRN) (n=5) ± standard error of the mean (SE), upon exposure to different concentrations (312.5, 625, 1250,2500 and 5000 µg/mL) of shoot extracts obtained from grass with endophyte (E+) and grass without endophyte (E-) at different ages (8,12,16,20 weeks, at exposure times of 24,48 and 72h.

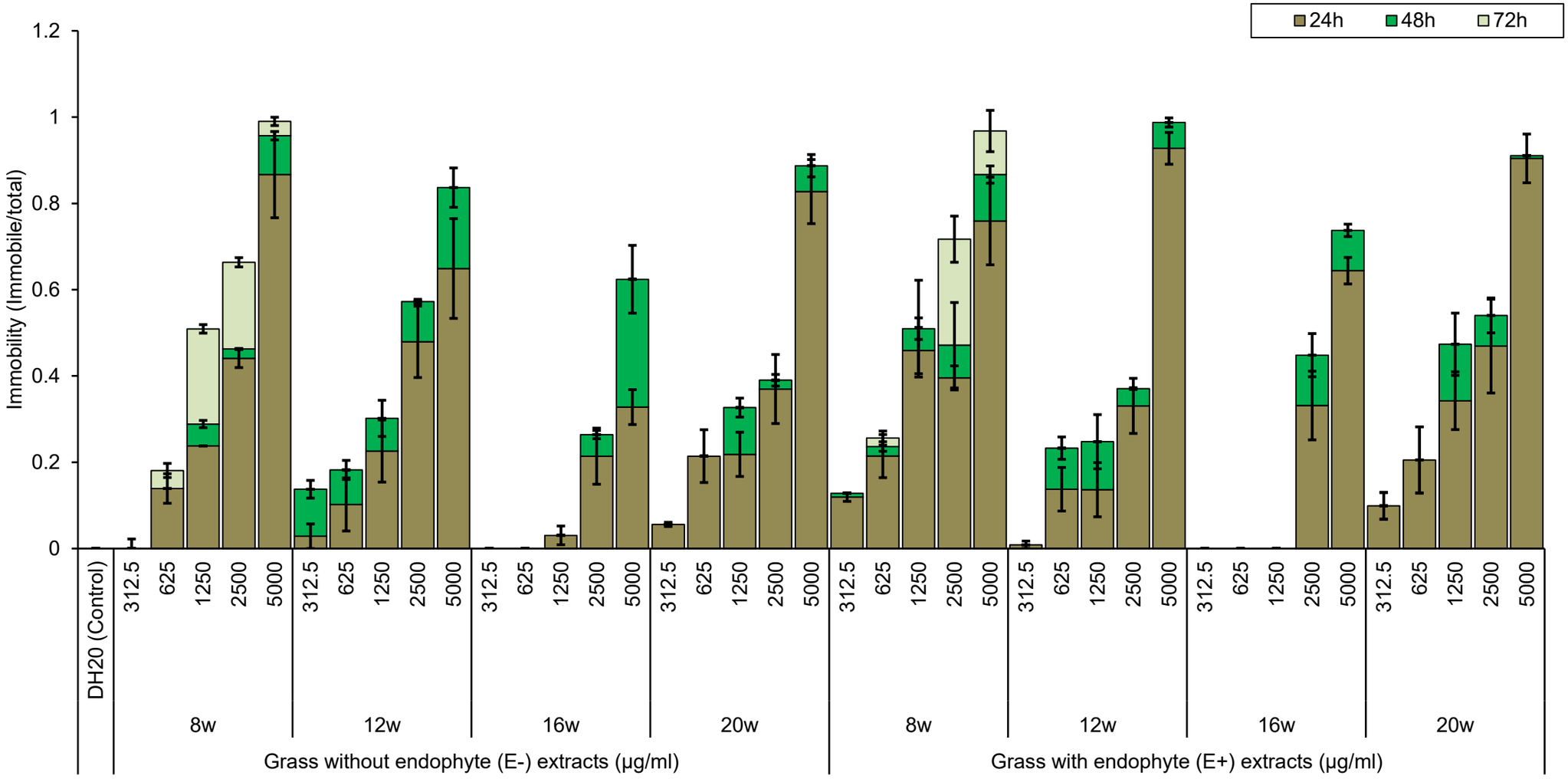

Figure 5.6 : Experiment 2, The average immobility of stubby root nematodes (SRN) (n=5) ± standard error of the mean (SE), upon exposure to different concentrations (312.5, 625, 1250,2500 and 5000 µg/mL) of shoot extracts obtained from grass with endophyte (E+) and grass without endophyte (E-) at different ages (8,12,16,20 weeks, at exposure times of 24,48 and 72h.

II. Root extracts.

The immobility of SRN recorded following exposure to root extracts was lower compared to what was observed in the shoot extracts. The endophyte status influenced immobility across the different concentrations, where immobility was higher in E+ groups compared to E-, notably at lower concentrations of 312.50 and 625 µg ml-1 with 16- and 20-week-old grass; E+ grass extracts increased immobility while E- had little to no effect on immobility. In general, little to no SRN immobility was recorded in response to root extracts obtained from younger roots compared to older roots in both E+ and E- extracts (Figure 5.7).

Increasing the concentration or exposure time to root extracts from 8-week-old E+ or E- plants had no effect on SRN immobility. Cumulative increase in SRN immobility was seen for concentrations from 1250-5000 µg ml-1 for both E+ and E- extracts at the 24 to 72 h exposure times. Lowest SRN immobility was recorded in response to 8-weeks-old extracts and ranged from 0-15% across all doses for both E+ and E- extracts, while the highest immobility rates were observed at the highest doses (5000 µg ml-1) of 16week-old E- extracts (98% immobility) and 20-week-old E+ extracts (89% immobility).

Dose dependent immobility was observed with 12-20-week-old root extracts for both E- and E+ extracts where concentrations above 1250 µl from 16-20-week-old plants caused significant immobility ranging from 28-100% for E- extracts and 15-96% for E+ extracts (p=0.001). Significant interaction effects were also recorded or the factors age: treatment and treatment: concentration (p=0.01 and 0.04) respectively.

In the second experiment, higher immobility was observed compared to experiment 1, but the trends were similar (Figure 5.8). Younger root extracts caused a lower rate of SRN immobilisation compared to the older root extracts. Eight-week-old root extracts caused less immobility compared to 12, 16 and 20-week-old E- root extracts; the immobility was not significantly different between 12, 16 and 20-weeks-old at the highest concentration of 5000 µg ml-1, however in E+ extracts, 16 and 20-weeksold extracts had higher immobilisation compared to 12-week-old extracts. A dose dependent effect was also recorded with little to no immobility recorded at lower concentrations of 312.50 and 625 µgml-1 for both E+ and E- extracts. Interaction effects were only recorded for treatment and age (p=0.02).

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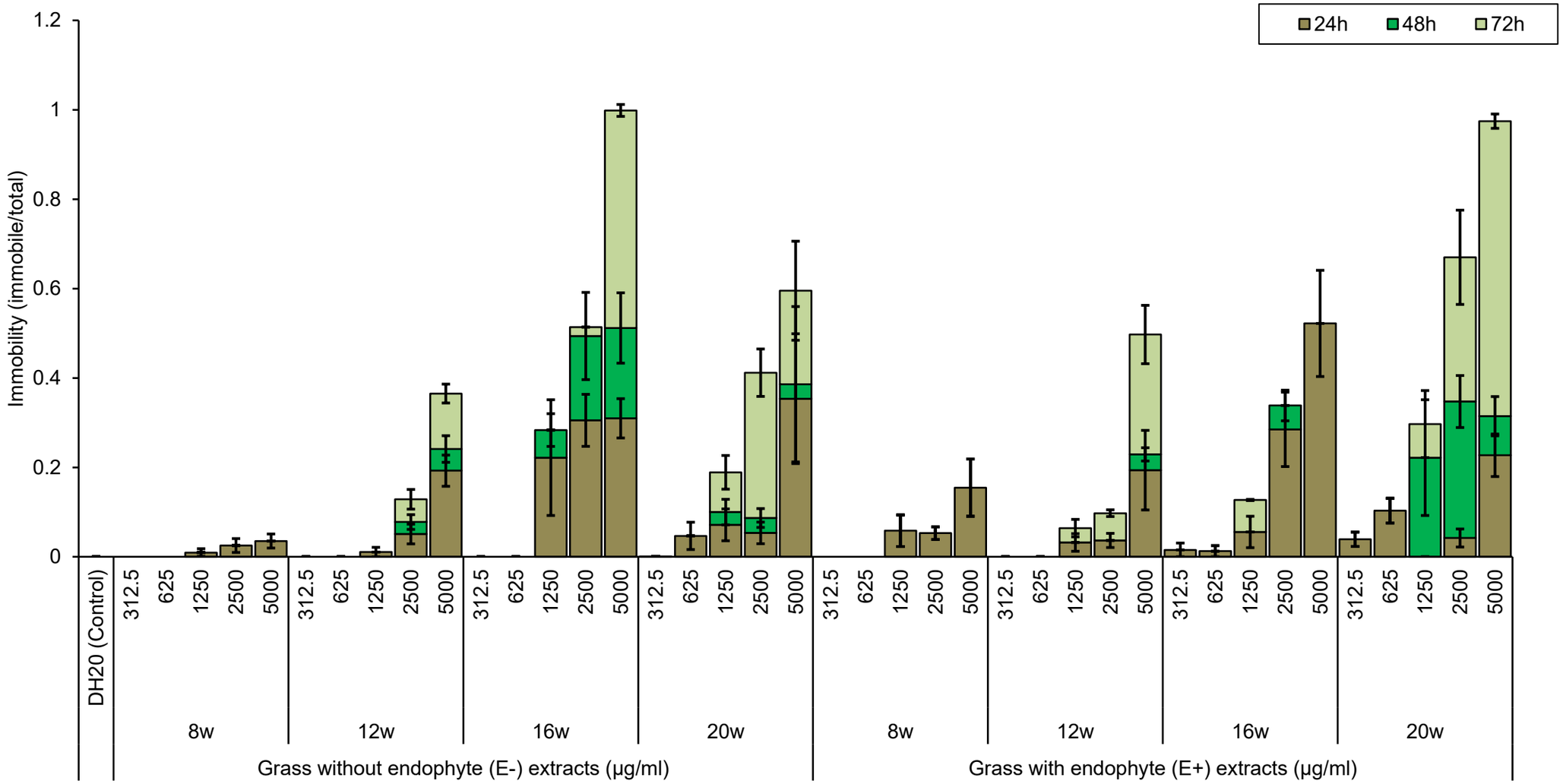

Figure 5.7: Experiment 1 Immobility of stubby root nematodes (SRN) upon exposure to different concentrations of endophyte (E+) and grass without endophyte (E-) at different ages, at exposure times of 24,48 and 72h.

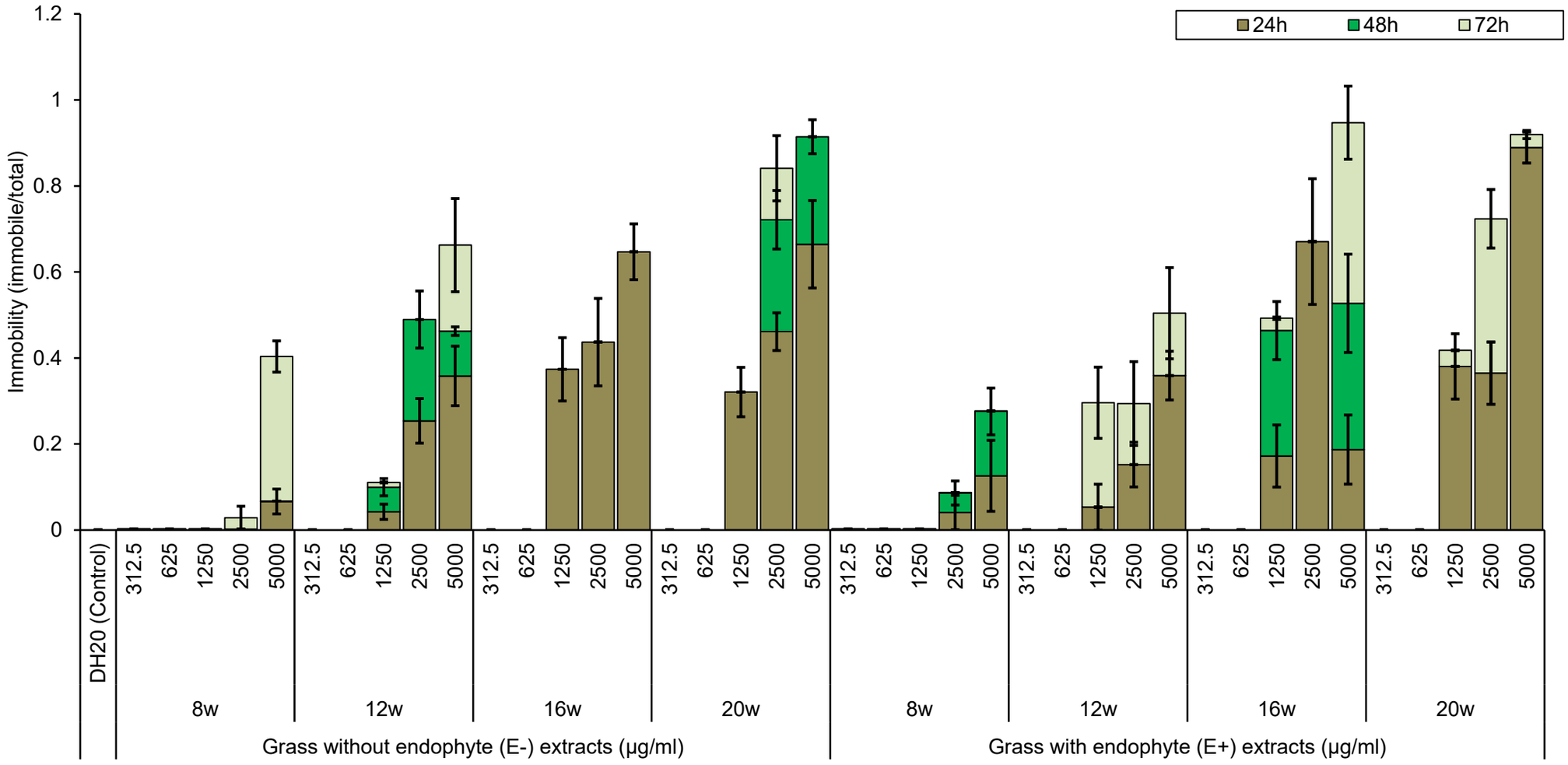

Figure 5.8: Experiment 2 Immobility of stubby root nematodes (SRN) upon exposure to different concentrations of root extracts obtained from grass with endophyte (E+) and grass without endophyte (E-) at different ages, at exposure times of 24,48 and 72h.

5.5.2 Effect on mortality of Stubby root nematodes.

Comparison of the LD_{50} values indicated that the presence of the endophyte had a significant effect on the mortality of SRN as compared to grass without endophyte (E-). The LD_{50} values of E+ extracts were significantly lower ($P<0.05$) when compared to E- extracts at all ages. (Table 5.1). The LD_{50} value for shoot extracts of endophyte grass (E+) was significantly lower at 8 weeks compared to all the other ages. The LD_{50} values for 12 weeks and 20 weeks were also significantly lower as compared to 16 weeks for E+ extracts. In the case of E- extracts, the LD_{50} values increased with an increasing age of the extracts, where 8-weeks-old plants had significantly lower LD_{50} values compared to 12, 16 and 20-weeks-old plants, indicating that extracts obtained from younger grass were more potent compared to extracts from older grass.

The reverse was observed in root extracts of E+, where mortality increased with increasing age of the grass. No mortality was recorded for 8-weeks-old root extracts for both E+ and E- grass, indicating that the immobility earlier observed was reversible as the nematodes recovered. For E+ root extracts the LD_{50} of 20 weeks was significantly lower than 12 weeks but was not significantly different from 16 weeks. The LD_{50} values of root extracts from grass without endophyte had a different trend where LD_{50} values increased with increasing age of the grass. The LD_{50} value for 20-week-old plants was five times higher compared to 12 and 16-week-old plants, while no significant differences were observed between the LD_{50} of extracts from 12 and 16-week-old plants. It was also clear from the LD_{50} values that root extracts from E+ grass were more potent compared to E- root extracts, where at 12 weeks E+ LD_{50} values were two times lower than E-, while at 20 weeks the LD_{50} value was almost 50 times lower when compared to E- extracts.

Experiment 2 showed a similar trend to experiment 1 for shoot extracts, however the calculated LD_{50} values for root extracts were higher compared to the first experiment (Table 5.2). Comparison of the LD_{50} values for E+ and E- indicated that the endophyte significantly increased the potency of the grass extracts as indicated by the significantly low LD_{50}values for 12–20-week-old shoot extracts in E+ extracts compared to E-. Extracts obtained from younger grass were more potent than older grass for both E+ and E- extracts, except for 16 weeks, which was higher than 20 weeks old shoot extracts in E+ extracts. For the shoot extracts of E- grass, LD_{50} values for 8- and 12- weeks-old extracts were significantly lower compared to the 16- and 20-week-old extracts. Like observations from experiment 1, the presence of the endophyte increased the potency of the root extracts, as revealed by the low LD_{50}values for the E- compared to E+ grass (Table 5.2). The LD_{50}values also decreased with increasing age in E+ extracts, where the lowest LD_{50} was recorded in oldest root extracts (20 weeks) and no mortality was recorded for 8-week-old root extracts. In E- extracts the opposite was true, where older root extracts had higher LD_{50}values as compared to root extracts from younger grass, just as recorded in the first experiment.

Table 5.1: Lethal dose values (LD_{50}) in µg/ml, standard error of the mean (Std.Error), lower and Upper limits of grass with endophyte grass (E+) and grass without endophyte (E-) shoot and root extracts obtained from grass at different ages on *Trichodorus primitivus* (Experiment 1).

Plant age			Shoots				Roots	
Grass with Endophyte(E+)	LD50	Std.Error	Lower	upper	LD50	Std.Error	Lower	upper
8 weeks	626.96[e]	93.58	442.325	811.60	nd	nd	nd	nd
12 weeks	1520.64[c]	151.13	1222.44	1818.83	6274.49[b]	3063.41	189.41	12359.58
16 weeks	7423.64[b]	3016.83	1471.18	13376.1	3399.12[a]	343.22	2717.37	4080.88
20 weeks	4274.92[b]	430.37	3425.83	5124.02	2846.62[a]	374.08	2103.55	3589.69
Grass without endophyte(E-)	LD50	Std.Error	Lower	upper	LD50	Std.Error	Lower	upper
8 weeks	860.192[a]	96.868	669.06	1051.32	nd	nd	nd	nd
12 weeks	4717.004[b]	389.162	3949.16	5484.85	5674.98[b]	710.99	4262.69	7087.27
16 weeks	9488[d]	543.13	8401.74	10574.26	6470.6[b]	1168.73	4149.07	8792.13
20 weeks	73450.11[c]	3317.74	66814.63	80085.59	140092.8[d]	8037.22	124,017.56	156166.44

nd-not detected.

Table 5.2: Lethal dose values (LD_{50}) in µg/ml, standard error of the mean (Std.Error), lower and Upper limits of grass with endophyte grass (E+) and grass without endophyte (E-) shoot and root extracts obtained from grass at different ages on *T. primitivus* (Experiment 2).

Plant age	Shoots				Roots			
Grass with endophyte (E+)	LD_{50}	Std.Error	Lower	upper	LD_{50}	Std.Error	Lower	upper
8 weeks	1410.7[a]	521.859	381.024	2440.368	nd	nd	nd	nd
12 weeks	1777.987[a]	280.57	1127.42	2337.99	6470.6[e]	1168.73	4149.07	8792.13
16 weeks	3059.765[b]	715.87	1647.3	4472.23	5403.67[e]	621.65	4168.85	6638.5
20 weeks	2534.14[d]	435.838	1672.46	3395.82	3399.12[c]	343.22	2717.37	4080.88
Grass without endophyte (E-)	LD_{50}	Std.Error	Lower	upper	LD_{50}	Std.Error	Lower	upper
8 weeks	1170.695[a]	185.065	805.547	1535.844	nd	nd	nd	nd
12 weeks	2717.33[b]	439.367	1850.422	3584.238	8660.95[a]	2631.25	3434.29	13887.61
16 weeks	7708.94[c]	2542.50	2623.94	12793.94	12345.12[d]	6027.06	373.11	24317.14
20 weeks	19966.7[e]	5000	9966.70	29966.70	21243.33[b]	20006.89	18497.9	60984.56

nd-not detected.

5.5.3 Nematotoxic effect of bruised Endophyte grass.

The extracts from regrowth shoot tissue obtained from wounded/bruised endophyte grass were more potent compared to the non-bruised control form the LD_{50} values comparison (Table 5.3). The lowest LD_{50}value was recorded in shoot extracts obtained from regrowth tissue at 11 days post bruising, it was two times lower than extracts from 30dpb and three times lower than the control non-bruised. The regrowth tissue extracts 3dpn and 7dpb also had LD_{50} values two times lower than the non-bruised control. No significant differences were recorded between 30dpb and the non-bruised control. There were also no significant differences between 3,7 and 11dpb LD_{50}.

Table 5.3: Lethal dose values (LD_{50}), standard error of the mean (Std.Error), lower and Upper limits of Endophyte grass extracts obtained from 8 weeks old shoots bruised at different time points (dpb) on *Trichodorus primitivus* (data pooled from two repeats) Means in same column followed by different letter are significantly different according to Tukey HSD (P≤0.05).

Days post bruising (dpb)	LD_{50}	Std.Error	Lower	upper
3dpb	804.652^{ab}	281.338	245.639	1363.665
7dpb	741.896^{ab}	179.494	382.02	1100.87
11 dpb	518.383^{a}	171.612	175.16	861.61
30dpb	1229.917^{bc}	340.574	681.15	1811.07
Control (non-bruised)	1731.277^{c}	426.895	883.047	2579.508

5.5.4 Phytochemical analysis.

5.5.4.1 Loline alkaloids in bruised and non-bruised treatments.

The artificial wounding and bruising not only led to the change in total lolines quantity but also caused a shift in the composition of the different lolines. The loline alkaloids N-formylloline (NFL), N-acetylloline (NAL) and N-acetylnorloline (NANL), were present after 3,7-, and 11-days post bruising (dpb) and absent in 30dpb and the control unbruised treatments, where NFL was the only loline detected. The loline alkaloid NFL was the most pre-dominant accounting for 89-91%, followed by NANL and the least was NAL. Bruising had a significant effect on the total loline content (Table 5.4). Bruised treatments had two to three times more lolines compared to the non-bruised control. The number of days post bruising also influenced the total loline alkaloids where after 3,7 and 11dpb loline alkaloids were significantly higher than 30 dpb.

Table 5.4: Effect of shoot bruising on loline content of 8 weeks old Endophyte grass (E+) at different days post bruising (dpb). Means in same column followed by different letter are significantly different according to Tukey HSD (P≤0.05)

Days post bruising (dpb)	NFL	NAL	NANL	Total lolines
3dpb	2366	151	191	2707 [a]
7dpb	3330	145	176	3650 [a]
11 dpb	3504	140	274	3918 [a]
30dpd	936	0	0	936 [b]
Control (Unbruised)	1011	0	0	1011 [b]

5.5.4.2 Loline alkaloids experiment 1.

Loline alkaloids: N-formylloline (NFL), N-acetylloline (NAL) and N-acetylnorloline (NANL), were tested in both endophyte infected (E+) and non-infected (E−) plants. No lolines were detected in E- plants. The trend in total lolines quantity and composition were influenced by age and variations were recorded in shoots and roots both in quantity and composition. The total lolines increased with increasing age, where at 20 weeks the lolines were twice the quantities at 8 weeks and significantly differed from all ages. In shoots, the loline NFL was the most predominant loline accounting for 84-92% of the lolines recorded for the different ages. At 8 weeks in shoots, only NFL was present, while in 12- and 16-weeks old NFL and NANL were present, all the lolines NFL, NAL and NANL were present in 20weeks old extracts. In roots only NFL was detected in 16-week-old grass and no lolines were detected in all the other treatments.

In the second experiment, the concentration of loline alkaloids was higher and detected in all treatments of E+ grass as compared to treatments in experiment 1. All the lolines NFL, NAL and NANL were present in shoots of all the E+ treatments except for 8 weeks old where only NFL was detected (Table 5.5). NFL was still the most pre-dominant loline followed by NAL and least was NANL. The total lolines followed the same trend where they increased with age and at 20 weeks, they were almost five times higher than 8 weeks. No differences were observed for 12 and 16 weeks, but both were three times higher than 8weeks, which were the lowest. Lolines. In the roots, like experiment 1, only NFL was detected, however in this case lolines were detected in all the treatments. Lowest lolines in the roots were detected in 8-week grass extracts while no significant differences were observed between 12,16- and 20-weeks old grass.

Table 5.5: Loline alkaloid concentrations µg/g dry matter (DM) in shoots and roots of at different ages of grass with endophyte grass (E+) and grass without endophyte (E-). Means in same column followed by different letter are significantly different according to Tukey HSD (P≤0.05).

Experiment 1								
Plant age	Shoots				Roots			
Grass with endophyte (E+)	NFL[a]	NAL	NANL	Total lolines	NFL	NAL	NANL	Total lolines
8 weeks	1011	nd	nd	1011[d]	nd	nd	nd	nd
12 weeks	1420	254	nd	1674[c]	nd	nd	nd	nd
16 weeks	1623	145	nd	1768[b]	144	nd	nd	144
20 weeks	2144	94	98	2336[a]	nd	nd	nd	nd
Grass without endophyte (E-)	NFL[a]	NAL	NANL	Total lolines	NFL	NAL	NANL	Total lolines
8 weeks	nd	nd	nd	nd	nd	nd	nd	nd
12 weeks	nd	nd	nd	nd	nd	nd	nd	nd
16 weeks	nd	nd	nd	nd	nd	nd	nd	nd
20 weeks	nd	nd	nd	nd	nd	nd	nd	nd

Experiment 2								
Plant age	Shoots				Roots			
Grass with endophyte (E+)	NFL	NAL	NANL	Total loline	NFL	NAL	NANL	Total lolines
8 weeks	1449	0	0	1449[a]	93.29	nd	nd	93 [a]
12 weeks	3716	408	162	4286[ab]	247.16	nd	nd	247 b
16 weeks	3682	405	172	4258[ab]	129.57	nd	nd	130 [ab]
20 weeks	5616	490	368	6475[b]	111.06	nd	nd	111.06 [ab]
Grass without endophyte (E-)	NFL	NAL	NANL	Total loline	NFL	NAL	NANL	Total lolines
8 weeks	nd	nd	nd	nd	nd	nd	nd	nd
12 weeks	nd	nd	nd	nd	nd	nd	nd	nd
16 weeks	nd	nd	nd	nd	nd	nd	nd	nd
20 weeks	nd	nd	nd	nd	nd	nd	nd	nd

NFLa = N-formylloline; NAL = N-acetylloline; NANL = N-acetylnorloline. Total loline = NFL + NAL + NANL. nd = not detected.

5.5.4.3 Total flavonoid and total phenol content

In extracts obtained from the shoots, the total flavonoid content (TFC) and total phenol content (TPC), reduced with increasing age from 8-20 weeks in both grass with endophyte (E+). and grass without endophyte (E-). No significant differences at different ages were recorded between extracts obtained from E+ and E- grass (Table 5.6). In E- and E+ shoot extracts, TFC was highest in week 8 and declined in week 12 ,16 and 20. The variation was recorded between all ages, where they were all significantly different from each other. In the roots extracts the TFC in E + roots were significantly higher in 20weeks old plants compared to 12- and 16-weeks old extracts, whereas the variation was not significant for 8 weeks old extracts.

On the other hand, for E- root extracts, the TFC were only significantly different between extract obtained from 8- and 16-weeks old grass, where they were higher at 8 weeks. Total phenolic content (TPC), in shoots of both E- and E+ grass extracts followed a similar pattern as TFC, where the content decreased with increasing age. This was also the case in E- grass extracts. However, in the TPC content in the shoot extracts obtained from E- grass was significantly higher compared to E+ extracts when compared at similar ages from 8-2o weeks old. In the roots, the TPC had an opposite trend to the shoot extracts where they declined with increasing age.

Root extracts obtained from 8– and 12-weeks old grass had significantly higher TPC compared to extracts from 16- and 20 weeks old grass. However, for root extracts obtained from E+ grass the TPC declined up to 16 weeks and increased at 20weeks, although the difference was not significant. When comparing the TPC at similar ages between E+ and E- grass, there were no significant differences. There was a negative correlation between the shoot biomass and the flavanoid content, where the increase in shoot biomass was correlated with lower TFC (R = -0.94). This was also the case for TPC in both shoots (R = -0.67) and roots (R = -0.79) (Table 5.6).

Table 5.6: Quantification of flavonoid and phenol contents derived from methanolic shoots and roots. crude extracts of 8-, 12- , 16- and 20-week-old grass with endophyte grass (E+) and grass without. Values are means (n= 3). Similar lower-case letters in each column indicate that means are not significantly different according to Tukey's multiple comparison at 0.05 level. QE= quercetin equivalent, GAE = gallic acid equivalent.

Age	Total flavanoid content (µg QE g^{-1})				Total phenol content (µg GAE g^{-1})			
	Shoots		Roots		Shoots		Roots	
	E+	E-	E+	E-	E+	E-	E+	E-
8 weeks	440.91^{a}	438.08 ^{a}	435.60^{abcd}	447.28 ^{ab}	1262.15^{b}	1346.20^{a}	1427.8^{a}	1607.2^{ab}
12weeks	426.04^{b}	430.64 ^{b}	428.87 ^{de}	409.06^{bcde}	1060.23^{c}	1238.60^{b}	1369.6^{ab}	1224.1^{abc}
16 weeks	414.37^{c}	412.24^{c}	411.89 ^{e}	416.14 ^{cde}	853.19^{e}	1022.20^{cd}	834.41^{de}	664.46^{e}
20 weeks	408.7^{d}	406.94^{d}	470.98 ^{a}	421.44 ^{abc}	955.54^{de}	1012.80^{c}	1255.5^{bcd}	692.16^{cde}

5.5.5 Shoot and root biomass.

The average shoot and root fresh weight significantly increased with increasing age of the grass, while no significant differences were recorded in shoot and root biomass when comparing similar ages for E+ and E- plants. The shoot biomass for E+ and E- grass at 8 and 12 weeks in Experiment 1 was lower compared to Experiment 2, while at 20 weeks, the shoot and root biomass in both E+ and E- grass was higher in Experiment 1 than in Experiment 2 (Table 5.7).

Negative correlations were recorded between the antioxidants measured and the shoot and root biomass. A negative correlation ($R=- 0.94$, $p<0.001$) was recorded for total flavonoid content and shoot biomass, while negative correlations were recorded between 1) total phenolic content and root biomass ($R = -0.79$, $p<0.001$) and 2) phenolic content and shoot biomass ($R=0.67$, $P<0.001$).

Table 5.7: Average fresh root and shoot biomass (grams) for *F. loliceum* Endophyte infected (E+) and non-infected (E-) grass grown under glass-house conditions, n=3. Means in same column followed by different letter are significantly different according to Tukey HSD (P≤0.05).

Experiment 1

	Shoots		Roots	
Age	E+	E-	E+	E-
8 weeks	42.34 ± 7^{e}	47.68 ± 15.06^{e}	15.74 ± 7.27^{a}	35.70 ± 11.82^{ab}
12 weeks	124.5± 7.02^{d}	157.04 ± 15.27^{d}	66.13 ± 15.89^{abc}	107.3 ± 14.44^{cd}
16 weeks	223.8± 31.79^{c}	352.63 ± 19.43^{bc}	88.2 ± 26.72^{bcd}	149.17 ± 4.32^{d}
20 weeks	601.67 ± 16.53^{ab}	694.58 ± 49.91^{a}	114.83 ± 14.52^{cd}	143.18 ± 10.33^{d}

Experiment 2

	Shoots		Roots	
Age	E+	E-	E+	E-
8 weeks	322.47 ± 20.08	398.1 ± 28.91	37.26 ± 0.96^{bc}	53.98 ± 6.82^{c}
12 weeks	456.70 ± 50.14	341.56 ± 100.12	126.96 ± 23.86^{abc}	70.87 ± 13.78^{abc}
16 weeks	394.57 ± 35.12	347.73 ± 15.73	197.77 ± 18.58^{a}	94.07 ± 38.07^{ab}
20 weeks	243.52 ± 123.19	377.1 ± 30.88	69.87 ± 42.84^{abc}	84.3± 14.28^{abc}

5.6 Discussion

Nematotoxic effects of root and shoot extracts.

In this study, root and shoot extracts of both grass with endophyte (E+) and without endophyte (E-), were able to immobilise *T. primitivus*. The presence of the endophyte *E. uncinata* did not change the bioactivity of the methanolic extracts. However, a comparison of the LD_{50} values of the shoot extracts indicated that E+ extracts were more potent than E-. Factors such as concentration of the extracts and the age of the grass associated with the extracts had the most significant effect on the immobility and final mortality recorded. In both shoot extracts of E+ and E-, high immobility and lower LD_{50} values were recorded from younger plants as compared to older plants.

This was opposite for root extracts in E+ plants where immobility increased with increasing age. On measuring the loline alkaloids, in shoots all the three loline alkaloids were detected i.e., (NFL, NAL, NANL) while only NFL was detected in the roots. The higher concentration of lolines in the vegetative tissue compared to the roots is expected as loline alkaloids are known to be initially synthesized in the vegetative tissue and subsequently translocated to the roots where the endophyte does not colonise (Meyer et al., 2020). The loline alkaloid NFL is the most abundant and is found in most organs of the grass as compared to the other loline alkaloids (Lee et al., 2021). No lolines were detected in E- grass and it also tested negative for the presence endophyte *E. uncinata* test.

The concentration of the alkaloids recorded in this study increased with increasing age of the plant and was not correlated with the mortality and the immobilisation observed in this study. Similarly for the roots there was negligible to no loline alkaloids detected. This was an indication that lolines were not the main drivers of the mortality observed in *T. primitivus*. These results are consistent with findings from a study where, despite the presence of lolines in E+ plants, there was no difference in *M. incognita* mortality between the E+ and E− root and shoot methanolic extracts obtained from greenhouse grown *Festulolium* spp. (Meyer et al., 2020). In some cases the presence of the endophyte has been shown to play a role in the motility of nematodes, for instance, in -vitro assay with methanolic root extracts obtained from the 22-week-old of tall fescue variety Jesup E+ verses E-, showed that the number of motile *P. scribneri*, after 72 h exposure period, were significantly lower in E+ compared to E- plants across all concentrations tested (111.5 -2400 µg ml-1).

However, some nematodes recovered after incubation in distilled water, indicating that the root extracts had a nematostatic effect (Bacetty et al., 2009a). With respect to root extracts activity, a nematostatic effect was also observed in this study where *T. primitivus* recovered during incubation in distilled water for 48 hours, and this explains the high LD_{50} values for root extracts. Categorization of extracts and compounds as nematicidal or nematistatic towards a specific species is usually a spectrum that is dependent on the compound concentration and exposure time. A compound can be nematostatic at lower concentrations and nematicidal at higher concentrations. Longer exposure time at lower concentrations can also render a compound nematicidal (Desmedt et al., 2020). In this study the exposure time did not play a critical role in increasing the mortality or immobilisation of *T.* primitivus as the counts did not change significantly from 24h-72h.

Different alkaloids associated with different endophyte-grass interactions differ in their mode of

activity, for instance, assays have been conducted using plant extracts as well as purified forms of the alkaloids. Loline, ergovaline and α-ergocryptine have been mostly documented to possess nematicidal activity while ergonovine have mostly been associated with nematostatic activity (Schouten, 2016). Other effects include repulsion activities, for example the presence of the endophyte in tall fescue with the endophyte *E. coenophiala* has also been associated with repulsion of *P. scribinieri,* when methanolic roots extracts were compared to E- plant roots which were an attractive effect instead (Bacetty et al., 2009b). The activity of plant extracts against organisms including PPNs, may be due to a major compound or a combination of different compounds (Chitwood, 2002).

In this study, despite high loline concentrations in E+ grass, mortality was also recorded for E- grass. The high loline concentrations in older grass (20-weeks) in the shoots did not correspond to high nematode mortality, whereas the younger shoots with lower loline concentration (8-weeks), had the highest mortality, as reflected in the LD_{50} values. This suggested that other compounds were involved in the pattern observed. Upon measuring the flavonoids and phenolic content, in shoot extracts for both E+ and E-, the younger plants had more phenolics and flavonoids compared to the older ones. These change in quantities with age correlated with the mortality we observed with respect to age of extracts tested which indicates that these compounds might be contributing to the SRN mortality recorded in this study, however, the direct effects of these phenolic and flavonoids were not tested towards *T. primitivus* in this study, but their correlation with the pattern of mortality recorded was of interest.

Previous studies have tested polyphenolics such as chlorogenic acid isolated from *Festuca* spp. colonised by *E. Coenophialum* and found that they have ability to immobilize *P. scribinieri*, however this effect was reversible (Bacetty et al., 2009). Irreversible effects of phenolic and flavonoid compounds on other plant parasitic nematodes have also been demonstrated. For instance, the phenolic compounds caffeic acid, syringic acid, and o-coumaric acid were active at 15 µg ml-1. in bioassays against *M. javanica* (Vouyoukalou & Stefanoudaki, 1998), methyl 4-hydroxybenzoate and methyl 4-hydroxycinnamate isolated from *Allium rayi,* were also shown to be active against *M.incognita*(Tada et al., 1988), similarly salicylic acid which is commonly known for inducing resistance in plants was shown to have direct toxicity to *M.incognita* and reduced the gallying index when applied at time of inoculation of nematodes in tomatoes (Maheshwari & Anwar, 1990).

Phenolic compounds have been reported to inhibit hatching in *P. penetrans* (Wuyts et al., 2006). The flavonoid coumestrol inhibited the motility of *P. scribneri* at 5–25 µg ml-1 but did not inhibit motility of *M. javanica* (Rich et al., 1977), similarly medicarpin was shown to inhibit motility of *P. penetrans* in vitro (Baldridge et al., 1998). The widely distributed flavonoid, quercetin, was recorded inhibiting the reproduction of *M. javanica* when applied as a soil drench at 400 µg ml^{-1} (Viglierchio, 1988). A negative correlation between high shoot biomass and flavanoid and phenolic concentration was recorded. Older plants had a higher biomass and might be one of the explanations to the lower concentrations recorded The change in phenolic content with age has also been demonstrated in other studies where the content of total phenols in sorghum grass declined with the maturity of the plant (Lanyasunya et al., 2007), similarly more phenolic compounds were isolated in younger tree bark residues as compared to older bark residues (Machrafi et al., 2006).

Effect of bruising foliage of endophyte grass on nematotoxicity.

In this study artificial wounding caused an increase in loline alkaloid concentration in endophyte infected grass compared to the control non-bruised grass. The increment in the loline alkaloid was highest in regrowth tissue 11 days post bruising, while regrowth tissue 30 days post bruising had significantly lower concentrations, indicating that the increase is temporary and declines as plant recovers from the attack/stress. following plant infection and contribute to plant defense strategies (Gantner et al., 2019). Unlike wounding caused by herbivores, artificial wounding has been shown to trigger volatile compounds release which drop after few hours and in some instances the response induced may take time to occur (Li et al., 2014), in this study at 11 days post bruising, the loline alkaloids were at their highest and this was seen to decline at 30 days post bruising.

Plants infected by pathogens, beneficial microbes or insects have been confirmed to contain elevated levels of phenolic compounds (Wallis & Galarneau, 2020). Similarly, flavonoid compounds have also been shown to accumulate in large quantities following herbivore attacks (Shen et al., 2022). The increased elevation in the loline alkaloids in E+ shoots was also reflected in their nematotoxic capabilities as shown in the low LD_{50} values in grass extracts 3, 7 and 11 dpb when compared to control unbruised. Wounding/bruising has been previously reported to affect the composition or concentrations of plant compounds, for instance feeding activity in the roots of Festulolium line u5 E+ inoculated with *M. incognita* was shown to elevate the total concentration of loline alkaloids as compared to uninoculated (Meyer et al., 2020).

The total loline concentration of meadow fescue colonised with *Neotyphodium siegelii* was also shown to increase almost twenty times from zero to 11 days post clipping (Craven et al., 2001). A similar result was also obtained when tall fescue colonised with *E. coenophialum* was artificially damaged, the loline concentration in damaged plants increased approximately two-fold for E+ plants (1.16%) compared to the control undamaged E+ plants (0.63%) (Bultman et al., 2004). Contrary responses have also been reported in insects where attack of meadow fescue by grass grubs (*Costelytra zealandica*), reduced the loline alkaloids in the crowns, but the total loline concentrations were unaffected. Patchett et al. (2008) indicated that the increase or decrease of alkaloids is different for the different interactions and other factors might be at play during the process. The phenolic compounds chavicol and demethyleugenol were shown to be in higher concentrations in wounded leaves of *Viburnum furcatum* when compared to unwounded plants. (Yoshizawa et al., 1993).

In conclusion, the change in the concentrations of phenol and flavonoid contents post-wounding was not analysed in this study, to help correlate with the mortality of the different treatments. Based on the results obtained from the first experiment on phenol and flavonoid content, it would have been interesting to measure how these compounds also change upon bruising, this should be further investigated in future studies. The elevation of the loline alkaloids upon wounding presents an opportunity to optimise the efficacy of the grass in suppression of the nematodes and needs to be evaluated at the field scale. Further studies should also focus on a comprehensive non-targeted metabolomics analysis to allow characterization of all the compounds present in grass with and without the endophyte, to help further selection and testing of the compounds potential nematicidal activity.

Chapter 6: General discussion.

Stubby root nematodes (*Trichodorus* and *Paratrichodorus* spp.) are economically important pests and have been reported to cause a root yield loss of up to 50% to the sugar beet crop (Cooke & Draycott, 1971). The formation of a fangy root system due to their feeding on sugar beet roots leads to accumulation of soil tare, which is a major challenge during sugar beet processing in factories, where the beets need to be washed to remove the excess soil (Wright et al., 2022).

The use of cover crops as a management strategy has been used for a long time in management of pests and pathogens. The modes of action vary depending on the plant family and sometimes species and determining how a cover crop is utilised in the management of these nematodes. In endoparasitic nematodes, which form an intimate relationship by creating feeding sites within the host, cover crops can be used as trap crops, where the cover crop is destroyed before the nematode finishes its life cycle.

Therefore, the utilisation of cover crops for nematode management considers the nematode lifestyle for appropriate targeting. Most ectoparasitic nematodes are not specialist feeders and feed on many plants, they migrate from one root to another and do not initiate an intimate relationship with the host. The active movement from one root system to another may limit the exposure of these nematodes as they may evade toxic metabolites released by allelopathic cover crops (Cook & Lewis, 2001b). For that reason, it is crucial to ensure compounds directly interact with these nematodes to achieve efficacy or explore other modes of action in cover crops that limit their feeding and reproduction.

This discussion synthesizes findings from a series of experiments conducted under controlled conditions and under field conditions to understand the effect of cover crops and their associated compounds in the suppression of SRN. These studies investigated the use of brassica and non-brassica cover crop species and evaluated some of their associated compounds. Brassicas contain glucosinolates which upon hydrolysis by the enzyme myrosinase, release isothiocyanates (ITCs) which have bioactive effects on pests and pathogens. In Chapter 3, *in-vitro* assays were conducted to evaluate the sensitivity of SRN to commercially available ITCs.

The ITCs selected were based on their association with the brassicas used for the field trials. The results of this study demonstrated that SRN are very sensitive to ITCs with mortality being recorded after 24 h exposure period. The lethal doses (LD_{50}) values were low (7-24 µg ml^{-1}) for all the ITCs tested. The potent concentrations recorded in the assay, are biologically relevant and achievable under field conditions (Bellostas et al., 2007; Ngala et al., 2015). Variation in toxicity of these ITCs to SRN, was observed where PEITC was more toxic compared to SITC and AITC. The variability in toxicity of different ITCs is influenced by their chemical structure, particularly the R side chain, which affects their biological activity. Studies have shown that aliphatic ITCs, such as allyl (AITC), methyl (MITC), and ethyl isothiocyanate (EITC), generally exhibit higher toxicity compared to aromatic ITCs like 2-phenethyl (PEITC) and benzyl (BITC) (Lazzeri et al., 1993; Lewis & Papavizas, 1971).

In this study, however, aromatic ITCs, specifically PEITC, demonstrated greater toxicity towards SRN compared to aliphatic ITCs such as AITC and sulforaphane (SITC). This observation contrasts with previous findings that aliphatic ITCs are more toxic to PPNs (Ashiq et al., 2021; Zasada & Ferris, 2003). This discrepancy may be attributed to variations in nematode species, environmental conditions, and specific bioassay methods employed in different studies. The

practical application of ITCs for nematode management in agricultural systems underscores the necessity for rapid action due to their volatility and short half-life in soil environments (Borek et al., 1995).

Effective concentrations of ITCs, achievable under field conditions, are contingent on the type of brassica used, its glucosinolate content, and prevailing soil properties. Brassicas such as *Brassica juncea* and *Raphanus sativus* have been identified as effective candidates for biofumigation due to their high glucosinolate content, which translates into higher ITC production upon tissue decomposition (Ngala et al., 2015) and in our field studies these brassicas were evaluated.

In Chapter 4, the effect of brassica and non-brassica cover crops on SRN densities were investigated under field conditions. The results in Chapter 3, correspond with the findings from the experiment conducted at Site 1, near Bury St. Edmunds, Suffolk, which investigated the efficacy of brassicas on SRN suppression. Decline in SRN densities were achieved as early as four weeks post brassica drilling for all the brassicas evaluated i.e., Indian mustard, oilseed radish and daikon radish. In this case, the glucosinolates may have been exuded through the young roots, which have been documented to possess high concentrations of glucosinolates during early growth.

The roots of brassicas such as oilseed rape are also known to release glucosinolates into the root rhizosphere (Choesin & Boerner, 1991; Paul Schreiner & Koide, 1993). Soil microbes in turn hydrolyse the glucosinolates into isothiocyanates by releasing the enzyme myrosinase (Dutta et al., 2019). Exudation of isothiocyanates from actively growing roots has also been reported (Elliott & Stowe, 1971) and is thought to be due to superficial cell damage during active root development when the plant is young (Ngala et al., 2015), a phenomenon known as partial biofumigation. The effect continued to be observed post incorporation of the brassicas, except for the daikon radish where there was a significant resurgence of SRN following drilling of winter wheat.

The oilseed radish used in this study (Terranova) was bred for resistance to beet cyst nematode and root lesion nematodes which may explain why it performed better than the daikon radish. At the site at Docking, the brassicas performed differently, and this may be due to factors such as the low amount of biomass accumulated; this was four times lower than site 1. The biomass accumulated by biofumigant brassicas is a key component to their efficacy. In the case of site 1, the biomass accumulated was close to the recommended 50 t ha^{-1} required for successful biofumigation (Lord et al., 2011). Site 2, however, had significantly lower biomass due to late seed sowing that led to poor establishment of the crop. This is an indication that the biofumigation process can be optimized for the suppression of this nematode, by ensuring good establishment and development of the brassicas.

The field experiment conducted at site 2 highlighted the varying impact of some of the non-Brassica cover crops in reducing SRN reproduction rates. *Phacelia* and opium poppy appeared to be less preferred hosts to SRN as compared to other cover crops like Italian rye grass and the nil-endophyte grass. In previous investigations of the host status of *M. hapla*, it was determined that *Phacelia* was categorized as a maintenance host, indicating that nematode densities did not fluctuate during the cropping season (Viaene & Abawi, 1998). Meanwhile, for *M. chitwoodi*, it was identified as a poor host (van Himbeeck et al., 2024), and as a fair host for *Ditylenchus dipsaci* (Augustin & Sikora, 1989), while it was considered a non-host for *H. schactii* (Gardner &

Caswell-Chen, 1993). Furthermore, it was observed that biologically enhanced *Phacelia* with *Pochonia chlamydosporia* successfully suppressed densities of *M. hapla* (Uthoff et al., 2024).

This indicates the potential use of *Phacelia* in nematode management. There is little information on the mechanisms behind the suppressiveness of *Phacelia* to plant parasitic nematodes, to facilitate optimisation of its use. However, *Phacelia* is known for its allelopathic properties that inhibit weed germination and growth (Wach, 2016; Kliszcz et al., 2023). Opium poppy has also previously been found to be a poor or non-host to several plant-parasitic nematodes.

For example, in field conditions, twelve species of nematodes in the tylechidae family were found in low frequencies ranging from 1-41% in poppy in the Afyon region, Turkey (Akgül & Ökten, 2001). Additionally, in pot experiment studies, poppy was determined to be a non-host for *Pratylenchus thornei* and *Merlinius brevidens* (Tobar et al., 1995). All members of the Papaveraceae family, in which opium poppy belongs, are known to produce and store different groups of benzylisoquinoline alkaloids, which have been extensively used in the pharmaceutical industry for their antimicrobial activities (Ismaili et al., 2017). Additionally, benzylisoquinoline alkaloids such as berberine and sanguinarine have been reported to reduce lipid accumulation in the nematode *Caenorhabditis elegans* (Chow & Sato, 2019). Berberine has also been shown to have antihelminth effects on *Strongyloides venezuelensis* in *in-vitro* experiments (Elizondo-Luévano et al., 2021), indicating the potential use of benzylisoquinoline alkaloids against nematodes.

Differences in SRN reproduction were also recorded in grass with endophyte (E+) which was two times lower compared to grass without endophyte (E-). The association between the fungal endophyte *Epichloë uncinata* and Festulolium hybrids leads to production of secondary metabolites known as lolines. These secondary metabolites play crucial roles in protecting plants against insect herbivory and environmental stresses (Lee et al., 2021). Loline alkaloids are water soluble and can be translocated to different host organs including the roots (Meyer et al., 2020). The translocation of these compounds to the roots and subsequent exudation might be one of the mechanisms responsible for the low reproduction in E+ grass as compared to E- grass during growth. Loline alkaloids exuded into the soil were not measured in this study, however previous studies have isolated some of the alkaloids e.g., ergot alkaloids associated with tall fescues in the soil. Alkaloids associated with grass-endophyte interactions have already been documented to cause mortality, paralysis and inhibit hatching of various nematode species (Meyer et al., 2020). The effects vary depending on the grass-endophyte combination which determines which alkaloids are produced as well as the mechanisms of suppression and the sensitivity of nematode species to the alkaloids produced.

In the experiment at site 3, the combination of cover crops in a mix (vitality mix) had the most pronounced impact on reducing SRN multiplication compared to the use of individual cover crops such as clover and stubble turnips. Specifically, clover appeared to increase the multiplication rate of SRN compared to all other cover crops, which was four times higher than that recorded for the vitality mix, three times higher than radish and vetch, and twice as high as oats and stubble turnips. In previous studies, cover crop mixtures have been found to be more effective in suppressing parasitic nematodes than single species cover crops.

In mixtures of cover crops, several mechanisms may occur based on the specific combinations involved, rendering them more effective compared to individual cover crops. Firstly, a potential dilution effect of allelochemicals may arise due to the reduced density of different species in the

mix, making it challenging for pests to locate their hosts (Boudreau, 2013). Secondly, the diversity in root architecture of the various crop species used creates a physical and visual barrier that complicates the host-finding process of pests (Ratnadass et al., 2012). Thirdly, the interactions between the plant species in the mixture may alter the morphological traits of the stand, further disrupting the pest's ability to locate hosts (Ratnadass et al., 2012). Additionally, depending on the species in the mixture, there may be changes in the chemical composition of the exudates released, making them either more attractive or repellent to the pest (Ratnadass et al., 2012).

Interestingly it has been demonstrated that the abundance of plant parasitic nematodes increased with the increasing C:N ratio of the aboveground biomass of the cover crops. Thus, brassica-legume mixtures may reduce nematode populations compared to pure crucifer crops, as their C:N ratio is generally lower (Couëdel et al., 2019; Cortois et al., 2017). A combination of vetch and radish yielded similar levels of plant-feeding nematodes as the vetch sole crop and more than the radish sole crop (Barel et al., 2018; Summers et al., 2014), indicating the importance of a legume in the mixture.

Additionally, black oat (*Avena strigosa* Schreb.) and white oat (*Avena sativa* L.) offer several benefits as rotational crops, including rapid growth, high biomass production, and extensive root development (Silva et al., 2021), and have been recorded to effectively suppress *Meloidogyne* spp. (Marini et al., 2016; Riede et al., 2015) and *Pratylenchus brachyurus* (Gabriel et al., 2018), however this suppression varies depending on the specific cultivar utilized (Machado et al., 2015). Cover crops such as oats, radish and vetch did not significantly differ from the vitality mix, suggesting their potential for individual sowing. It has also been demonstrated that cover crops which increased nematode numbers when planted individually yielded similar results when planted in mixtures with other cover crops (Bhan et al., 2010), the combination of these cover crops in the mixture could explain the overall reduced SRN densities. Similarly, no differences in the suppression of potato cyst nematodes were observed when mixing Indian mustard, white mustard, and rocket compared to sole crops. Mixtures of white and Indian mustard are commonly used to suppress plant-feeding nematodes, but their efficiency compared to sole crops is unclear (Kokalis-Burelle et al., 2013; Kruger et al., 2013).

In addition to variations in SRN densities observed due to cover cropping, it was evident that the population densities were influenced by multiple factors. In site 2, several controls were included to account for other factors that may contribute to SRN decline or increase. Firstly, incorporation of cover crops into the soil involves various mechanical operations which include flailing, rotavating and sealing. Stubby root nematodes are known to be more sensitive to mechanical handling and tillage compared to other PPNs (Bor & Kuiper, 1966), this was also confirmed in this study, where there was a post incorporation effect leading to SRN decline in all plots except in the undisturbed fallow plots. Secondly SRN are known to be polyphagous in nature and parasitize on weeds as alternative hosts. A sterile fallow included in the study had fewer SRN as compared to undisturbed fallow control where no weeds were managed, indicating the importance of weed management in fallows. The weed pressure in this site was higher and could have masked the efficacy of some cover crops. Cover crops such as *Phacelia* are known to have allelopathic effects to weeds (Uthoff et al., 2024), where they inhibit weed germination and growth. This characteristic of *Phacelia* might have contributed to the low SRN densities observed in plots with *Phacelia*.

Environmental factors such as soil temperature and amount of rainfall also influenced the SRN densities recorded in the field trials. The sampling dates that took place during cover crop growth were characterised by significant differences in rainfall and soil temperature, and this had a significant effect on the SRN densities. The highest amount of rainfall at sampling was recorded in January 2023, and a positive correlation was observed between high rainfall and increasing nematode densities. This observation agrees with previous studies which reported that densities of SRN were positively correlated to high rainfall in May (Cooke, 1973). The strong negative correlation between soil temperature and SRN densities in our study can be explained by the behavior of trichodorids, which tend to move deeper in the soil profile during dry conditions, as they are highly susceptible to desiccation (Winfield & Cooke, 1975).

The use of cover crops had a notable impact on the quality characteristics of sugar beet roots during harvest. Root fanging symptoms and soil tare were markedly lower in plots with reduced SRN reproduction. There was a positive correlation between the densities of stubby root nematodes at the time of sugar beet drilling and the presence of root fanging. Consequently, plots with high nematode pressure exhibited increased root fanging, which in turn was positively associated with soil tare due to the accumulation of dirt in the roots.

The feeding activity of SRN on young sugar beet seedling roots result in the development of stubby lateral roots (fanging), which eventually turn grey-brown and black as they deteriorate. The susceptibility of young sugar beet to SRN infestation was demonstrated in a study where higher densities of *T. cylindricus* or *P. pachydermus* were found around young seedlings (1500 l^{-1}) compared to larger plants (600 l-1) at Gayton, Thorpe, England (Whitehead & Hooper, 1970). This finding explains the positive correlation observed between root fanging and initial SRN densities at sugar beet drilling at the site at Docking.

Additionally, high SRN densities at the time of sugar beet drilling were positively correlated with increased rainfall and lower soil temperature, which may have significantly contributed to the extent of root fanging. Similar observations were made, linking severe damage to young sugar beet seedlings with high total rainfall in May. The soil type where SRN predominantly occurs is characterized by high sand content, which means high drainage capacity of the soil, leading to a lot of fluctuations in soil moisture, and SRN hence follow this moisture gradient. Therefore, when soil is at field capacity, SRN are found more in the rhizosphere and hence more docking disorders are evident. Moreover, higher rainfall can result in the leaching of soil nutrients, such as nitrogen and manganese, which has been associated with a high incidence of Docking disorder symptoms.

The effect of cover cropping on sugar beet root yield was not recorded in this study. This could be due to various reasons. One is that the sugar beet variety used in this study is a known tolerant variety to the beet cyst nematodes (*Heterodera schactii*), secondly, previous research has indicated that sugar beet affected by Docking disorder may recover later in the season, potentially explaining why no significant differences in yield were observed despite the visible root fanging symptoms at harvest.

Following differences in SRN reproduction observed between E+ and E- grass in the field experiment at Docking Norfolk, (Site 2), further work (Chapter 5) was undertaken to compare the nematotoxic effects of crude extracts obtained from grass with endophyte (E+) and grass without endophyte (E-). The experiment was designed to mimic the incorporation of the root and shoot biomass under field conditions, where the nematodes would be exposed to compounds from the

incorporated biomass as they decompose. *In-vitro* tests demonstrated that SRN were equally sensitive to extracts from both E- and E+ shoots in early stages at 8 and 12 weeks old where the LD_{50} values were lower than older plants. Despite the presence of loline alkaloids in E+ plants, there was no significant difference in nematode mortality between E+ and E- grass extracts in their early stages. However, as the plant was aging at 20 weeks differences in SRN mortality between E+ and E- were evident where the LD_{50} values were lower in E+ grass compared to E-.

The LD_{50} values were however lower in younger shoots, and older roots, indicating their high nematicidal activity. This was despite high loline concentrations being recorded in older shoots as compared to younger shoots. The loline alkaloids and the endophyte presence were not detected in E- grass hence the mortality observed indicated the role of other compounds. The opposite effect was recorded in root extracts where lower LD_{50} values were recorded in root extracts from older roots. This could be partly explained by the development difference between shoots and roots and the redistribution of alkaloids as the plant is growing. In efforts to monitor the phytochemical present in the grasses that might be associated with observed nematotoxic effects, three groups of compounds, namely lolines, phenolic and flavonoid compounds were measured to elucidate any compound patterns associated with the observed age-dependent bioactivity.

Upon measuring total flavonoid and phenolic content, there were no significant differences in the quantities between E- and E+ grass. In both E- and E+ shoots the phenols and flavonoids varied with age, with high amounts being observed in the young shoots as compared to older shoots. Previous investigation of extracts from tall fescue with and without endophyte also indicated that polyphenols identified in extracts as chlorogenic acid, 3,5-dicaffeoylquinic acids, caffeic acid, and two unidentified compounds, but these were not correlated with endophyte status, qualitatively or quantitatively (Bacetty et al., 2009). In previous studies phenolic and flavonoid compounds have been associated with induced plant resistance to nematodes, nematicidal activity, impaired motility and hatching inhibition (Chitwood, 2002).

On the other hand, lolines alkaloids increased with age, suggesting a shift in compound composition as the grass ages. No loline alkaloids were detected in E- grass suggesting that the observed effects were caused by other compounds such as phenols and flavonoids. For root extracts, all the alkaloids measured increased with increasing age. The loline alkaloids were very low and in 8 weeks old plants in the first experiment no lolines were detected in the roots. This is due to the natural distribution of lolines in tissues in grass-endophyte interactions. Higher concentrations are mainly found in the shoots as they are the points of synthesis, and the concentrations observed in roots are because of translocation through the xylem as loline alkaloids are water soluble.

This study did not involve fractionation of the individual compounds to assess their direct effects on the SRN, however the age variations in the flavonoids and phenols followed a similar pattern as the mortality we observed where younger shoots with high phenol and flavonoid content being associated with the highest mortality while older shoot extracts with lower quantities had corresponding lower mortality. However, it is possible that the observed effect is a result of combined effects of multiple compounds, and this needs further testing to verify which compounds are actively involved in the mortality.

The high mortality obtained in the younger shots at 8 weeks old led to investigation into how efficacy could be increased. Herbivore damage and wounding on the grass with endophyte is

known to stimulate the levels of lolines (Bultman et al., 2004). The next study explored the effect of wounding and cutting of the grass on elevation of these alkaloids and evaluation of how this translated into higher nematotoxic effects. The application of artificial wounding led to an increase in the concentration of loline alkaloids in endophyte-infected grass as compared to non-bruised control grass. The highest increment in loline alkaloid concentration was observed in regrowth tissue 11 days after bruising, while regrowth tissue 30 days post bruising exhibited significantly lower concentrations, indicating a temporary increase that diminishes as the plant recovers from the attack. This effect translated also into increased mortality where regrowth tissue of grass extracts obtained 11 days post bruising had the lowest LD_{50} value which was significantly lower than the control.

Many plants demonstrate heightened chemical and structural responses following damage by herbivores. However, this is not a universal reaction, as some plants lacking chemical defenses tend to become more vulnerable to damage or attack. Phenolic compounds such as lignin, coumarins, furanocoumarins, flavonoids, and tannins are notably elevated following plant infection and contribute to plant defense mechanisms. Similarly, flavonoid compounds have been shown to accumulate in significant quantities following herbivore attacks. The increase in loline alkaloids in endophyte-infected shoots also reflects their nematotoxic capabilities, as evidenced by the low LD_{50} values in grass extracts post bruising compared to unbruised control. Previous reports have indicated that wounding or bruising can alter the composition or concentrations of plant compounds. For instance, feeding activity in the roots of Festulolium line u5 E+ inoculated with *M. incognita* elevated the total concentration of loline alkaloids compared to uninoculated plants. Similarly, the loline concentration in meadow fescue colonized with *Neotyphodium siegelii* increased from 0.1% to 1.9% of plant dry mass from zero to 11 days post clipping. Contradictory responses have also been observed, as the attack of meadow fescue by grass grubs reduced the loline alkaloids in the crowns, although the total loline concentrations were unaffected, indicating that the increase or decrease of alkaloids varies depending on the specific interactions and other influencing factors. The changes in the concentrations of other compounds such as phenols and flavonoids following wounding were not examined in this study to establish a correlation with the mortality of the different treatments.

However, previous studies have also demonstrated that wounding can elevate the concentration phenolic compounds such as chavicol and demethyleugenol in wounded leaves of *Viburnum furcatum* compared to unwounded plants (Chitwood, 2002).

In conclusion, the integrated use of brassicas with high glucosinolate content, grass-endophyte associations producing nematicidal secondary metabolites, and strategic agronomic practices provide a robust framework for managing nematode populations. This approach leverages the natural defense mechanisms of plants and the influence of environmental factors, offering a comprehensive and sustainable strategy for nematode management in agricultural systems. Tailoring nematode management strategies to specific local conditions and crop systems is essential for optimizing their effectiveness, recognizing that there are numerous factors under field conditions that may influence the efficacy of management strategies, which need to be taken into consideration. By doing so, this will enable selection of suitable strategies across diverse agricultural contexts, ensuring that farmers can effectively suppress nematode populations, improve crop health and enhance overall agricultural productivity.

Further work.

Results from this study have demonstrated that brassicas can be used in management of SRN through in-vitro work that is supported by one of the field studies at site 1 in Bury St. Edmunds. However, variations were recorded in Docking, where brassicas did not establish well and, alot of multiplication was recorded on Indian mustard in this site. There is need to evaluate more brassica cultivars to enable selection of those that do not support multiplication to optimize the biofumigation effect. The observations of the cover crops *Phacelia* and opium poppy as less preferred hosts for SRN need to be repeated under controlled glass-house conditions to verify their host-status and underpin the mechanisms of action, especially for *Phacelia* which is already known to be allelopathic to weed species. In studies on nematotoxic effects of grass with and without endophyte, more work is needed in profiling all alkaloids present in grass species with and without the endophyte and subsequent section of alkaloids with bioactivity against SRN.

This would require exposing the nematodes to pure forms of these compounds to determine potential candidates. This would help optimise their use once the active compounds have been verified and tested. Investigation of the fate of these compounds in the soil and the quantities available is also crucial as it has direct implications to the soil dwelling nematodes. The fact that lolines are water soluble and are translocated from leaves to roots also indicates that these compounds are continuously exuded from the roots to the soil. Plants are known to utilise 40% of their synthesized carbon in root exudates hence are worth investigating for nematode management (Badri & Vivanco, 2009). This study also only reports the direct effects of these compounds on nematode mortality. Other mechanisms such as repulsion and induced plant resistance might be at play and need investigating. The wounding of the grass with endophyte was shown to elevate alkaloids, leading to higher nematotoxic effects. This finding presents an opportunity to further explore how the change in alkaloid concentration when the plant is wounded can be utilised under different circumstances to effectively suppress nematodes.

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