# An investigation into the anthelmintic properties of seed extracts from endophyte containing pasture grasses

A thesis

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by

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The study investigated the *in vitro* anthelmintic properties of extracts from seeds containing the endophytes AR1, AR37, wild type in perennial rye grass (RG), and the loline producing endophytes namely Meadow Fescue (MF and U2) and Tall Fescue (TF and AR542) with Nil endophytes rye grass (Nil RG) and Nil endophytes tall fescue (Nil TF) used as controls. The *in vitro* efficacy of the extracts was determined on the gastro-intestinal parasite species *Teladorsagia circumcincta* and *Trichostrongylus colubriformis.* For loline producing endophytes, the LD50 for *T. colubriformis* egg hatching for AR542 and Nil TF were not different from each other ($P>0.05$) but significantly differed from meadow fescue ($P<0.05$). Within the rye grass varieties, the LD50 for *T. circumcincta* egg hatching for AR1 and wild-type endophyte were not different from each other ($P>0.05$) but differed significantly from AR37 and Nil RG ($P<0.05$). For *T. circumcincta* larvae incubated in loline producing endophytes, there was no observable difference in the immobility of larvae incubated in either AR542, nil Tall fescue or meadow fescue ($P>0.05$). For rye grass varieties, LD50 for larval immobility was significantly greater in nil RG and wild-type RG than either AR1 or AR37. For *T. colubriformis* larvae incubated in loline producing endophytes, there was no observable difference in the immobility of larvae incubated in either AR542 or nil Tall fescue but differed from meadow fescue ($P>0.05$). For rye grass varieties, LD50 for larval immobility was significantly greater in Nil RG compared with AR1, AR37 and wild-type endophyte and AR1 and AR37 were significantly greater than wild-type endophyte. Re-dilution significantly reduced the percentage of immobile *T. circumcincta* L3 from 59% at 15hrs to 24% ($P<0.001$) and significantly reduced the percentage of immobile *T. colubriformis* L3 from 63% at 15hrs to 39% ($P<0.001$) indicating that effects were reversible. In conclusion, the apparent anthelmintic properties demonstrated *in-vitro* in these studies indicated that seed extracts, independent of endophyte type can have deleterious effects on nematode viability.

# Acknowledgements

I would like to take this time to especially thank my supervisor, Dr Andrew Greer for providing me with the opportunity to do this project. He took a chance with me and encouraged me even when I doubted myself. I would also like to thank Prof Grant R Edwards, my associate supervisor for his feedback and all his contributions for making this project a success. I would also like to thank Dr. Micheal Cripps, Prof Grants Edwards, valuable information on endophytes and providing me with the seeds used in my experiments. I can't forget about Mr Robin McAnulty who taught me how to collect nematode egg, procedures for the assays and all the assistance he gave me in the laboratory. I would also like to thank Dr Brian Patchett of Cropmark for providing seeds for meadow fescue and Dr Micheal Cripps for providing seed for Tall fescue (Nil endophyte and P542 endophyte). I would also like to thank all the laboratory technicians who helped me with sourcing some laboratory consumables like centrifuge tubes, filters and others who helped me with freeze drying of samples.

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# Chapter 1

# Introduction

## 1.1 Parasitism

Parasitism is a non mutual symbiotic relationship between species where one of the species, the parasite benefits at the expense of the other, the host. In animal production systems, internal parasites of major significance fall into three main groups namely, nematodes (round worms), cestodes (tapeworms) and trematodes (flukes) with nematodes being the most significant (Pomroy, 1997a). Parasites reduce host biological fitness and production by exploiting them for resources necessary for survival for example food, water, warmth and habitat (Athanasiadou & Kyriazakis, 2004). Due to these significant effects there is need to control these parasites. The most common method of controlling nematode parasites in grazing animals is the use of synthetic chemotherapeutic drugs (anthelmintics) (Waller, 2003) for example albendazole, mebendazole, diethylcarbamazine, ivermectin and praziquantel. The use of synthetic anthelmintics is faced with some challenges such as development of resistance, public health and environmental concerns with development of resistance being the main one (Waller et al., 1995b). Given these challenges there is search for alternative methods.

## 1.2 Fungal Parasites

Fungal endophytes are microscopic fungi that naturally occur in plants (Stepek, Behnke, Buttle, & Duce, 2004) whilst other novel ones are the result of inoculations. Fungal endophytes have been isolated from a broad range of plants including grasses, herbs and trees and from various plant tissues (Backman & Sikora, 2008). They colonise tissues such as leaves, stems, young inflorescence and seeds (Kuldau & Bacon 2008) establishing long-term mutual associations with plants. The associations are persistent, with the fungal endophyte benefiting from inhabiting the grass interior where there is a reliable source of nutrients, water, and isolation from competition and vertical transmission to the next generation and, in turn, it assists the grass resistance to insect herbivory, drought and also protect it from overgrazing in a relationship known as symbiosis (Muller & Krauss, 2005). In plants such as bananas, tomatoes and rice, fungal endophytes such as *Fusarium oxysporum* and *Trichederma atroviride* have been observed to reduce numbers or activities of nematodes

such as *Radopholus similis,* Helicotylenchus *multicinctus* and *Meloidogyne incognita*. Most of the pastures in New Zealand and the world over namely rye grass, meadow fescue and tall fescue contain fungal endophytes such as AR37, AR1, wild endophyte, AR542 and U2. These endophytes produce alkaloids such as peramines, lolitrems, ergovaline and lolines which have been reported to have insecticidal properties (Siegel et al., 1987). Given that fungal endophytes in plants have anti parasitic, there is a possibility that fungal endophytes in pastures have the capacity to assist in animal internal parasite control. The project aims to identify if grass fungal endophytes have anti parasitic properties against some common internal parasites of grazing animals.

# Chapter 2

# Literature Review

## 2.1 Internal parasites

Parasites are organisms that live either on the internal (endo-parasites) or on the external (ecto-parasites) of animals or plants. They can be found on all-living organisms and, in natural conditions, they generally exist in comparative harmony with their hosts, with outbreaks of clinical parasitism relatively rare (Brunsdon, Charleston, Cumberland, Vlassoff, & Whitten, 1975). Farm animals are hosts to a large number of endo-parasites of which the most economically significant are gastro-intestinal nematodes. Gastro-intestinal parasitism is a major disease syndrome of pastoral ruminants because the environments which favour pastoralism such as high rainfall , tall grass and warm conditions also favour the survival and development of the free-living stages of helminth parasites (Sykes, 1997).

Although there are different species of nematode parasites that affect livestock, there are only a few parasite species that cause major problems in grazing livestock notably *Haemonchus contortus, Ostertagia circumcincta, Trichostrongylus colubriformis* and *Nematodirus spathiger* (Urquhart, Armour, Duncan, Dunn, & Jennings, 1996). Nematode infections in grazing livestock are almost always a mixture of species (Waller, 2006). Proliferations of the gastro-intestinal parasites depends on environmental conditions for example where conditions are warm and moist like some New Zealand summers and autumns, *Haemonchus* are the most significant in sheep (Vlassoff, 1998). The *Ostertagia* species are the most significant in spring and summer, *Trichostrongylus axei* are significant in late summer and autumn, *Nematodirus* species are significant in early spring through summer and *Cooperia* species are common in autumn (Vlassoff, 1998). In the South Island of New Zealand the two major species as reported in ewes have been reported to be *Teladorsagia circumcincta* with 56% prevalence followed by *Trichostrongylus colubriformis* and *T. vitrinus* together at 25% prevalence (Herve, McAnulty, Logon, & Sykes, 2003).

## 2.2 Life cycle of helmith parasites

Development of effective and sustainable control programmes against helminth parasites is reliant on a good understanding of their life cycle both within and outside the host animal

(Familton & McAnulty, 1997 ; Vlassoff, Leathwick, & Heath, 2001).

With the exception of *Nematodirus*, the gastro-intestinal nematodes have a similar basic life cycle with no intermediate hosts (Familton & McAnulty, 1997 ; Pomroy, 1997a). Adult females lay eggs which pass out in the faeces (Figure 1). First stage larvae (L1) develops inside each egg then hatches and feeds on bacteria in the faeces. The period to hatching depends on availability of moisture and temperature with warm moist conditions speeding the process. The L1 larvae develop into L2 larvae and retain their sheaths to form infective third stage (L3) larvae. The sheaths become protective cuticles and development ceases until the L3 larvae are ingested by a host. These L3 larvae are about 1 mm in length and migrate out of the faeces with moisture films onto herbage. Development from eggs to larvae depends on the weather (temperature and moisture) and can take from one to ten weeks. The L3 larvae are ingested with the herbage by a host and are carried in the ingested food to the specific part of the gastro-intestinal tract that they normally inhabit then attach themselves on the walls. They moult in response to $CO_2$ concentration, temperature and pH (Vlassoff et al., 2001) entering the mucosal lining into the gland crypts and develop through two more larval stages and return to the lumen as adults. During this time, they grow from about 1 mm as L3s to adults of about 7-30 mm or more in length depending on the species. Male and female adults mate and the female produces eggs which pass through the lumen of the gastro-intestinal tract and then excreted via faeces thereby completing the lifecycle.

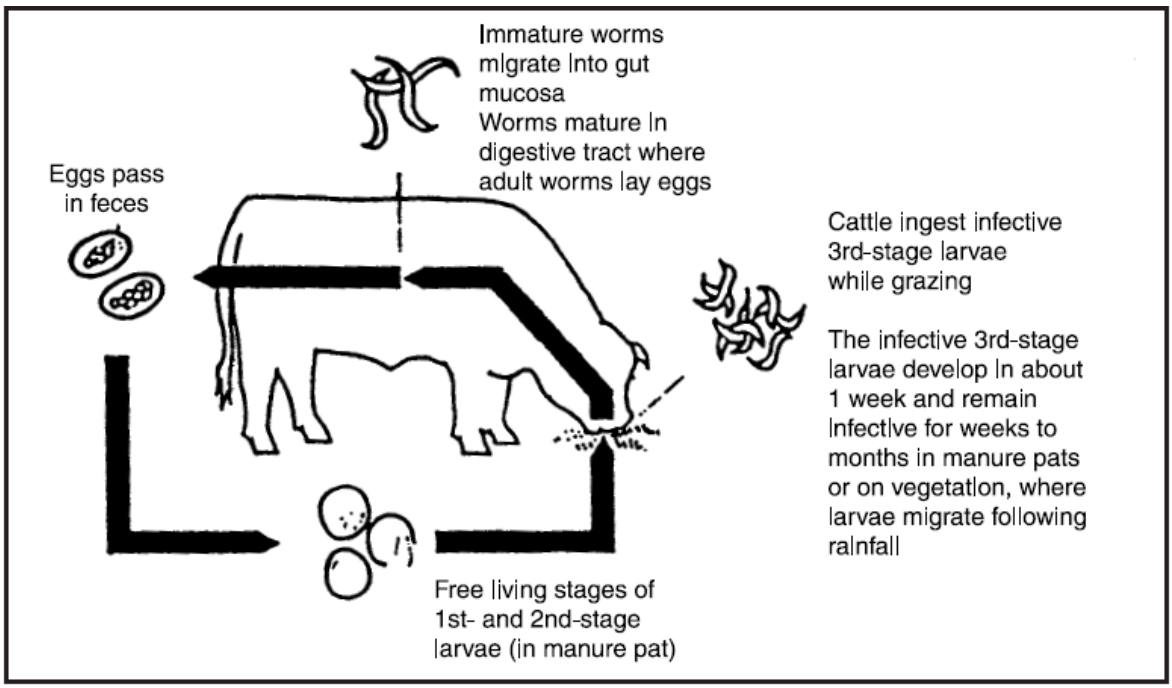


**Figure 1: A typical life cycle of gastro-intestinal parasites.**

Source: Gadberry, Pennington & Powell

## 2.3 Effects of internal parasites

Gastro-intestinal parasites are one of the greatest causes of lost productivity in grazing animals (Perry & Randolph, 1999). They reduce nutrient availability to the host through reductions in voluntary food intake and or reductions in efficiency of nutrient absorption (Athanasiadou & Kyriazakis, 2004). The depression of appetite in chronic subclinical infections can range from about 15 – 20 % (Coop & Kyriazakis, 1999), an effect which is reversible with intakes returning to normal in *T. colubriformis* infected sheep within a few days following anthelmintic treatment (Kyriazakis, Anderson, Oldham, Coop, & Jackson, 1996). Parasites also cause metabolic impairment to the animal and this is influenced by the level of larval challenge and the number and species of the worms which establish (van Houtert & Sykes 1996) and is also modified by host factors such as age, breed, nutritional and immune status (Sykes, 1997 ; Coop & Kyriazakis, 1999). Gastro-intestinal parasites also reduce the gross efficiency of utilisation of ME for growth in animals due to the reduction in appetite as a greater proportion of the energy intake is used for maintenance (Coop & Kyriazakis, 1999). This was supported by (Sykes, 1983) who stated that the efficiency of use of ME in sheep with *T. colubriformis* infections was reduced from 0.26 to 0.1 and with *O. circumcincta* from 0.19 to 0.14.

In parasitised ruminants, nutrients including protein are diverted away from production processes such as skeletal growth, wool and milk production and muscle deposition into responses essential for homeostatic maintenance such as plasma, blood protein synthesis, mucus production, repair of the gastro-intestinal tract, mucosal integrity and maintenance of host defences (MacRae, 1993). The combined effects of increased loss of host protein, repartitioning of nutrients and increased requirement of the gastrointestinal tract for protein synthesis at the expense of muscle growth may reduce production by as much as 25-35% for weight gain, 10-25% for wool growth and 20-30% for milk production, even in subclinical infections (Kahn & Watson, 2001). However, the nutrition of the animal strongly influences the magnitude to which infection reduces production and also the extent to which the animal can develop resistance to infection (Coop & Kyriazakis, 1999). Nematodes such *Haemonchus contortus* and *H. placei* are blood sucking hence cause anaemic conditions which again cause poor performance in animals and even death.

## 2.4 Control of internal parasites

Conventional methods of controlling nematode parasites of grazing animals have been the use of synthetic chemotherapeutic drugs (anthelmintics) (Waller, 2003). Other methods which can be used to reduce the effects of these are the use of genetic resistant hosts, improved host nutrition, plant secondary metabolites and biological control (Waller & Thamsborg 2004) and are outlined below.

### 2.4.1 Anthelmintics

Anthelmintics are synthetic chemicals used to control parasites and are grouped according to their chemical structure. This grouping is a pointer to the mode of action, general spectra of activity, persistent of activity and residue profile which affects meat and milk withdrawal periods (McKellar 1997). Livestock producers have relied greatly on the use of these anthelmintics because of their efficacy, low cost and broad spectrum of activity (Waller, 1993 ; Morley & Donald, 1980).

### 2.4.2 Problems of anthelmintics

As the concept of sustainability spreads wider in the public consciousness there is increasing pressure on animal production systems to reduce the reliance on anthelmintics due to a number of problems (Niezen, Charleston, Hodgson, McKay, & Leathwick, 1996). The most

serious being the rapidly escalating problem of anthelmintic resistance which is posing a serious threat to the future of chemotherapeutic control of animal parasitic nematodes (Waller, Dash, Barger, Le Jambre, & Plant, 1995a ; Waller et al., 1995b). This was also supported by (De & Sanyal 2009) who pointed out that anthelmintic resistance is escalating globally and threatening the survivability of small ruminant farming. Resistance has been mainly caused by the repeated treatment with anthelmintics to achieve adequate parasite control. Another problem associated with anthelmintics use is the presence of chemical residues in livestock products such meat and milk (Soder & Holden, 2005 ; Waller, 1993) which is of concern to public health authorities. Due to public health awareness consumers are demanding products that comply with stricter standards for chemical residues (Soder & Holden, 2005). As a result of this awareness there is a growing niche market for "organically" grown produce and a desire for some farmers to cater for this market and take advantage of the high premiums it offers (Niezen et al., 1996). Environmentalists are also voicing concern on the effects of anthelmintics on the environment (Halling-Sørensen et al., 1998). The main concerned is that anthelmintics will find their way in water sources where they can kill marine life. The anthelmintics can also find their way into humans through the consumption of these marine animals, again causing a public health concern. As the world becomes wary of the ecological damage caused by synthetic anthelmintics, research continues for the discovery of selective, safe, effective and sustainable methods which rely less on synthetic anthelmintics to control internal parasites (Soder & Holden, 2005).

### 2.4.3 Genetic resistant hosts

According to (Waller & Thamsborg 2004) genetic resistance is the ultimate in sustainable parasite control as it is a low cost, permanent solution requiring no additional resources. This resistance has found greater favour in tropical animals (Baker, 1998) because of a combination of harsh environmental stress, malnutrition and large larval challenge. These harsh conditions have necessitated natural selection resulting in survival of the fittest (Baker et al., 2003). According to (McEwan, Mason, & Baker, 1992), within breed selection for nematode resistance is also making good progress in temperate regions of the world even in Merino sheep which are typically seen to be generally very susceptible. Nevertheless the benefits of selection for resistance to gastro-intestinal parasites may be reduced as a "trade off" between parasite resistance and performance has been noticed (Waller & Thamsborg 2004 ; McEwan et al., 1992). For example in New Zealand McEwan et al., (1992) and Morris,

Vlassoff, & Bisset, (1997) observed undesirable genetic correlations of 0.80 and 0.57 respectively for faecal egg count (FEC) and wool weight in Romney yearlings affected by *Trichostrongylus* species. Nevertheless, McEwan et al., (1992) also observed favourable (negative) correlation of -0.42 between FEC and live weight gain in Romney sheep. Some inconsistent results were also observed for example; desirable genetic correlations ranging from -0.20 to -0.26 were observed between FEC and live weight whilst undesirable correlation of up to 0.21 were observed in Australian Merino resource flocks (Baker, 1998). Colditz, (2008) noted that this inconsistency of results in the relationship between resistance and performance may be due to the differences in the relative selection pressure applied for productive traits, allele frequencies in population of sheep, the composition of parasitic challenge or environment in which the measurements were taken.

### 2.4.4 Nutrition

According to (Donaldson, 1997; Donaldson, van Houtert, & Sykes, 1997; Sykes, 2000 ; van Houtert, 1997) the importance of nutrition, especially dietary protein, for development of the immune response is very significant. Studies have shown that the development of host immunity responds dramatically to dietary protein in a number of situations. In a study, (van Houtert, Barger, Steel, 1995) exposed young Merino sheep with natural as well as artificial infection of 6000 *T. colubriformis* and 2000 *H. contortus* every fortnight. These animals received small amounts of supplements based on fishmeal (376g CP/kgDM), sunflower meal (241g CP/kgDM) or oat grain (82g CP/kgDM) for 14 or 28 weeks. The unsupplemented control group was also infected and results showed that supplementation overcame the impact of nematodes on live weight gain (LWG) and fish meal was more effective as it supplied a greater level of metabolisable proteins. Donaldson et al., (1997) also demonstrated the impact of protein supplementation on worm numbers in lactating ewes. The ewes were infected with both *T. colubriformis* and *Teladorsagia circumcincta* which were fed diets that provided different levels of energy and protein. Three weeks after lambing the worm burdens of ewes fed the greatest protein diet were reduced by 87% while the high energy diet did not cause a significant reduction in worm burdens (Figure 2) indicating the importance of protein relative to energy.

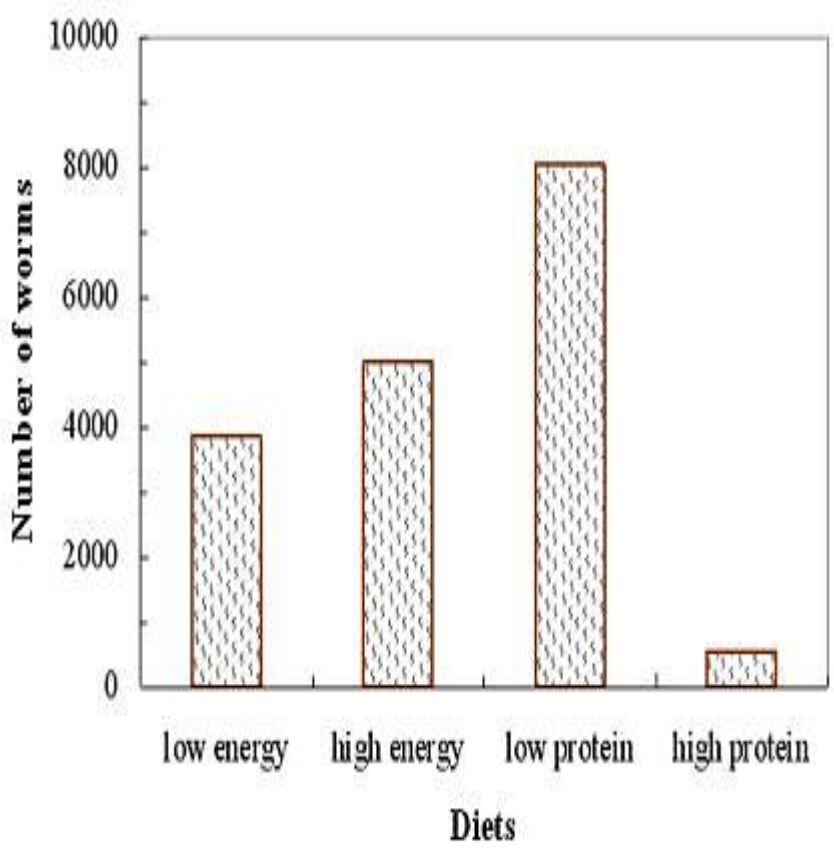


**Figure 2: Number of worms recovered from Coopworth ewes 3 weeks after lambing when fed diets to provide for low and high levels of energy and protein.**

Source: ( Donaldson et al., 1997)

### 2.4.5 Herbal control (Plant secondary metabolites)

Some herbs such as Chicory *(Cichorium intybus)* (Knight, Moss, Fraser, & Rowarth, 1996) and papaya (Behnke, Buttle, Stepek, Lowe, & Duce, 2008) produce secondary metabolites which have been shown to have anthelmintic properties. Waller & Thamsborg, (2004) pointed out that a large and diverse range of herbal de-wormers are utilised in places such Africa and Asia but they alluded to the fact that there is lack of scientific validation of the purported anthelmintics effects of these. Compounds such as cysteine proteinases in papaya latex and other plants such as pine apple and figs have been shown by Behnke et al., (2008) as having anthelmintic properties. Chicory contains some compounds such as sesquiterpene lactones which, according to Hoste et al., (2004), have demonstrated anti-parasitic properties in *in-vitro* experiments. In a UK experiment Athanasiadou, Gray, Tzamaloukas, Jackson, & Kyriazakis, (2007) investigated the use of chicory in parasite control in organic ewes and their lambs. They observed FEC of lambs from undrenched ewes grazing chicory were

significantly lower (340epg) than those lambs from undrenched ewes grazing grass/clover (590epg) but had similar abomasal worm counts and intestinal worm counts as those grazing grass at 12 weeks of age. In an *in-vitro* experiment in Ethiopia, Eguale, Tilahun, Debella, Feleke, & Makonnen, (2007) used extracts from seeds of the annual herb *Coriandrum sativum* on *Haemonchus contortus* eggs and adults. The extracts were screened and found to contain plant secondary metabolites linalool, campor, geranyl acetate, geraniol, coumarins and quercetin 3 glucoronide. Results showed that the extracts caused a reduction in egg hatching, FEC and overall worm burden and caused adult worm mortality. The effective dose (ED) 50, for egg hatching was 0.12mg/ml for the aqueous extract and 0.18mg/ml for the hydro-alcoholic extract as compared with 0.04um/ml for albendazole. The worm burden was reduced by 8.96% by a dose of 0.45g/kg live weight (LW) and 26% by a dose of 0.90g/kg LW compared with 98% by a dose of 3.8mg/kg LW for albendazole. Sainfoin (*Onobrychis viciifolia Scop*) a legume forage was found to contain tannins and flavinol glycosides by Barrau , Fabre, Fouraste, & Hoste, (2005) who confirmed in *in-vitro* assays the anthelmintic effects of these condensed tannins. They also observed that the flavonol glycosides namely rutin, nicotiflorin and narcissin significantly inhibited larval migration. In another experiment to investigate the effects of plant secondary metabolites Akhtar & Ahmad, (1992) supplemented parasitised goats with an extract from *Mallotus philippinensis* a plant very common in south eastern Asia and is rich in glycosides. It resulted in an 80% reduction in the mixed nematode parasitic burden compared with unsupplemented controls.

Even though some results from plant secondary metabolites studies have been promising the benefits have been inconsistent and according to Waller & Thamsborg (2004) it might be related to many factors such as soil types, climate, season, level of grazing or cutting and cultivar which affect production (quantity and composition) of the secondary metabolites in plants. Further research might be necessary to test the anthelmintic effects of different herbs in different places and how effective they can be.

### 2.4.6 Biological control

Biological control for any target organism is aimed at exploiting its natural enemies so as to reduce the number of target organism in the environment to a level less than would happen in the absence of the enemy (Waller 2006). Nematophagous fungi such as *Duddingtonia flagrans* use nematode larvae as their main source of food (Waller 2006). These fungi are

ingested by ruminants and survive passage through the gastro-intestinal tract and colonise faecal material where they produce specialised hyphael trapping devices with networks of adhesive knobs or constricting and non-constricting rings on the mycelium (De & Sanyal 2009). These structures are used to trap and destroy free living stages of parasitic nematodes in manure and reduce faecal egg count in grazing livestock during the course of the grazing season.

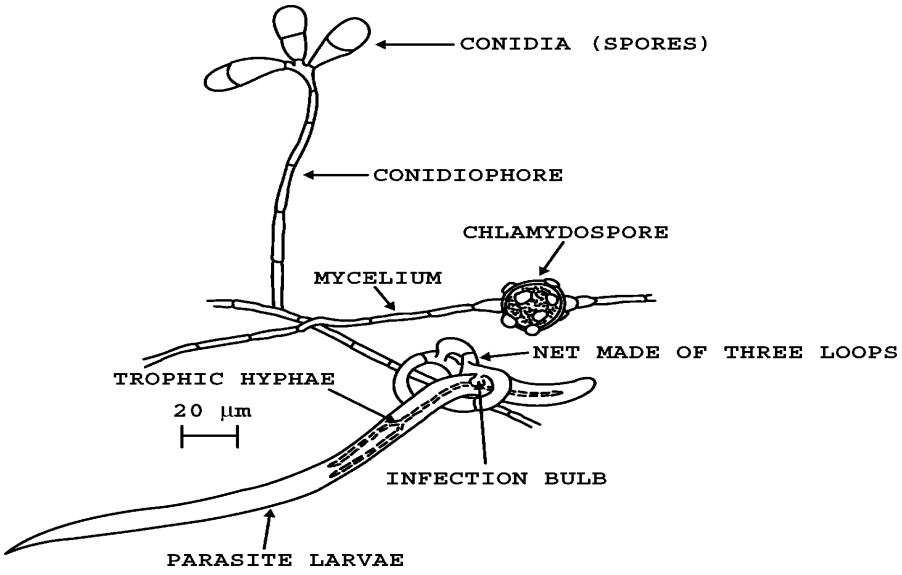


**Figure 3: Nematophagous nature of Duddingtonia *flagrans***

Source: (De & Sanyal 2009)

This fungus can be given to animals as part of feed supplements or incorporated into feed blocks for grazing or non-grazing animals. To achieve optimal results the fungal spores need to be continuously shed in the animal dung at the same time that contamination of the pasture with parasite eggs occurs (Waller & Thamsborg 2004). Greater opportunities for using this would occur if effective methods for *D. flagrans* delivery were available (Waller 2006). In an experiment by Gronvold et al., (1993) *D. flagrans* isolates resulted in a 74- 85% reduction in larvae transmitted to the herbage surrounding cows manure piles but however results can be variable with reductions of as low as 50% also reported. Despite some promising results, further studies on the influence of climatic conditions are still required. The need to continuously feed animals with the fungi makes it a less attractive method in large production settings. Another problem with bio-control agents like *D. flagrans* have

been the inconsistent performance due to biotic and abiotic factors such interaction with non-target organisms, damage by non-target organisms, physical and chemical composition of the rhizosphere (Meyer & Roberts, 2002). Besides their biological use in helmintic control there are some current synthetic anthelmintics which have fungal origin for example Emodepside, Parahercuquamide. Currently fungi as fungal endophytes have found extensive use in the prevention and control of nematodes in plants.

## 2.5 Fungal endophytes

Fungal endophytes are microscopic fungi that naturally occur in plants (Stepek, Behnke, Buttle, & Duce, 2004) and can be classified as beneficial, neutral or detrimental depending on the nature of their interaction with their host (Backman & Sikora, 2008). Fungal endophytes have been isolated from a broad range of plants including grasses, herbs and trees and from various plant tissues (Backman & Sikora, 2008). Some grass endophytes belong to the family Clavicipitaceae (Ascomycota) and have a sexual and an asexual life cycle (Schardl, Leuchtman, & Spiering 2004). The sexual form, *Epichloe* has a parasitic nature and causes choke disease in plants, where the stromal mycelium subsume the inflorescence (Craven, 2000) and suppress flower and seed production and this form is transmitted horizontally to new host plants (Muller & Krauss, 2005). During their sexual stage they form a dense stroma around the reproductive stems of the host below the developing seed head thus preventing normal maturation (Easton, 1999).

The asexual form belong to the genus *Neotyphodium* and it actively colonises grass tissues such as leaves, stems, young inflorescence and seeds (Kuldau & Bacon 2008) establishing long-term mutual associations with grasses causing no visible symptoms, being transmitted vertically through the seeds of the host plant (Grewal & Richmond 2003 ; Schardl et al., 2004 ; Kuldau & Bacon 2008). The associations are persistent, with the fungal endophyte benefiting from inhabiting the grass interior where there is a reliable source of nutrients, water, and isolation from competition and vertical transmission to the next generation and, in turn, it assists the grass resistance to insect herbivory, drought and also protect it from overgrazing in a relationship known as symbiosis (Muller & Krauss, 2005).

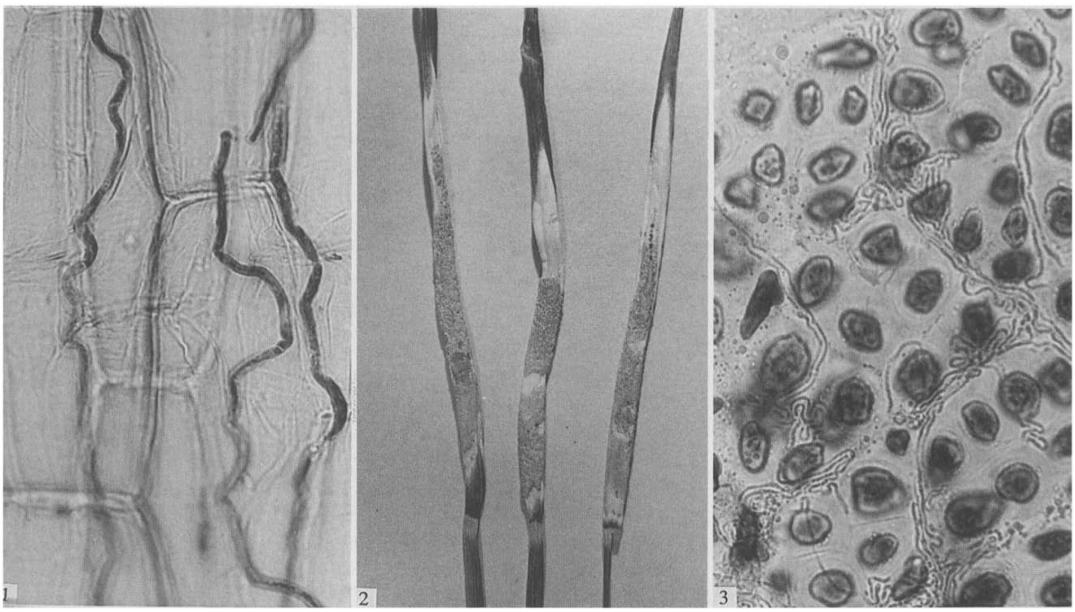

**Figure 4: Endophytic fungi of grasses: 1, Endophytic hyphae of *Neotyphodium coenophialum* in leaf sheath of tall fescue grass. 2, Stroma of Epichloe typhina infecting *Daetylis glomerata*. 3, Endophytic hyphae of *Neotyphodium lolii* in the aleurone layer of perennial ryegrass seed**

*Source* (Clay 1989)

### 2.5.1 Endophyte life cycle

The endophyte hyphae are concentrated in the leaf sheath of the vegetative plant (Musgrave, 1984). These grow in and around the basal meristem of the grass tillers and as the new leaves form, the endophyte grows to a slight extent into the blade and, more abundantly, into the leaf sheaf (Schmid & Christensen, 1999 ; Kemp, Bourke, & Wheatley, 2007) with the hyphae elongating as the leaf sheath extends. When a tiller becomes reproductive the endophytes grow within the elongating stem into the developing inflorescence, the flower and the developing seed and embryo as it forms (Philipson & Christey, 1986). At germination, the endophyte hyphae outside the embryo appear to play no further part in invasion of the already infected embryo and that growing within the embryo, grow with the seedling and hence the new plant (Fletcher, Hoglljnd, & Sutherland, 1990).

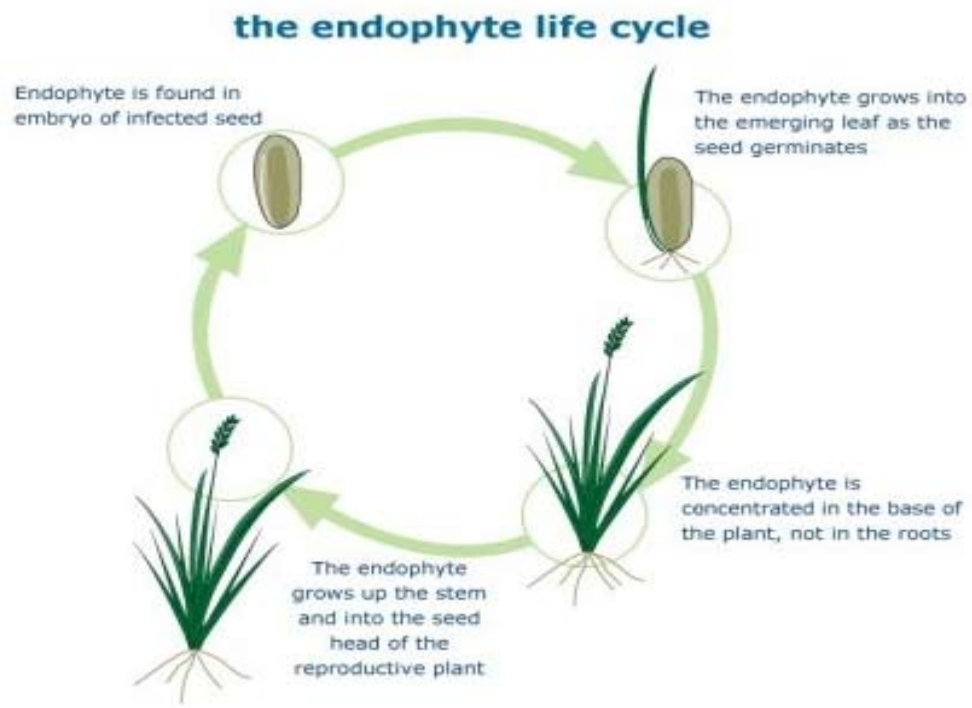


**Figure 5: The endophyte life cycle**

Source: (Agricom web site)

### 2.5.2 Mechanism of endophyte action in plants

According to (Sikora, Schäffer, & Dababat, 2007 ; Paparu, Dubois, Coyne, & Viljoen, 2010) certain endophyte strains activate enzymatic host-plant defence mechanisms following inoculation. This causes some enzymes to become upregulated only when the plant has been challenged by pests such as nematodes, in a phenomenon called priming, which is desirable as it enables endophyte enhanced plants to conserve energy in the absence of pests (Dubois, Coyne, & Felde, 2011). Many fungal endophytes produce secondary metabolites and some of these compounds are anti-fungal and antibacterial and strongly inhibit the growth of other microorganisms including plant pathogens (Gao, Dai, & Liu, 2010). Some endophytes produce alkaloids which are pest repellent (Stewart, 2005) and some produce lytic enzymes that hydrolyze a variety of polymeric compounds including chitin, proteins, cellulose and hemi-cellulose, hence, have the function to suppress plant pathogen activities directly and have the capacity of degrading the cell walls of pathogenic fungi and oomycetes (Gao et al., 2010).

Endophyte infection increased root and shoot dry matter in selected genotypes of perennial ryegrass (Lewis & Day, 1993), tall fescue (De Battista, Bouton, Bacon, & Siegel, 1990) and

meadow fescue (Schmidt & Osborn, 1993) and also show an increase in growth rate and length of root growth (Richardson, Hoveland, & Bacon, 1993) which plays a role in drought protection and nutrient acquisition. In response to drought stress, some genotypes of endophyte infected tall fescue have been seen to lower stomatal conductance (Elmi & West, 1995). This mechanism was suggested to help endophyte infected plants to reduce water loss via transpiration and survive drought thereby increasing persistence. Lolines have been reported to affect osmotic potential of grasses hence reduce the effects of drought stress (Malinowski, Leuchtmann, Schmidt, & Nosberger, 1997a ; Malinowski, Leuchtmann, Schmidt, & Nosberger, 1997b). Endophytes can alter the growth, morphological and physiological characteristic of the host plant thereby influencing the persistence and survival rate of affected plants (Clay, 1994).

### 2.5.3 Seed storage and endophyte viability

Commercial seeds have up to 98% endophyte and to protect the endophyte in seeds it is important to store seeds in cool dry places. According to (Rolston, Hare, Moore, & Christensen, 1986) temperatures exceeding 37$^{o}$C for one to two weeks can eliminate the endophyte from the seed even though the seed itself retains its germinability. In comparison, storing seeds at around 0$^{o}$C and 30% relative humidity maintain endophyte viability hence maintain alkaloid levels and seeds held at 5$^{o}$C and 60% relative humidity will suffer a loss of its viable endophyte infection in a few months (Hare, Rolston, Christensen, & Moore, 1990 ; Rolston et al., 1986). Given this endophyte sensitivity to temperature and relative humidity it is very important to store grass seeds in appropriate conditions before planting.

### 2.5.4 Effects of endophytes on plant nematodes

Many studies have been carried out to investigate the effects of endophytes on plant nematodes in crops such as bananas, tomatoes, rice and pasture grasses such as perennial ryegrass and tall fescue.

#### 2.5.4.1 Effects of endophytes on bananas

In bananas, many studies have investigated the burrowing nematode *Radopholus similis* which is considered to be one of the major pathogens affecting banana production and the cause of banana root rot (Niere, Speijer, & Sikora, 1999). In an *in-vitro* experiment by (Niere et al., 1999) different fungal endophytes from the species *Fusarium oxysporum* were used in

shoots of the banana cultivar Gros Michel affected with the nematode *R. similis*. Plant growth measurements were taken every four weeks and at the end of the experiment results showed that isolates of *Fusarium oxysporum* resulted in enhanced height of 19 week old plants. There were also differences in nematode multiplication detected between endophyte inoculated and control plants, for example in roots of plants inoculated with different endophytes (Table 1). Treatment V5w2 significantly reduced the number of nematodes by 62% in the root segment compared with the control plants at 19 weeks. The other treatments resulted in a non-significant reduction in nematode numbers of 26% by 1114w1 in 19 week old plants. In the 38 week old plants fungal endophytes reduced nematodes numbers by between 51-89 % of the initial inoculums. Treatments 1113w3, 1114w1 and V5w2 significantly reduced *R. similis* compared with the control.

**Table 1: Multiplication of *R*. similis in root segment of 19 and 38 week old Gros Michel 6 week after nematode inoculation (Niere et al., 1999)**

| Endophyte treatment | Nematode multiplication as % of initial inoculums | |
|---|---|---|
| | 19 week old plants | 38 week old plants |
| PDB only (Control) | 101 | 133 |
| V4w5 | 213 | 49 |
| 1113w3 | 129 | 11 |
| 1114w1 | 74 | 14 |
| V5w2 | 38 | 33 |

Niere et al., (1999) used an isolate from the endophyte *F. oxysporum* on the nematodes *R. similis* and *Helicotylenchus multicinctus* in bananas. Results showed a non-significant reduction of 50% female nematodes compared with control but the males and juveniles were not affected. Further, fungal endophyte inoculation of tissue cultured banana plants accounted for reduced nematode numbers in the roots of all endophyte treated plants compared with the control plants 4 months after transplanting into the field (Table 2). The number of nematodes was significantly lower in plants treated with vw5w2 compared with the control which was reduced by 75%. The other treatments did not show a significant difference but still showed a trend for reduced larval development compared with the control. Even though there are variations between endophytes effects, results clearly show that endophytes can affect nematode numbers, with the best performing endophytes reducing nematode numbers by between 50-89% in both *in-situ* and *in-vivo* conditions.

**Table 2: Density reductions of spiral nematode *Helicotylenchus multicinctus* in roots of endophyte free (control) and *F. oxysporum* endophyte isolates inoculated tissue banana plants cultivar Grass Michel 4 months after transplanting to nematode infested field (Niere et al., 1999).**

| Treatment | Nematode /100g roots | % reduction compared with control |
|---|---|---|
| Control | 14063 | - |
| V4w5 | 8260 | 41.3 |
| 1113w3 | 9063 | 35.6 |
| 1114w1 | 4060 | 71.1 |
| Vw5w2 | 3524 | 75 |

In green house experiments Vu, Hauschild, & Sikora, (2006) investigated the ability of 4 mutualistic endophyte fungal isolates to induce systemic resistance in bananas to the burrowing nematode *R. similis*. The isolates used were two of *F. oxysporum*, one of *F. diversisporum* isolated from bananas and another of *F. oxysporum* isolated from tomatoes. The roots of the bananas seedlings were inoculated with the 4 endophytic fungi and the nematode *R. similis* and results showed that root penetration was reduced by 29-39% and 22- 41 %, 5 and 15 days respectively after nematode inoculation.

A number of other studies performed in bananas using strains of the non-pathogenic *F. oxysporum* have shown to have a direct toxic effect on cultures of the nematode *R. similis* (Schuster, Sikora, & Amin, 1995). Schuster et al., (1995) isolated endophytic fungi from the internal root tissues of different crops and when culture filtrate of the endophyte fungi isolated were tested, 11.8- 26.5 % of these isolates caused more than 90% inactivation of nematode species tested and also enhanced plant growth. This was supported by (Schardl et al., 2004) who reported endophytes provided many complex and indirect benefits to the host such as improved persistence under herbivore and drought pressure, more vigorous growth, increased seed and tiller production and enhanced root growth. Kiewnick & Sikora, (2006) carried out studies in growth chambers to evaluate the potential to control root knot nematode *Meloidogyne incognita* by the fungal agent *Paecilomyces lilacinus* strain 251(PL251). It was observed that pre-planting soil treatment reduced root galling by 66% and the number of egg masses by 74% and the final nematode population in the roots by 71% compared with the non-inoculated controls.

In Guatemala trials (Felde, Pocasangre, & Sikora, 2005 ; Sikora & Pocasangre, 2004) noticed that some soils have suppressive effects on nematodes *(R. similis)* in bananas. A number of isolates of the endophytes *F .oxysporum* and *Trichederma atroviride* were isolated from the endorhiza of bananas growing in these *R. similis* suppressive soils and also gave high levels of biological control activities of nematodes in green house trials. The work from these authors demonstrated that the effects in these fields were plant based and not just limited to the soil–borne micro flora.

#### 2.5.4.2 Effects of combined inoculations in bananas

Field trials using single and multiple inoculants containing the isolates of both *F. oxysporum* and *T. atroviride*, (Felde et al., 2006) have observed that inoculation led to a strong reduction in the number of *R. similis* in the roots and also promoted growth in the first year after planting. Results clearly show an increasing reduction in the nematode number and density from single to dual to multiple inoculations (Table 3). This supported results by (Speijer, 1993) who also observed a reduction in the nematode penetration in banana roots when the endophytes *F. oxysporum* and *Pratylenchus good-eyi* were simultaneously inoculated in bananas. Results also showed that different isolates differ in their effectiveness against the nematode.

**Table 3: Percentage reductions in *R. similis* numbers in bananas eight weeks after inoculation with different endophyte isolates (Felde et al., 2006)**

| Endophyte isolates inoculated | % Reduction in total number of nematodes(*R. similis)* |
|---|---|
| Single (MT- 20) | 44 |
| Single (S2) | 49 |
| Single (P12) | 53 |
| Dual(MT-20 & S2) | 61 |
| Dual (S9 & P12) | 63 |
| Multiple(MT-20 , S2S9 , P12) | 65 |
| Control | 0 |

Results in banana experiments are variable in terms of nematode control, suggesting that control of nematodes using endophytes needs to be part of an integrated approach. It also highlighted the fact that there are different isolates of endophytes with varying degrees of effectiveness.

#### 2.5.4.3 Endophytes in Tomatoes

Studies in tomatoes by (Dababat, 2007 ; Dababat & Sikora, 2007) using the endophyte *F. oxysporum* isolate 162 reported that greater than 56% of root-knot juveniles were repelled from tomato plants inoculated with the endophyte. Of major importance were their findings that root exudates extracted directly from rhizosphere soil of non-inoculated and inoculated plants also affected attraction. In an *in vitro* investigation, 80% of the nematodes placed in a sand-filled chamber migrated to the side containing exudates from the control plant (non-inoculated) and away from the exudates taken from *F. oxysporum*-treated plants. These results seem to reinforce those obtained in trials using fungal culture filtrates in plants.

#### 2.5.4.4 Endophytes in paddy rice

In studies involving fungal and bacterial endophytes isolated from paddy rice in Vietnam, (Padgham & Sikora, 2006 ; Padgham & Sikora, 2007), a number of endophytic fungi with biological control activity towards the nematode *M. graminicola* were reported. The majority of the fungal isolates obtained were of the species *Fusarium* with a few being of the *Trichoderma* species. Seed treatment with these different isolates led to a significant decrease in root knot galling which was accompanied by root growth promotion (Figure 6). Results also show that the different isolates also differ in their effectiveness towards the nematodes.

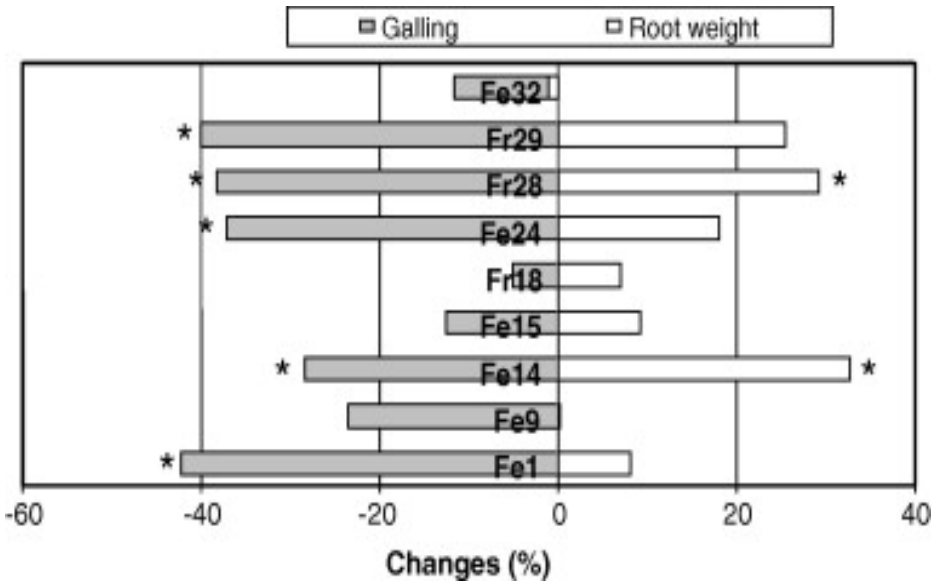


**Figure 6: Effects of *Fusarium* isolate on galling severity of *M. graminicola* and root biomass *in-vivo* rice seedling test, Fe: putative endophytic *Fusarium* species, Fr: rhizosphere *Fusarium*. The percentage reduction and the root weight changes are expressed relative to the non-fungal –inoculated control. Columns with * show significant differences from the control based on LSD test (P=0.05)**

Source: (Padgham & Sikora, 2007)

### 2.5.5 Effects of endophyte metabolites on plant nematodes

A number of studies have investigated the effects of endophyte metabolites on plant nematodes. Bacetty et al., (2009), reported that root extracts including ergot, loline alkaloids from endophyte infected tall fescue were nematicidic to *Pratylenchus scribneri* a nematode pest of tall fescue. *In-vitro* bioassay and green house studies were used to test for the effects of root fractions and alkaloids on motility and mortality of the nematodes. The results showed that endophyte–infected tall fescue grass is not host to *P. scribneri* with a very low root population of 3 to 17 nematodes per pot compared with 4866 and 8450 nematodes per pot in endophyte–free grasses. The *in-vitro* bioassay showed that root extracts from the endophyte infected grasses were nematicidic and concentration dependent. In particular the alkaloid ergovaline was very nematicidic even at low concentrations of 5 and 50 µg/ml and α-ergocryptine was more nematicidic at concentrations above 50 µg/ml while orgocornine and ergonovine were not nematicidic at most concentrations. Loline (N-formylloline), a pryrrolizidine was nematicidic from 50 to 200 µg/ml and more so at 100 and 200 µg/ml, (Table 4). The results also indicated that the effectiveness increases with time with greater nematicidic activity observed at 72 h than 24 h of incubation (Table 4). At the lowest concentration, 5 µg/ml, ergovaline treatments

negatively affected nematode with 100% reduction of motility after 72 h. At the 72 h time period, nematode motility ceased in the loline treatment in excess of 5 µg/ml and α-ergocryptine treatments in excess of 50 µg/ml. Motility in nematodes exposed to ergonovine was severely decreased from the least concentration of 5 µg per ml to the greatest concentration at 250 µg/ml at which point all motility was prevented. Ergocornine treatments caused a significant decrease in nematode motility but complete cessation of motility was not observed even at the greatest concentration of 250 µg/ml (Table 4).

**Table 4: Motility bioassay of *Pratylenchus scribneri* following exposure to serial dilutions of ergot and loline alkaloids (Bacetty et al., 2009).**

| Alkaloid treatment | Percentage (%) nematode motility at different concentrations | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 24hrs | | | | 72hrs | | | |
| | Concentration of alkaloids in (µg/ml) | | | | | | | |
| | 5 | 50 | 100 | 250 | 5 | 50 | 100 | 250 |
| Control | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Loline (NFL) | 28 | 14 | 0 | 0 | 10 | 0 | 0 | 0 |
| Ergonovine | 17 | 15 | 10 | 3 | 17 | 7 | 3 | 0 |
| Ergocornine | 45 | 45 | 45 | 41 | 31 | 28 | 21 | 13 |
| α-Ergocryptine | 31 | 14 | 0 | 0 | 10 | 5 | 0 | 0 |
| Ergovaline | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

When synergistic effects of the alkaloids in questions were tested on their toxicity on *P. scribneri* results showed that some combinations are more effective than others and overall more effective than individual alkaloids (Table 5). This supported results which were observed when combinations of endophytes were used in controlling nematodes in bananas which showed more effectiveness with combinations as compared to single endophytes (Felde et al., 2006).

**Table 5: Motility bioassay of *Pratylenchus scribneri* following exposure to ergot and loline alkaloid combinations (Bacetty et al., 2009).**

| Alkaloid treatment | Percentage (%) nematode motility at different concentrations | | | |
|---|---|---|---|---|
| | 5 µg /ml | 50 µg /ml | 100 µg /ml | 250 µg /ml |
| Control | 100 | 100 | 100 | 100 |
| Loline + Ergocornine | 19 | 5 | 5 | 0 |
| Loline + α-Ergocryptine | 29 | 5 | 0 | 0 |
| Ergocornine + α-Ergocryptine | 33 | 19 | 5 | 0 |
| Loline + Ergocornine + α-Ergocryptine | 14 | 0 | 0 | 0 |

Bacetty et al., (2009) carried out further experiments aimed at determining if root extracts of tall fescue effected the chemoreceptor activity and if any of the specific alkaloids (ergovaline, ergotamine, ergonovine, α ergocryptine) and loline alkaloids produced by endophyte altered *P. scribneri* chemotaxis. Movements of nematodes were tracked by using thin agar plates after the endophyte extracts were inoculated at two positions in the plates (Figure 7). Results showed that nematode behaviour is affected by the metabolites, more so with increasing time after the start of experiment. When the purified alkaloids produced in *N. coenophialum* infected tall fescue were used in the same chemotaxis bioassay systems, all of them produced significant effects compared with comparable controls. The results were as shown in the plate diagrams below.

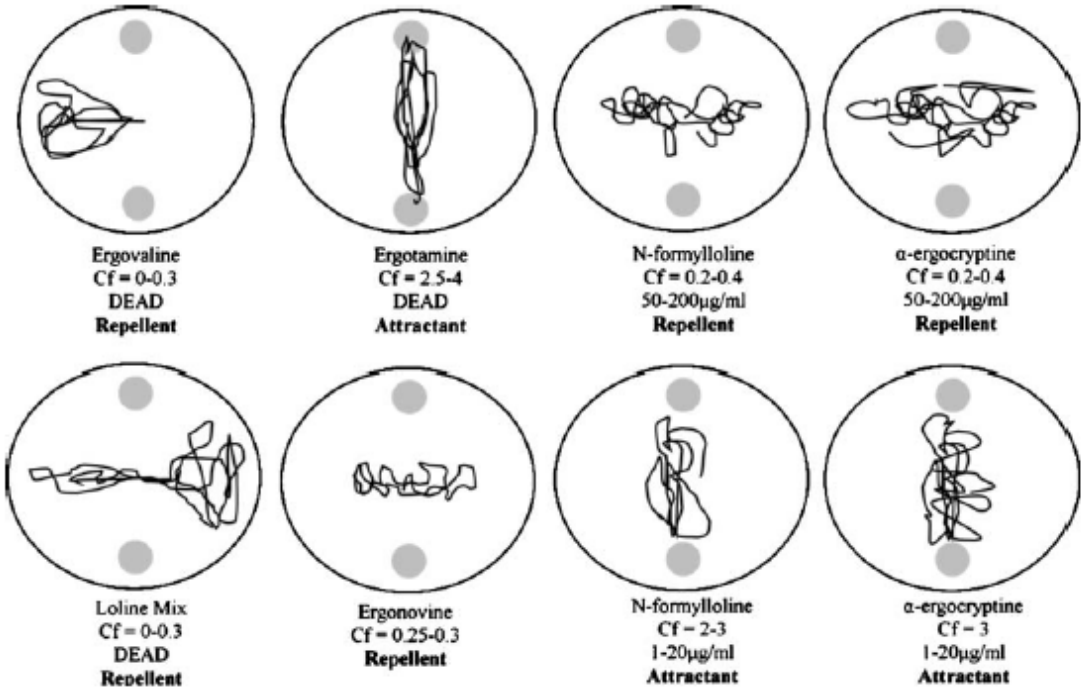


**Figure 7: Selected chemotaxis patterns displayed by *Pretychuncus scribneri* exposed to purified ergot and loline alkaloid concentration made during a 2 hour observation period**

Source: (Bacetty et al., 2009)

The results showed that the metabolites were effective attractants or repellents to the nematodes with ergovaline, ergotamine and lolines extracts also effective at killing the nematodes. Mortality in other metabolites was low, being affected by concentration. Panaccione, Kotcon, Schardl, Johnson, & Morton, (2006) also investigated the effects of ergot alkaloids, ergovaline and setoclavine using *P. scribneri* in perennial ryegrass. The results showed that ergovaline reduced nematode numbers, further supporting the effectiveness of ergovaline reported by (Bacetty et al., 2009), also suggesting that the nematode reduction was related to the infection status of the grass.

### 2.5.6 Pasture fungal endophytes

Perennial ryegrass *(Lolium perenne)* pastures are naturally infected with the *N. lolii* endophyte (Siegel, Latch, & Johnson, 1987) and according to Latch, (1983) most sown cultivars have 65-95% of the plants infected with the endophyte and 80% of rye grass seeds are infected with either AR1 or AR37. The *N. lolii* endophyte produces the alkaloids peramines, lolitrems and ergovaline and the peramines have been shown to deter Argentine stem weevil (Rowan & Gaynor, 1986).

**Table 6: A summary of different insects affected by different rye grass pasture endophytes (Stewart, 2005)**

| Pests | Endophytes | | | |
|---|---|---|---|---|
| | AR1 | AR37 | NEA2 | Endo 5 |
| Argentine stem weevil | √ | √ | | |
| Pasture mealy bug | | √ | | √ |
| Black bettle | √ | √ | √ | |
| Root aphid | √ | √ | √ | |
| Porina | √ | √ | √ | |

Perennial ryegrass staggers, a neurological disorder of grazing cattle, sheep and deer has been associated with feeding of perennial ryegrass pastures containing the wild type endophyte (Fletcher & Harvey, 1981). This condition is very common in summer and autumn and is commonly associated with warm ambient temperature and grazing of short grass swards (Siegel & Bush, 1997). According to Gallagher, White, & Mortimer, (1981) and Gallagher & Hawkes (1985) the main suspects of perennial rye grass staggers are lolitrem neurotoxins such as lolitrem B. Besides staggers, animals grazing *N. lolii* infected perennial rye grass also exhibited reduced weight gain, probably associated with reduced herbage intake (Fletcher & Barrel, 1984). According to Barker et al., (1985) endophytes free perennial rye grass was suggested in New Zealand after the health problems associated with some endophytes were apparent. These were, however, too susceptible to insect predation for example by the Argentine stem weevil and drought, hence, have poor persistence. This supported findings by the authors that biotic and abiotic stresses are the main reasons for the inability of endophyte free perennial grass cultivars to survive in New Zealand. Generally perennial ryegrass ecotypes and cultivars increase in endophyte infection with time as endophyte free plants are gradually removed by insect pests.

Tall fescue (*Festuca arundicaea*) is naturally infected by the endophyte *N. coenophialum* (Siegel et al., 1990). This endophyte produces the alkaloids lolines, peramines and the ergo peptides alkaloids mainly ergovaline. Like in perennial rye grass, some of these alkaloids in tall fescue have been reported to have insecticidal properties (Siegel et al., 1987). For example *N-acetylloline* (NAL) and *N-formylloline* (NFL) have been reported to confer some insect resistance in the grass (Johnson, Bush, & Siegel, 1986 ; Jackson et al., 1984 ; Kennedy & Bush 1983). *N. coenophialum* in tall fescue has, nevertheless, been linked to fescue toxicity (summer syndrome), a thermoregulatory syndrome of cattle (Bacon, Porter, Robbins, &

Luttrell, 1977). The disease occurs during hot summer-autumn conditions and may include any or all of the following: rough hair coat, rapid breathing, excessive salivation, nervousness, elevated body temperature and increased sensitivity to heat and the animals seeking to stand in the shade and water (Mueller, 1985). Several alkaloids, namely loline, NAL and NFL (Siegel & Bush, 1997) and the ergo peptide ergovaline (Garner, Rottinghaus, Cornell, & Testerci, 1993) have been suggested to contribute to fescue toxicity and heat stress but (Bacon , Lyons , Porter, & Robbins, 1986) pointed out that many symptoms of fescue toxicosis are consistent with signs of ergot poisoning ,hence, suggesting ergovaline as the cause of fescue toxicity. There is also associated reduced food intake, poor weight gain and reduced milk production in affected animals (Hemken, Jackson, & Boling 1984). This endophyte is also associated with fescue foot which is a gangrenous condition occurring mainly in winter and may result in rough coat, sore hooves and in severe cases the hooves mainly the rear, tips of ears and tails may slough off (Hemken et al., 1984). When it was recognised that endophyte infected grass was responsible for animal toxicosis it was suggested that removal of the endophyte would lead to alleviation of symptoms and improved animal productivity (Fletcher & Harvey, 1981). Discovery of this toxicity and endophyte association led to a movement in places such as the USA to establish endophyte free fescue. These pastures however proved to have poor persistence and grow less than infected pastures, particularly under heat and drought stress (Easton, Lee, & Fitzgerald, 1994). The result was that with endophyte, farmers needed to care for the animals and without it needed to care for the plants and according to (Easton et al., 1994) more farmers tend to be more competent in caring for the animals hence endophyte infected pastures are becoming the norm.

Endophyte infected tall fescue cultivars such as AR542 has been shown to have increased biomass above ground, tiller numbers, seed production and stress tolerance in grass host and promoted persistence in pastures (Schardl et al., 2004). For example in a USA experiment endophyte infection was seen to promote persistence in pasture grasses compared with endophyte free grass pastures (Figure 8).

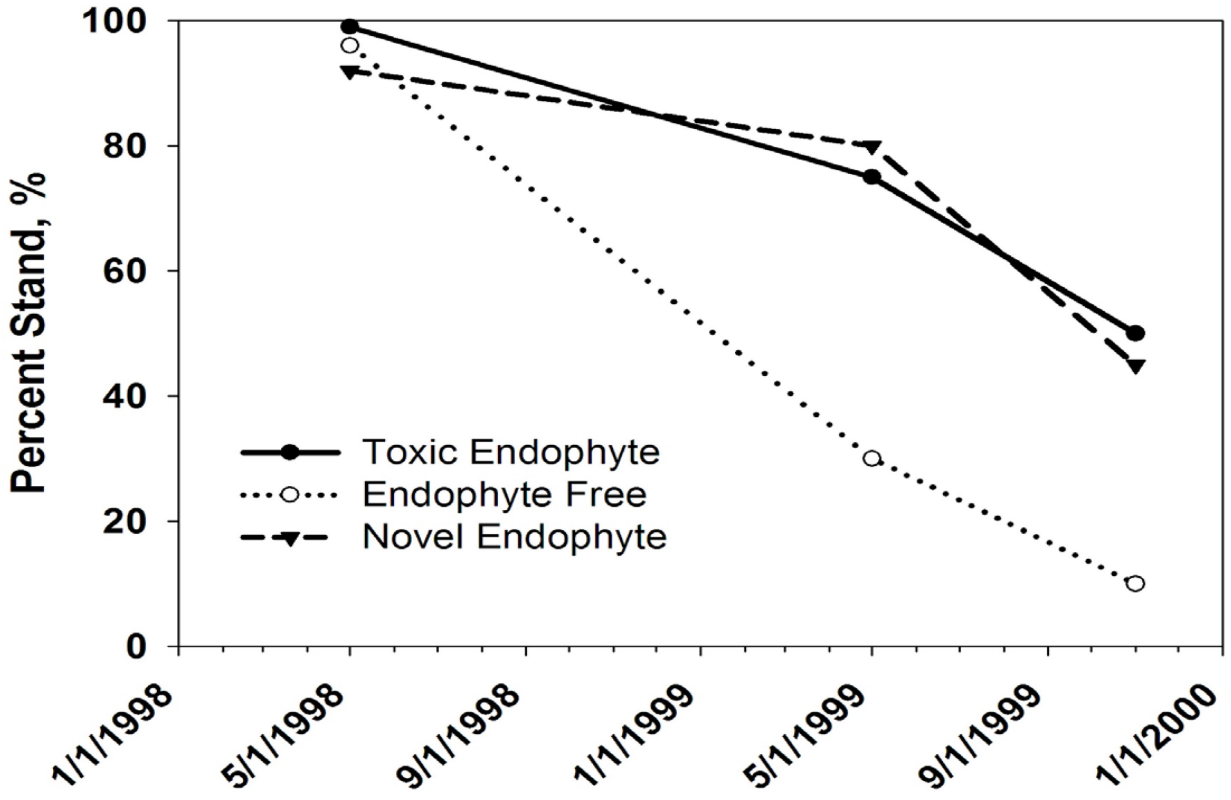


**Figure 8: Stand persistence of novel endophyte-infected ("Jesup MaxQ™"), toxic endophyte-infected, and endophyte- free tall fescue.**

Source: (Bouton et al., 2000)

Meadow Fescue (*Festuca pratensis*) is a temperate pasture grass naturally infected by the endophyte *N. uncinatum* (Popay, Townsend, & Fletcher, 2003). In contrast to the two other pasture grasses previously mentioned (perennial ryegrass and tall fescue), in New Zealand endophyte infected Meadow fescue only produce loline alkaloids such as NFL, NAL, N-metylloline, N-acetylloline, norloline and N- formylnorloline (De-wen, Jin-yi, Patchett, & Gooneratne, 2006 ; McLeod, Rey, Newsham, Lewis, & Wolferstan, 2001).

**Table 7: Summary of Alkaloids produced in common grass endophyte associations (Cook, Lewis, & Mizen, 1991)**

| Grass | Endophyte | Ergopeptines | Lolitrems | Peramines | Lolines |
|---|---|---|---|---|---|
| Perennial ryegrass | *N. lolii* | Yes | Yes | Yes | No |
| Tall fescue | *N. coenophialum* | Yes | No | Yes | Yes |
| Meadow fescue | *N. uncinatum* | No | No | No | Yes |

Loline alkaloids in grasses are produced only when the endophytes *N. coenophialum and N. uncinatum* species are present in a host-endophyte symbiosis (Siegel et al., 1990). Meadow fescue plants produced 1254 µg/g of NAL and 4360 µ*g/g* of NFL with its natural endophyte *N.uncinatum*. When the distribution of loline alkaloids in tall fescue and meadow fescue were measured, by Burhan, (1984) and Justus, Witte, & Hartmann, (1997) spikelets of tall fescue were shown to contain the greatest levels of total loline (4566µg/g) with lesser amounts in leaf lamina (74µg/g) and leaf sheath (181µg/g) (Burhan, 1984). In the study of (Justus et al., 1997) meadow fescue spikelets were observed to contain the greatest concentration of total lolines (150µg/g). The NAL and NFL concentrations in Tall fescue increased during the season(s) with the greatest accumulation in summer and declined in winter (Bush, Fannin, Siegel, Dahlman, & Burton, 1993). Justus et al., (1997) showed that in vegetative tissue of meadow fescue, the greatest concentrations are found in the young leaves in early spring and in late summer after disappearance of reproductive stems.

### 2.5.7 Factors affecting alkaloid production

Levels of alkaloids production depends on several environmental factors (Lane et al., 1997b ; Easton, 1999) such as temperature, sunlight and rainfall. Nitrogen and phosphorus fertilizers were also observed to alter the level of alkaloids in tall fescue. For example, increasing N fertilizer from 134kg/ha to 334kg/ha increased concentration of total ergo peptide alkaloids by 60-80% in different years (Belesky, Stuedemann, Plattner, & Wilkenson, 1988). This was also supported by Lyons, Plattner & Bacon, (1986) who observed that the total ergot alkaloids increased 2.5 fold in infected tall fescue following application of nitrogen fertilizer. Furthermore, these authors observed that added nitrogen was associated with increased growth of endophytes in the plant and increased symptoms in animal toxicosis. In contrast no association was observed in lolines and nitrogen application in an experiment by (Kennedy & Bush 1983). This was also supported by (Burhan, 1984) who, in a glasshouse pot experiment, showed that N fertiliser had no effect on loline concentration in tall fescue but phosphorus (P) increased loline concentration by threefold (Bush et aI., 1993). Concentration of NAL and NFL in three Kentucky 31 tall fescue lines increased in response to water deficit (Belesky, Stringer, & Plattner, 1989). This was also supported by (Kennedy & Bush, 1983) who observed that when GI-307, a *Festuca-Lolium* line was exposed to severe water stress, NAL concentration increased by five-fold compared with control levels after nine weeks and NFL was twice the control levels after a twelve week period. Water deficit

was also associated with increased ergovaline and lolitrem B concentrations and lolitrem B levels were also increased in higher nitrogen levels (Lane et al., 1997b). Temperature was also observed to have a significant effect (Robbins, Sweeney, Wilkinson, & Burdick, 1972). For example NAL and NFL concentration increased by 200-300% in tall fescue plants growing at 21°C day/ 15°C night for 10 weeks compared with plants grown at 32°C day /27°C night and 16°C day /5°C night when alkaloid levels tended to be constant or decrease slightly (Kennedy & Bush, 1983). Ergovaline and lolitrem B also tended to be in low concentrations in early spring and increase with rising temperature (Lane, Tapper, & Davies, 1997c). Overall, alkaloids are the properties of the endophyte; however the plant does exercise some genetic control of the growth of the endophyte within it and the endophyte production of alkaloids (Ball, Prestidge, & Sprosen, 1995 ; Latch, 1994).

Physical activities such as harvesting/ clipping have been observed to affect the levels of alkaloids production in grasses. For example, green house studies by Burhan, (1984) showed that loline concentrations increase in re-growth tissues following clipping (Table 6). Measurement of endophyte, NAL and NFL levels in tall fescue-ryegrass hybrids in plants that were cut every three weeks showed that the alkaloid concentration increased with each harvest, NAL and NFL in the first harvest was relatively low and that subsequent NAL and NFL concentrations of harvest increased with time. High NAL and NFL concentrations were found in subsequent regrowth from plants clipped six or seven weeks after seeding. Results also showed that NAL and NFL concentration increased as plants matured with but there was no increase in endophyte density.

**Table 8: Mean for each harvest of endophyte and NAL and NFL concentration in shoot tissue of tall fescue (Burhan, 1984)**

| | Loline alkaloid concentration (µg/g DM) | | | |
|---|---|---|---|---|
| Harvest* | 1 | 2 | 3 | 4 |
| endophyte | 183 | 543 | 92 | 102 |
| NAL | 77 | 410 | 681 | 872 |
| NFL | 239 | 1389 | 2315 | 2316 |

*Harvest interval 3 weeks

Peramines, ergot alkaloids and lolitrems are in relatively low concentrations of around 0.2 - 22µg/g dry weight and lolines are found in concentrations as high as 825µg/g plant dry weight (Jones, Buckner, Burrus, & Bush, 1983). Leaf sheath and blades of *N. lolii* infected

perennial rye grass have been reported to contain lolitrems at concentrations as high as 4.9 and 0.37 mg/g plant dry weight, respectively, and the amount of *N. lolii* mycelium in infected plants has been positively correlated with resistance of Argentine Stem Weevil and with the time of the year when ryegrass staggers occur (Fletcher & Harvey, 1981 ; Barker, Prestidge, & Pottinger, 1985).

The location of alkaloid production in the plant reflects in part the location of the endophyte (Ball et al., 1995 ; Keogh et al., 1996). Peramine and, to a lesser extent lolitrem B moves through the developing seedling offering protection against insect attack before the renewed post germination endophyte activity begins to generate fresh peramines (Ball, Prestidge, & Sprosen, 1993). Low concentrations of peramines, ergovaline and lolitrem B have been found in root tissue (Ball, Barker, Prestidge, & Lauren, 1997a ; Ball, Bernard, & Gwinn, 1997). Concentrations of peramines, ergovaline and lolitrem B vary in plants parts as different tissues mature. For example lolitrem B was reported to be greater in older than in young leaves while those of peramine are the reverse (Keogh, Tapper, & Fletcher, 1996). The concentrations of peramines is maintained when the leaf is mature until senescence and as senescence approaches peramine decreases in the leaf while the concentration of lolitrem B remains high. Concentrations also vary as the structure of the pasture canopy evolves, with changes in levels as proportions of the reproductive tissue in the plant increases to a first peak at maximum seed head emergence. The levels reduce in the post reproductive regrowth and increase again through the summer in response to increasing water stress and increasing temperature along with accumulation of older leaves and, under continued grazing, an increase in the proportions of leaf sheath to leaf blade. Ergovaline, and to lesser extent lolitrem B, were observed to remain in senescence leaves and also in leaves killed by defoliation or rapid desiccation but peramines break down as the leaves senesce (Keogh et al., 1996).

The stability of endophyte alkaloids was investigated in ryegrass infected with wild-type and the AR37 by (Hume, Hickey and Tapper 2007). Herbage was cut and then dried in the field under a simulated hay drying regime in early and mid-summer. The alkaloids measured for were peramine, ergovaline and lolitrem B for wild-type, and epoxy-janthitrem for AR37 and the measured alkaloid concentrations were as shown (Table 9).

**Table 9 : Endophyte alkaloid concentration (ppm) for ryegrass herbage cut directly from trays in glass houses before being placed in the field to dry in experiments carried out in early summer and mid-summer. Epoxy-janthitrems are relative and in arbitrary units (Hume, Hickey and Tapper 2007).**

| Endophyte Strain | Endophyte alkaloid | Early summer | Mid summer |
|---|---|---|---|
| Wild type | Lolitrem B | 5.1 | 3.0 |
| Wild type | Ergovaline | 0.15 | 0.26 |
| Wild type | Peramine | 29.8 | 23.3 |
| AR37 | Epoxy-janthitrem A1 | 0.56 | 0.40 |
| AR37 | Epoxy-janthitrem A2 | 0.57 | 0.39 |
| AR37 | Epoxy-janthitrem A3 | 1.15 | 0.76 |
| AR37 | Epoxy-janthitrem B | 2.00 | 1.25 |
| AR37 | Total | 4.27 | 2.80 |

Peramine, lolitrem B and total janthitrems generally exhibited a similar pattern of linear or gradual decline. Generally the decline in these alkaloids was not immediate, particularly for lolitrem B in mid-summer which remained high up to 4 days after cutting. For peramine and total janthitrems, a 50% decline occurred within 5 to 7 days in both experiments, despite the contrasting weather conditions in early and midsummer. Ergovaline showed quite variable and erratic responses over time possibly due to the low concentrations in the cut herbage and was not detectable from Days 5 and 10 onwards in the early and mid-summer experiments, respectively. Roberts, Kallenbach, Rottinghaus and Hill, (2011) conducted a trial over 2-years to also determine the change in concentrations of ergovaline and total ergot alkaloids in tall fescue when was conserved as silage, hay, and ammoniated hay. They observed that compared to concentrations in the original pasture, both ergovaline and total ergot alkaloid concentrations were lower in the hay, with ammoniation of the hay resulting in even lower concentrations. In the silage, ergovaline concentrations were lower than original pasture, while total ergot alkaloids were higher. Their results also supported findings by (Hume, Hickey and Tapper 2007). Nevertheless these results show that dead leaves in pastures, hay and silage still contain significant source of toxins which may still be toxic to animals (Clark, Thom, & Waugh, 1996).

Lolitrem B and ergovaline are concentrated in the lower 20 - 40mm of pasture or the 1000-12000kgDM/ha residual and in the stem and seed head (Clark et al., 1996), therefore, if grazing residuals are maintained above 1000-1200kgDM/ha the amount of alkaloids consumed is reduced, hence reducing the likelihood of harm on the grazing animals. Supplementary feeding with other feeds was suggested to avoid deep grazing. Seed head

development in pastures should also be avoided as high concentrations of alkaloids accumulate.

### 2.5.8 Effects of endophytes in grass nematodes

The effects of endophytes on plant nematodes have been investigated in perennial ryegrass. (Stewart & Grant, 1993) carried out an experiment to evaluate the development of the nematode *Meloidogyne naasi* on endophyte-infected and endophyte-free perennial ryegrass. Plants infected with endophyte had fewer galls (87) as compared with 134 in endophyte free plants. Similarly, endophyte-infected plants hosted fewer female nematodes (113) as compared with 195 in endophyte-free plants and no juveniles nematodes were seen in all the treatments. These results are also in agreement with those found by (Ball et al., 1997) who found that roots of endophyte-free grass contained greater number of nematodes compared with endophyte-infected perennial ryegrass. These findings, nevertheless, contrasted with those of Cook et al., (1991) who reported no differences in infection by *M. naasi* in perennial rye grass free or infected with *N. lolii* in a pot trial. The contrast between the other results and those of Cook et al., (1991) using the same species of ryegrass, endophyte and nematode, add emphasis to the comment by Kirkpatrick, Barham, & Bateman, (1990) that differences among endophyte isolates or interactions between host genotype and fungal isolate are important. Reduced numbers of female *M. naasi* in endophyte-infected plants would have resulted from one or more of the following: reduced invasion, increased emigration of juveniles or death of nematodes in the roots. Since gall counts as well as female numbers were lower in endophyte-infected plants it seemed likely resistance occurred early in the plant nematode interaction. It is possible that endophyte toxins in the roots repel or kill nematodes as toxins have been reported in roots by (Gallagher, Smith, di Menna, & Young, 1982 ; Sifegel, Dahlman, & LBush, 1989) or alternatively, the presence of the fungus may enhance plant resistance.

Studies in tall fescue also supported a positive association between presence of endophytes and reduced numbers of parasitic plant nematodes in the grass roots and soils surrounding the roots. In an experiment with tall fescue, (Pedersen, Rodriguez-Kabana, & Shelby, 1988) investigated the effect of *N. coenophialum* infection on ecto-parasitic nematodes namely *Helicotylenchus dihystera* and *Paratrichodorus minor*. Results showed significant difference in cyst nematode numbers between endophyte-infected and endophyte-free tall fescue in the root fractions. *N. coenophialum* infected grass also had reduced spiral nematode

population in the soil sampled around the grass roots. Another trial by Elmi, West, Kirkpatrick, & Robbins, (1990) supported these findings by showing that endemic *N. coenophialum* infection suppressed reproduction of root knot nematode *Meloidogyne graminis*. Kimmons, Gwinn, & Bernard, (1990) compared population densities of *P. scribneri* in the roots and soils surrounding tall fescue plants with and without endophytes and observed fewer nematodes in the endophyte infected grass roots than the endophyte-free roots and fewer nematodes were also found in the soil surrounding the endophyte infected grass (Figure 9).

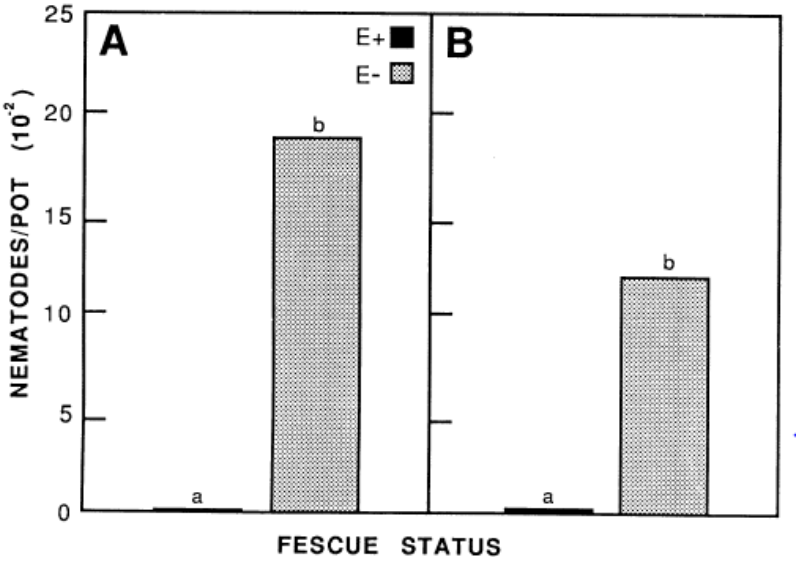


**Figure 9: Mean numbers of *P. scribneri* on endophyte-infected (E+) and endophyte –free (E-) tall fescue. A, is experiment 1, B is experiment 2, and columns with same letters do not differ at P=0.05 (using independent t test).**

Source: (Kimmons et al., 1990)

Nevertheless, (Kimmons et al., 1990) showed that the levels of *Helicotylenchus pseudorobustus*, an ecto-parasitic nematode was not significantly different in pots of endophyte infected and endophyte free tall fescue. The reproduction of *Meloidogyne* species nematode on white clover was not affected by the presence or absence of endophytes in the same trials. These findings seem to suggest that some nematode species are not affected by the endophytes and that the plant species also affect the nematicidic effect of the endophytes as the same nematode affected in fescue was not affected in white clover. West, Izekor, Oosterhuis , & Robbins, (1987) observed a positive association between endophyte infection (*N. coenophialum*) and tolerance to drought conditions in tall fescue (cultivar Kentucky 31). They suggested that the effect might have been partially enhanced by

resistance to parasitic nematodes like *P. scribneri and Tylenchorhynchus ewingi*. This was supported by a trial by (West, Izekor, Oosterhuis, & Robbins 1988) which showed that endemic N. coenophialum infection of tall fescue conferred resistance against *P. scribneri* and *T. actutus*. In another experiment in tall fescue, (Pedersen et al., 1988) investigated the effect of *N. coenophialum* infection on ecto-parasitic nematodes namely *Helicotylenchus dihystera* and *Paratrichodorus minor*. Results showed significant difference in cyst nematode numbers between endophyte infected and endophyte free tall fescue in the root fractions and the *N. coenophialum* infected grass also had reduced spiral nematode population in the soil sampled around the grass roots. Further investigations by (Elmi et al., 1990) supported these findings by showing that endemic *N. coenophialum* infection suppressed reproduction of the root knot nematode *Meloidogyne graminis*.

### 2.5.9 Effects of lolines

Loline producing endophytes like tall fescue and meadow fescue have been observed to reduce Argentine stem weevil (ASW) adult feeding (Patchett, Chapman, Fletcher, & Gooneratne, 2008a) and ASW aviposition (Jansen et al., 2009), foliar and root aphid population (Schmidt & Guy, 1997), feeding and growth of grass grub larvae (Popay et al., 2003 ; Patchett, Chapman, Fletcher, & Gooneratne, 2008b), bird cherry oat aphids (Wilkinson et al., 2000), grass grub and porina larvae (Popay & Lane, 2000). These effects were supported in the observations by (Siegel & Bush, 1997) who pointed out that lolines are broad spectrum insectides. Control of these insects, while minimizing any effect on animal health and productivity, is of significant economic impact to farmers, the livestock industry and the country. These lolines have no known effects on grazing mammals (Siegel & Bush, 1996 ; Schardl, Grossman, Nagabhyru, Faulkner, & Mallik, 2007 ; Patchett 2007), even though their concentration in endophyte infected grasses are 100-1000 times higher than other alkaloids (Siegel, Dahlman, & Bush, 1989).

## 2.6 The basis of the proposed research

The foregoing information shows that endophytes play a very important role in plant nematode control. Specific studies with purified alkaloids showed that they are effective against soil and plant nematodes. However there is little information as to whether endophytes in grass pastures have anthelmintic properties to animal nematodes. Society nowadays is demanding that agriculture production systems implement environmentally

sustainable systems of production that have low chemical usage. Helminths continue to be a common limiting factor in livestock production systems, decreasing the quality and quantity of livestock products and requiring periodic treatment with anthelmintics. Reduced dependence on anthelmintics will follow development of alternative parasite control measures. This current study will be conducted to explore the potential anthelmintic properties of seed extracts infected with a variety of known endophytes on gastro-intestinal species.

## 2.7 Specific objective

To determine if the aqueous extracts from seeds infected with different pasture endophytes infected have the potential to inhibit the development of common gastro-intestinal nematode parasites under *in-vitro* conditions.

# Chapter 3

# Materials and Methods

A series of *in-vitro* experiments were performed using extracts of seeds from a variety of endophytes infected grasses on gastro-intestinal parasite eggs and larvae. The gastro-intestinal parasites used were from strains of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* which were collected from 2 mono-specifically infected sheep at Lincoln University.

The seeds were from a nil endophyte rye grass and other rye grasses infected with the endophytes namely AR1, AR37 and wild endophyte. Seeds from tall fescue infected endophyte P542, a nil endophyte tall fescue and an endophyte infected cultivar of Meadow Fescue (U2) were also used.

**Table 10: The endophyte /grass type which were used in the experiments and the known alkaloids they contained**

| **Endophyte/grass type** | **Likely alkaloids** |
|---|---|
| Nil endophyte rye grass (Nil RG) | Nil |
| AR1 | Peramines |
| AR37 | Epoxy-janthitrems |
| Wild-type endophyte (Wild-type endo) | Peramines, lolitrems ergovalines |
| Tall fescue (P542) | Lolines |
| Nil endophyte tall fescue (Nil TF) | Nil |
| U2 (Meadow F) | Lolines |

## 3.1 Preparatory stages

### 3.1.1 Collection of water soluble seed extracts

For each endophyte tested, water soluble extract from the seeds was collected as described below. For each seed type approximately 200 g seeds were ground through a 1mm sieve and then incubated in 3 litres of nanopure water at 4$^{o}$C for 3 hours. Following incubation the resultant solution was sieved and centrifuged at 1200 g for 10 minutes at 4$^{o}$C to remove large debris. The supernatant was collected and passed through reducing sieve sizes to remove large particles. The extracts were finally filtered through a 0.2 µm filter in order to sterilise and prevent any biological activity during incubation steps. Each water soluble extract was then frozen at -20$^{o}$C prior to freeze drying (lyophilisation). Lyophilised extracts were homogenised and then stored at -20$^{o}$C in a container with desiccation pellets until required. For each assay, only the amount of extract required for each analysis was rehydrated each time.

### 3.1.2 Harvesting of gastrointestinal parasite eggs

Nematode eggs were collected using a method adapted from (Coles et al., 1992) and (Hubert & Kerboeuf, 1984) as follows. Faeces were collected from lambs that had mono-specific infections of either *T. colubriformis* or *T. circumcincta*. The lambs were housed on wooden slats and fed hay, Lucerne chaff and commercial concentrate pellets and had free access to water. Faeces were collected using improvised harnesses and collecting bags. From a fresh daily collection of faecal material from each mono-specifically infected animal 100 g were of faeces immersed in 250 mls of water and then homogenised using a stomacher for 20 seconds to form a slurry suspension.

The slurry suspension was filtered through a sieve of pore sizes 150 µm using a pressurised water tap with the contents collected on a 38 µm pore mesh sieve. The material retained on the 38 µm sieve contained the eggs and smaller faecal debris and this material was then placed into 50 ml centrifuge tubes and centrifuged at 1000 rpm for 2 minutes. The supernatant was removed using a vacuum line leaving sediment of approximately 1 ml material containing faecal debris and eggs. The sediment was then re-suspended in 10 mls of saturated sodium chloride. The faecal debris, eggs and saturated sodium chloride mixture was shaken gently and centrifuged for 2 minutes at 1000 rpm allowing all eggs to float to the surface. The supernatant which contained the eggs was collected using a vacuum line then

washed in a 20 µm sieved container for 15 minutes in running tap water to remove the salt solution.

The washed eggs were centrifuged for 2 minutes at 1000 rpm with the sediment representing concentrated eggs. The concentration of eggs was estimated by counting the number of eggs in 5 aliquots of 50 µl of the suspension on a microscope slide. Eggs were subsequently diluted to a final concentration of 100 eggs per 50 µl and were refrigerated until used. This entire process was repeated for faeces containing each species of gastro-intestinal parasite on each day the tests were run and the eggs were used within 4 hours of collection.

## 3.2 Actual Experiments

### 3.2.1 Egg hatch assay

Eggs were collected from sheep faeces as described above and the Egg Hatch Assay was performed according to the method adapted from (Marie-Magdeleine, Mahieu, D'alexis, Philibert, & Archimede 2010). Briefly, the ground powdered extract obtained from freeze dried seed extracts were mixed with distilled water then vortexed before being pipetted to give serial dilutions. The dilutions used in the assays were; 1:1, 1:8, 1:64, 1:512, 1:1024 and 1:2048. Two hundred (200) µl of each of these dilutions for each of the different seed extracts were pipetted on each well on a 48 multi-well plate and tap water was used as control in an arrangement shown in Table 11. Approximately 100 eggs in 50 µl of the egg suspension were pipetted into each well then incubated. This was repeated for *T. colubriformis* and *T. circumcincta* for each group of eggs separately and dilution for each seed extract was replicated four times.

**Table 11: Arrangement of the multi-well plate**

| Dilutions | Extract type | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AR542 | Nil TF | Meadow F | AR37 | Nil RG | Wild Endo | AR1 | water |
| 1:1 | | | | | | | | |
| 1:8 | | | | | | | | |
| 1:64 | | | | | | | | |
| 1:512 | | | | | | | | |
| 1:1024 | | | | | | | | |
| 1:2048 | | | | | | | | |

Lugol's iodine solution was also added to the unused eggs suspension so that the initial hatch rate can be calculated and factored in the final inhibition percentage. The multi well plates containing seed extracts and eggs were gently shaken then incubated under humidified conditions at 25$^{o}$C for 48 hrs.

After 48 hrs incubation, a drop of Lugol's iodine solution was added to each well to prevent further hatching. All the unhatched eggs and L1 larvae in each well were counted at X 100 magnification. The percentage inhibition of egg hatching was calculated after factoring in percentage hatch rate of the initial collected eggs.

### 3.2.2 Larval development assays

#### 3.2.2.1 Larval development assay in faecal material

Faeces were collected from two sheep, one infected with *T. colubriformis* and the other with *T. circumcinta*. Three faecal egg counts were performed to ensure that the faeces contained sufficient eggs. The faeces were thoroughly mixed and 50 g was taken then homogenised. The homogenised faeces were then loosely put into a 24 multi-well plate. Serial dilutions were made for each seed extract from the lyophilised samples prepared in the preliminary stages with 1 ml inoculated into each well with each concentration used in triplicate. The plates with their lids removed to improve aeration were placed in trays wrapped in holed plastic bag to ensure free air movement as well as reducing dying of faeces. The plates were incubated at 25$^{o}$C for 10 days to allow for egg hatching and larval development. After every 3-4 days water was irrigated on the plates to keep the faeces moist and the faeces were perforated using a pipette tip on day 4 and 7 to improve aeration. After 10 days of incubation the contents of each well were soaked with water draining any larvae to the

bottom of the well. The solid faecal material in each well was removed leaving some extract at the bottom of the well. The wells were observed for live/active larvae (L3) using an inverse microscope at magnification 40X. Due to difficulties of extracting all larvae in each well, reliable quantification of larvae was not possible so results were noted as larvae present or not present.

#### 3.2.2.2 Larval development assay in faecal aqueous extract

Faeces were collected from two sheep one infected with *T. colubriformis* and the other with *T. circumcinta*. A portion of faecal material collected was soaked in water for about an hour then broken up. The faeces were passed through a sieve to collect a bacteria rich extract for larvae to feed on. One millilitre of the faecal extract was pipetted into each well on a 24 multi- well plate. 30 µls of egg suspension containing 100 eggs collected as described above were seeded into each well. The plates were then incubated at 25$^{o}$C for 48 hrs to allow eggs to hatch.

After the 48 hrs serial dilutions were made for each seed extract then 400 µl inoculated into each well with each concentration used in triplicate. The plates had their lids removed to improve aeration then wrapped in holed plastic bag to ensure free air movement as well as reducing evaporation of well contents. The plates were incubated at 25$^{o}$C for 8 days to allow for larval development from eggs to L3. Each day the plates were removed from the plastic container for about 15-20 minutes and moved in a swirling motion to improve aeration and any crusting on the surface was removed. At the end of the incubation, with minimum disturbance the supernatant of each well was decanted leaving the larval rich sediment. The sediment was diluted with water to allow larval counting under the microscope. The numbers of immobile and mobile larvae were counted.

### 3.2.3 Larval motility assays

#### 3.2.3.1 Larval mobility inhibition test

Nematode eggs hatch then develop into L3 inside faecal materials on the ground. The L3 then migrate or move out of the faecal material prior to ingestion by grazing animals and wait for passing animals to feed on the grass. The larval mobility inhibition test is therefore very important in that substances that can inhibit larval migration have the capacity to reduce the number of L3 which can migrate out of the faecal material and being ingested.

The larval motility assay was conducted on *T. colubriformis* and *T. circumcincta* L3 larvae of

sheep following a method adapted from (Marie-Magdeleine et al., 2010). L3 larvae were collected from sheep faeces after faecal culturing at 25$^{o}$C for 10 days followed by active migration method and concentrated by sedimentation and Baermann's technique at Johnstone Memorial Laboratory (JML). Approximately 100 L3 in 50 µl suspension per well were exposed to serial dilutions of 400 µl of the test extracts from 1 g extract to 8mls of water to a dilution of 1 to 2048 and the plates incubated at 25$^{o}$C. The motility of the L3 was observed using an inverse microscope at approximately 24 hrs after exposure as immobile or mobile. The percentages of immobile L3 in the experiment were used as the criterion for the anthelmintic activity of the extracts.

#### 3.2.3.2 Effect of extract exposure time on immobility

Larval mobility and immobility was assessed using a method adapted from (Bachaya, Iqbal, Khan, Sindhu, & Jabbar, 2009). Briefly, an extract of AR542 was made into an aqueous serial dilution of 1 part extract to 4 parts water, 1:8 and 1:16. Each dilution was halved to produce a total of 6 tubes with 6 ml of solution. In 3 tubes approximately 5000 *T. colubriformis* L3 larvae suspension with was placed and in the other 3 tubes 1 ml of *T. circumcincta* L3 suspension with about 5000 L3 was added. Each tube was thoroughly mixed before four aliquots of 20 µl were pipetted onto a microscope slide. The numbers of immobile and mobile larvae were counted on the four aliquots. This was repeated after every hour for three hours and then after 15 hrs the following day. This was repeated for all the 6 tubes.

#### 3.2.3.3 Effect of redilution on immobility

After 15 hrs of larval exposure to the extract, the tubes with extracts were diluted with water to half the concentration of the original 1 part extract to 4 parts of water, 1:8 and 1:16 then the tubes incubated for 6 hrs at 25$^{o}$C. After the 6 hrs each tube was thoroughly mixed before aliquots of 20 µl were put on to a slide then observed for immobile and mobile L3 using a microscope so as to calculate the percentage of immobile larvae for nematode at each rediluted concentration.

### 3.3 Calculations and statistical analysis

The percentage (%) of eggs that hatched was calculated using the formula:

1) ((Number of larvae)/ (number of unhatched eggs + number of Larvae)) * 100

The percentage (%) of immobile larvae was calculated as:

2) ((Number of immobile larvae) / (number of immobile +mobile larvae))*100.

Statistical analysis was performed for each nematode species separately using Genstat Statistical Software (Version 13.3 VSN international Ltd). For the comparison of efficiency across several dilutions for egg hatch assays and larval motility LD95, LD90, and LD50 was calculated and compared across all seed types using Probit regression analysis with time and log % hatched and motile as fitted terms. Post-hoc comparisons of the LD50 values were completed using a Tukeys test with 5% significance. Differences in larval immotility following time periods or redilution were assessed using ANOVA with nematode species, concentration and re-dilution as factors.

# Chapter 4

# Results

## 4.1 Experiment 1

### 4.1.1 Egg hatch assay

Overall, eggs incubated in water demonstrated minimal inhibition, with a mean percentage eggs hatching ranging from 85 to 95%. For all extracts and both nematode species understudy, the concentration of 1:1 completely lysed the eggs, consequently results are not shown.

For *T. circumcincta* the percentages of eggs hatching at each concentration less than 1:1 are given in Figures 10 and 11. For all extracts, the percentage of eggs hatching increased with decreasing extract concentration. Mean LD50 values with 95% confidence intervals for each extract are given in Figure 12. Mean LD50 values were greatest for Nil RG being 0.019, followed by AR1, wild-type endophyte, AR3, AR542, Meadow fescue 0.018, 0.017, 0.016, 0.016, 0.014 and lastly Nil TF being 0.012. LD50 concentrations were least for Meadow F and Nil TF which were significantly less than AR1, AR37, AR542, Nil RG and wild endophyte. Furthermore LD50 for AR542 and AR37 were less than AR1 and Nil RG and Wild-type endophyte but were not different from each other.

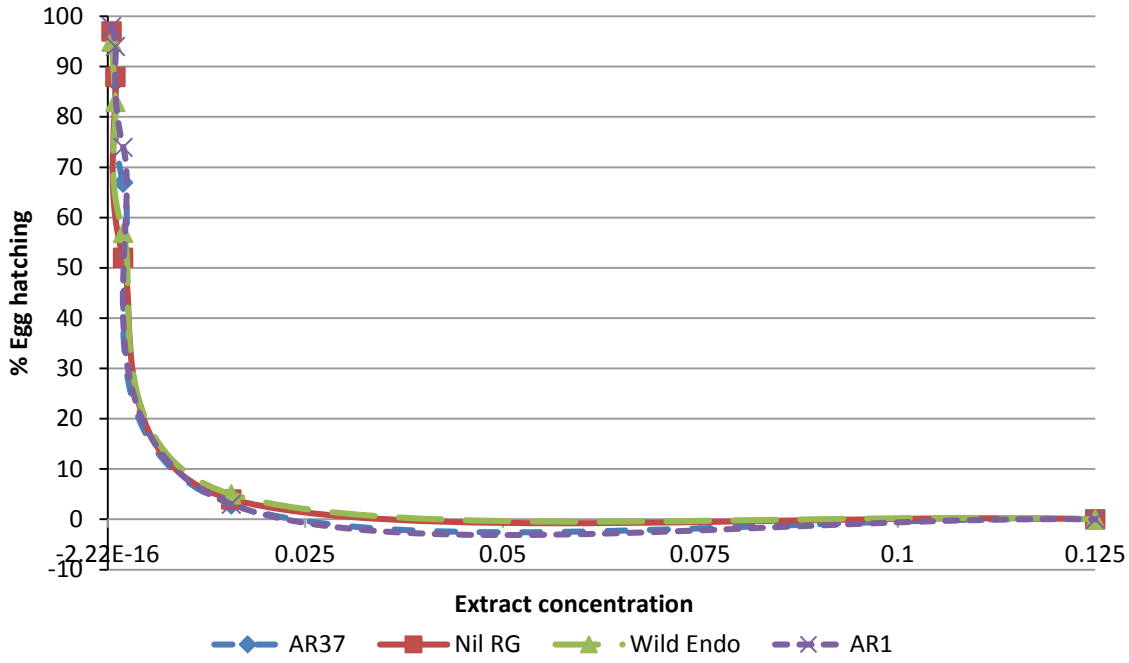


**Figure 10: Percentage of *Teladorsagia circumcincta* eggs hatching following incubation for 24hrs in different extract concentrations of AR37, Nil RG, Wild–type Endo and AR1 seeds.**

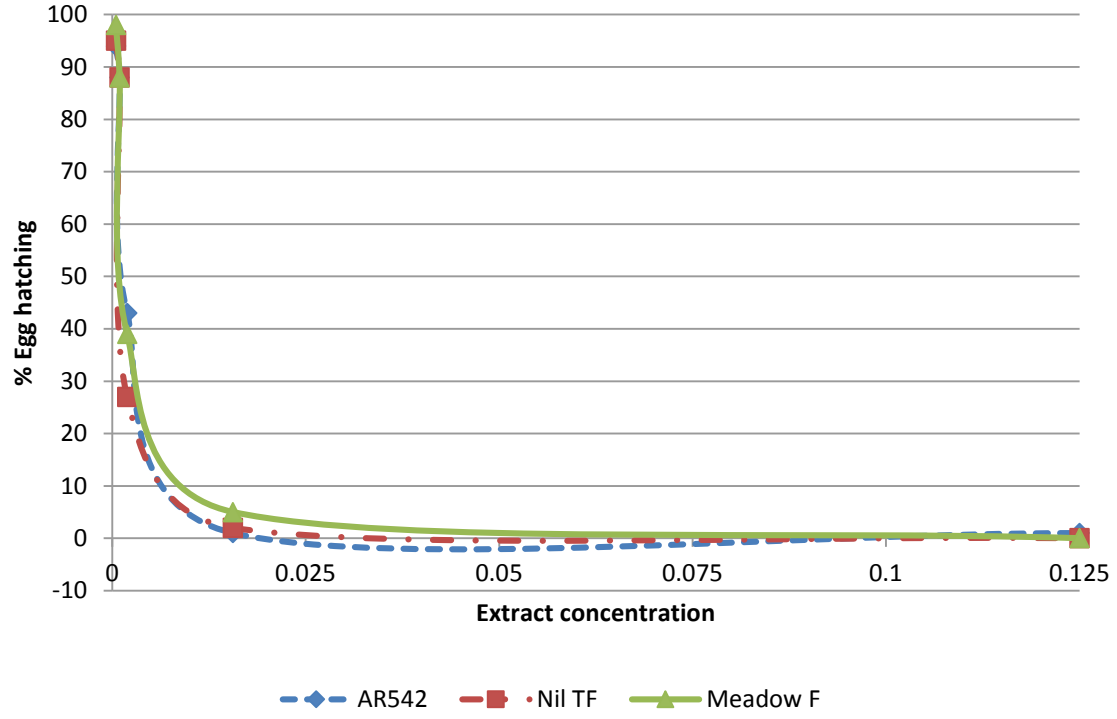


**Figure 11: Percentage of *Teladorsagia circumcincta* eggs hatching following incubation for 24 hrs in different extract concentrations of AR542, Nil TF and Meadow F seeds.**

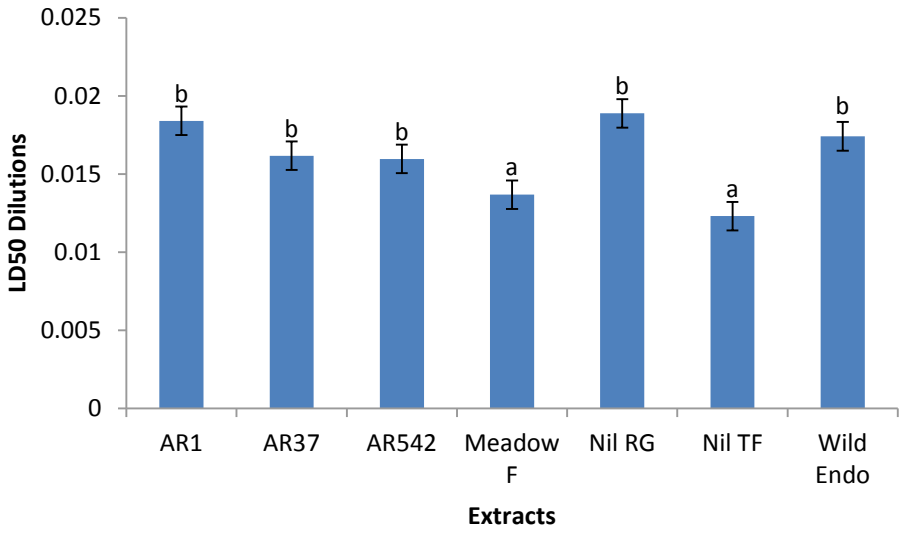


**Figure 12: Mean LD50 dilutions for *Teladorsagia circumcincta* for the seed extracts from AR542, Nil TF, Meadow F, AR37, Nil RG, Wild–type endophyte and AR1 endophytes. Bars with similar superscript are not significantly different (P>0.05)**

For loline producing endophytes, the LD50 for *T. circumcincta* egg hatching was greater (P<0.05) for AR542 compared with either nil Tall fescue or meadow fescue, the latter two not differing from each other (P>0.05) Within rye grass varieties, no difference in the LD50 for *T. circumcincta* egg hatching was observed between AR1, AR37, wild-type or nil-ryegrass.

For *T. colubriformis* the percentage eggs hatching at each concentration less than 1:1 is given Figures 13 and 14. For all extracts, the percentage of eggs hatching increased with decreasing extract concentration. Mean LD50 values were greatest for Wild-type endophyte being 0.008 followed by AR1, AR37, Nil RG, meadow fescue with AR542 0.008, 0.007, 0.007, 0.007, 0.006 respectively and lastly Nil Tall fescue with 0.006. Hatching percentage LD50 values with 95% confidence intervals for each extract is given in Figure 15. LD50 concentrations were least for AR542 and Nil TF which were significantly less than AR1, wild-type endophyte, AR37, Nil RG and meadow fescue. Furthermore LD50 for AR37, Nil RG and meadow fescue were significantly less than AR1 and Wild-type endophyte.

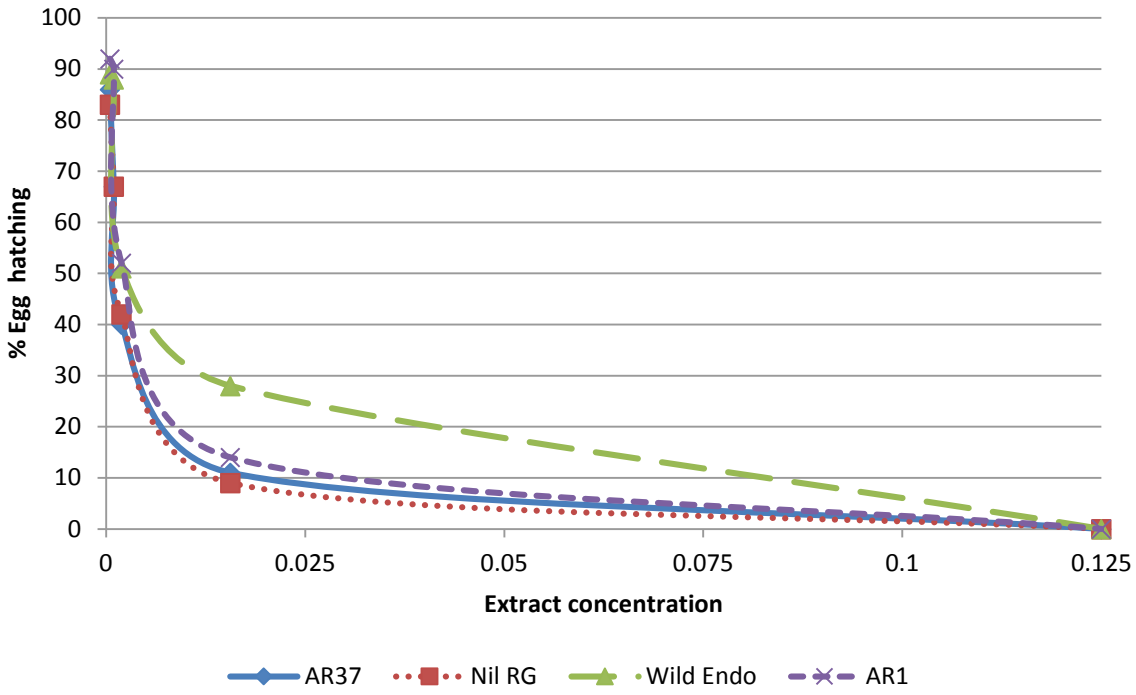


**Figure 13: Percentage of *Trichostrongylus colubriformis* eggs hatching following incubation for 24 hrs in different extract concentrations of AR37, Nil RG, Wild-type endophyte and AR1 seeds.**

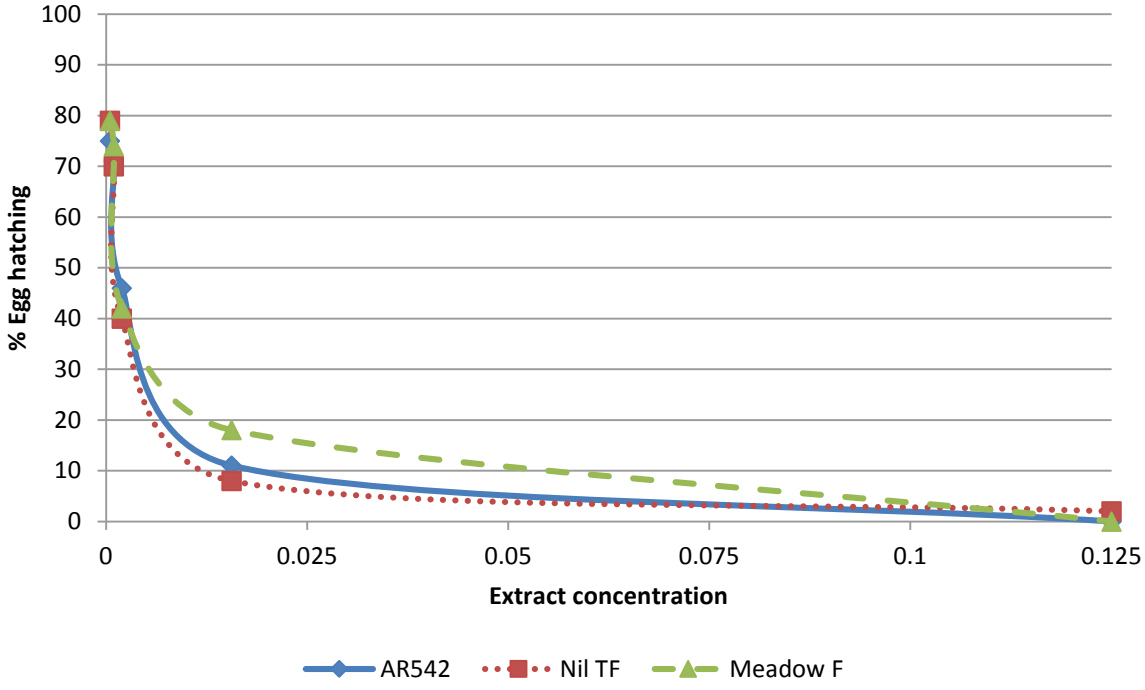


**Figure 14: Percentage of *Trichostrongylus colubriformis* eggs hatching following incubation for 24 hrs in different extract concentrations of AR542, Nil TF and Meadow F seeds.**

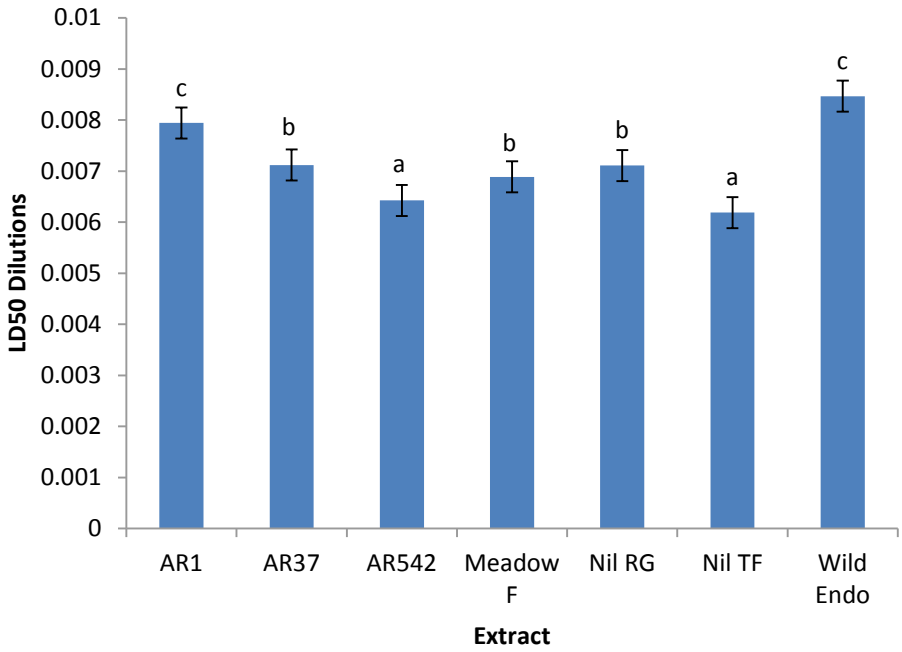


**Figure 15: Mean LD50 values for *Trichostrongylus colubriformis* for the seed extracts from AR542, Nil TF, Meadow F, AR37, Nil RG, Wild–type endophyte and AR1 endophytes. Bars with similar superscript are not significantly different (P>0.05)**

For loline producing endophytes, the LD50 for *T. colubriformis* egg hatching for AR542 and Nil TF were not different from each other (P>0.05) but were significantly lower from meadow fescue (P<0.05).

Within rye grass varieties, the LD50 for *T. circumcincta* egg hatching for AR1 and Wild-type endophyte were not different from each other (P>0.05) but were significantly greater than with AR37 which was significantly greater than Nil RG (P<0.05).

## 4.2 Experiment 2

### 4.2.1 Larval development assay in faecal extract

For the plates inoculated with either *T. colubriformis* or *T. circumcincta* eggs, the eggs failed to hatch or develop through to L3 larvae. There were 95% unhatched eggs and it was observed there was a few L1s in the *T. colubriformis* plates.

### 4.2.2 Larval development assay in faecal material

For the 2 nematodes understudy active L3 larvae were observed in all wells for all the extracts at different concentrations and about 50% unhatched eggs were also observed in

each of the wells but quantification and comparisons were not possible.

## 4.3 Experiment 3

### 4.3.1 Effect of extract concentration on immobility

For *T. circumcincta* the percentage immobility at each dilution less than 1:1 is given in Figures 16 and 17. For all extracts, the percentage immobility decreased with increasing extract dilution. For *T. circumcincta,* mean LD50 dilutions was greatest for wild-type endophyte being 0.004, followed by Nil RG, Meadow fescue, AR542, Nil Tall Fescue AR37, AR1, being 0.003, 0.002, 0.002, 0.002, 0.002 and 0.001 respectively. Mean LD50 values with 95% confidence intervals for each extract is given in Figure 18. LD50 dilutions were greater and significantly similar for both Nil RG and Wild-type endophyte relative to AR1, AR37, AR542, Meadow fescue and Nil TF, all of which were similar.

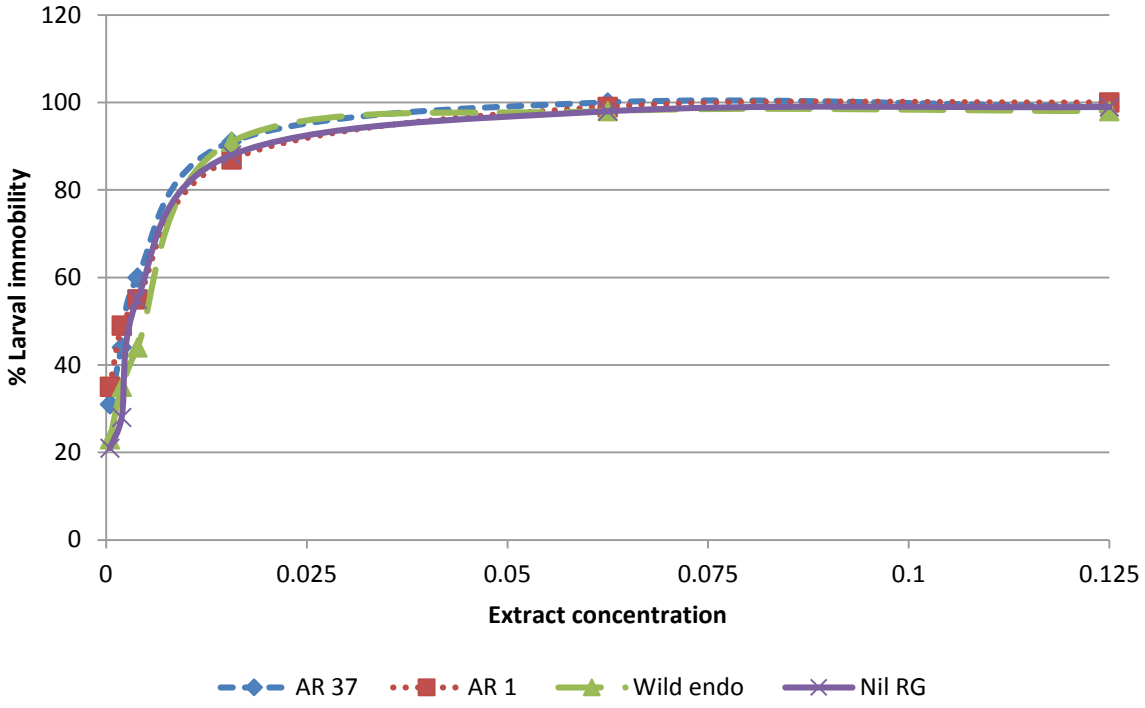


**Figure 16: Percentage of *Teladorsagia circumcincta* larval immobility following incubation in different extract concentrations of AR37, Nil RG, Wild–type endophyte and AR1 seeds.**

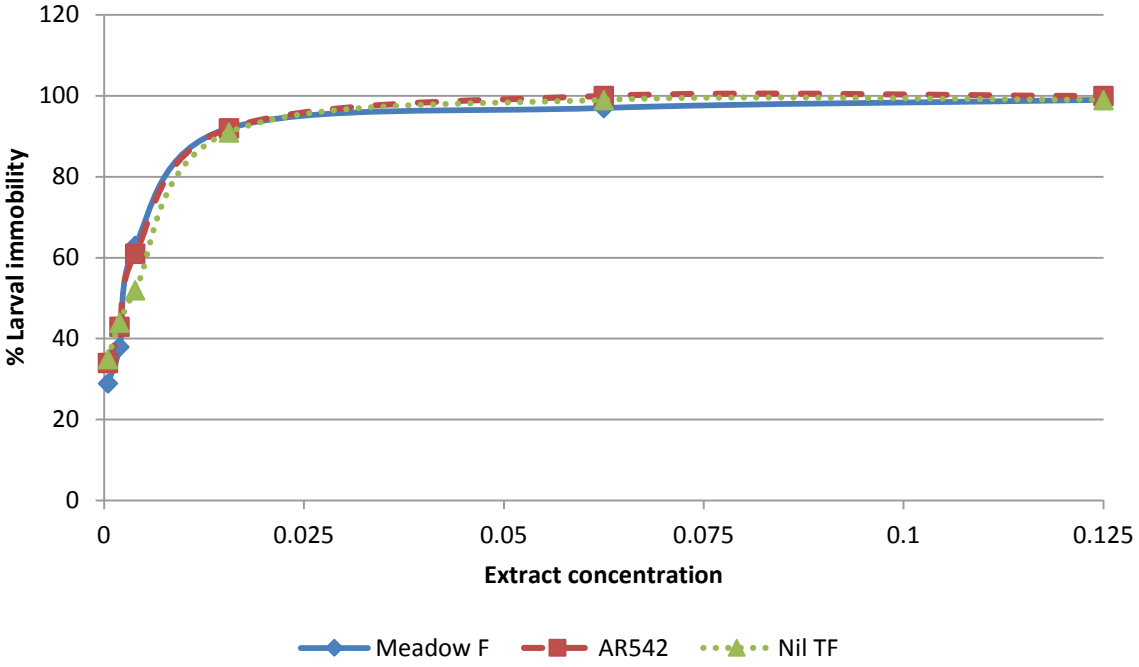


**Figure 17: Percentage of *Teladorsagia circumcincta* larval immobility following in different extract concentrations of AR542, Nil TF and Meadow F seeds.**

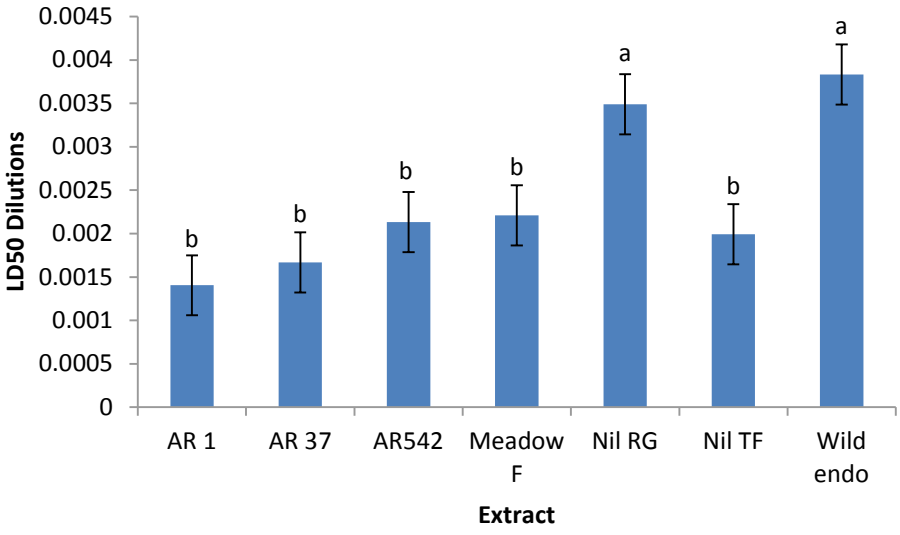


**Figure 18: Mean immobility LD50 dilutions for *Trichostrongylus colubriformis for* the seed extracts from AR542, Nil TF, Meadow F, AR37, Nil RG, Wild-type endophyte and AR1 endophytes. Bars with similar superscript are not significantly different (P>0.05).**

For *T. circumcincta* larvae incubated in loline producing endophytes, there was no observable difference in the immobility of larvae incubated in either AR542, nil Tall fescue or

meadow fescue (P>0.05). For rye grass varieties, LD50 for larval immobility was significantly greater in nil RG and wild-type endophyte than either AR1 or AR37.

For *T. colubriformis* the percentage immobility at each concentration less than 1:1 is given in Figures 19 and 20. For all extracts, the percentage immobility decreased with decreasing extract concentration. For the *T. colubriformis,* mean LD50 values were greatest for AR542 being 0.004 followed by Nil RG, Nil TF, AR37, AR1, Wild-type endophyte, 0.004, 0.004, 0.003, 0.003 respectively and 0.002 lastly Meadow fescue with 0.00179. Mean LD50 values with 95% confidence intervals for each extract is given in Figure 21. Statistical significance in the different LD50 concentrations was observed only for Nil RG, AR542 and Nil TF relative to AR1, AR37 and relative to wild-type endophyte and Meadow F. LD50 dilutions were higher and significantly similar for Nil RG, AR542 and Nil TF relative to AR1 and AR37 which were significantly similar and also relative to Wild–type endophyte and Meadow fescue which were significantly similar.

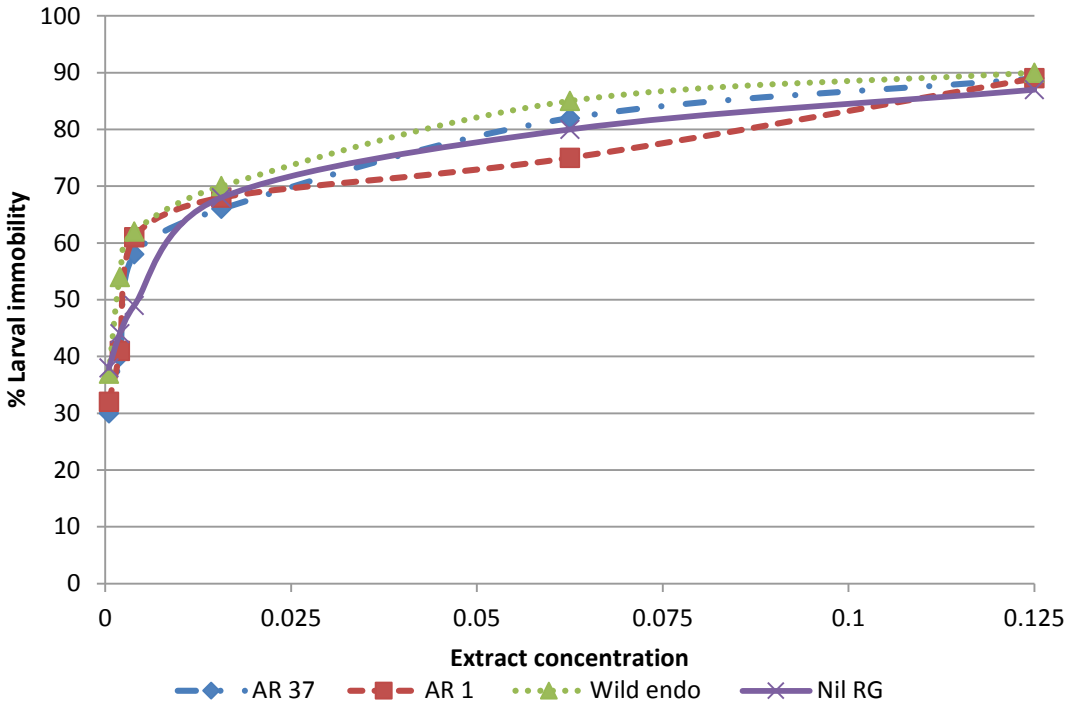


**Figure 19: Percentage of *Trichostrongylus colubriformis* larval immobility following incubation in different extract concentrations of AR37, Nil RG, Wild–type endophyte and AR1 seeds**

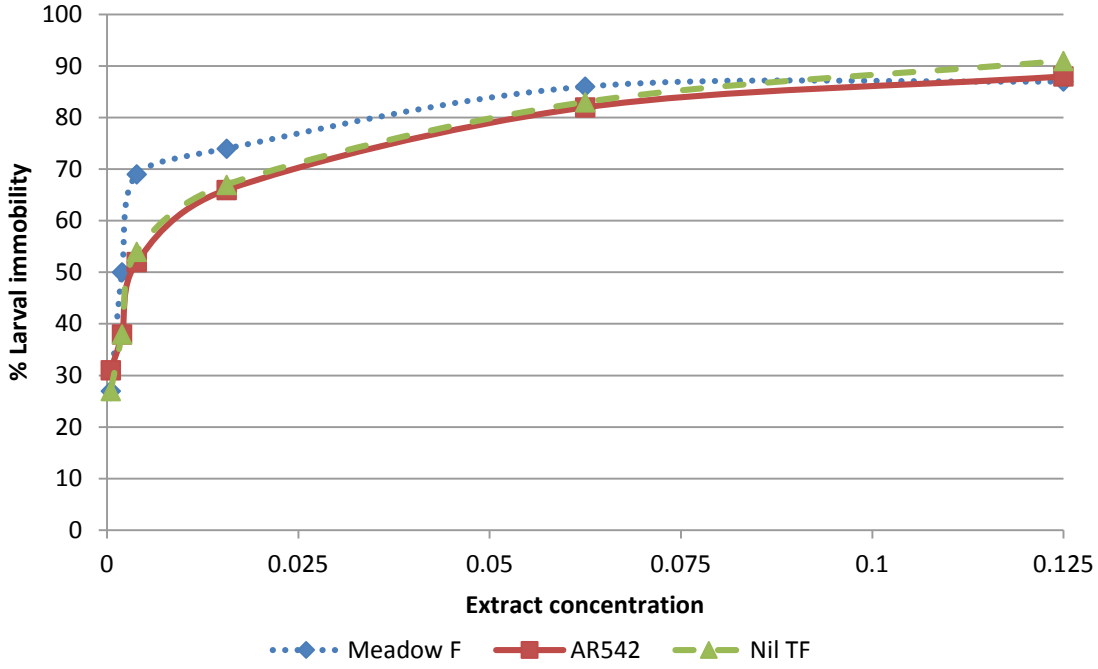


**Figure 20: Percentage of *Trichostrongylus colubriformis* larval immobility following incubation in different extract concentrations of AR542, Nil TF and Meadow F seeds**

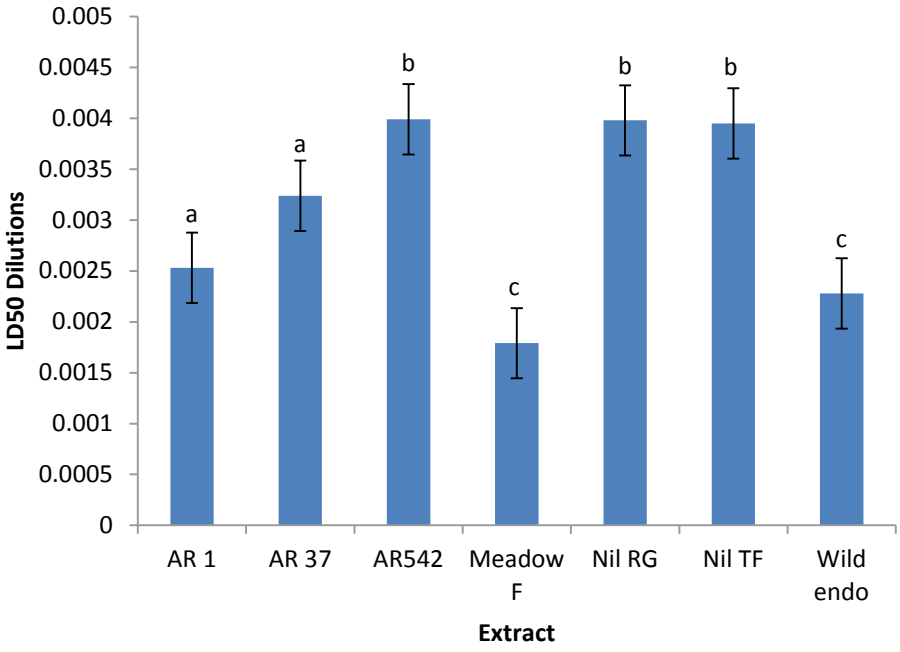


**Figure 21: Mean immobility LD50 dilutions for *T. colubriformis* for the seed extracts from AR542, Nil TF, Meadow F, AR37, Nil RG, Wild-type endophyte and AR1 endophytes. Bars with similar superscript are not significantly different (P>0.05)**

For *T. colubriformis* larvae incubated in loline producing endophytes, there was no observable difference in the immobility of larvae incubated in either AR542 or nil Tall fescue but differed from meadow fescue (P>0.05).

For rye grass varieties, LD50 for larval immobility was significantly greater in Nil RG as compared to AR1, AR37 and wild-type Endo and AR1 and AR37 were significantly greater than wild-type endophyte.

### 4.3.2 Effect of extract exposure time on immobility

For *T. circumcincta* mean immobility was 17% at time 0, 50% at time 1 hr, 57% at time 2 hrs, 67% at 3hrs and 73% at 15 hrs. Percentage immobility of *T. circumcincta* L3 at 1; 8 AR542 dilution was not significantly different at time 0, 1 hr, 2 hrs or 3 hrs from the previous time but was significantly different after 15 hrs.

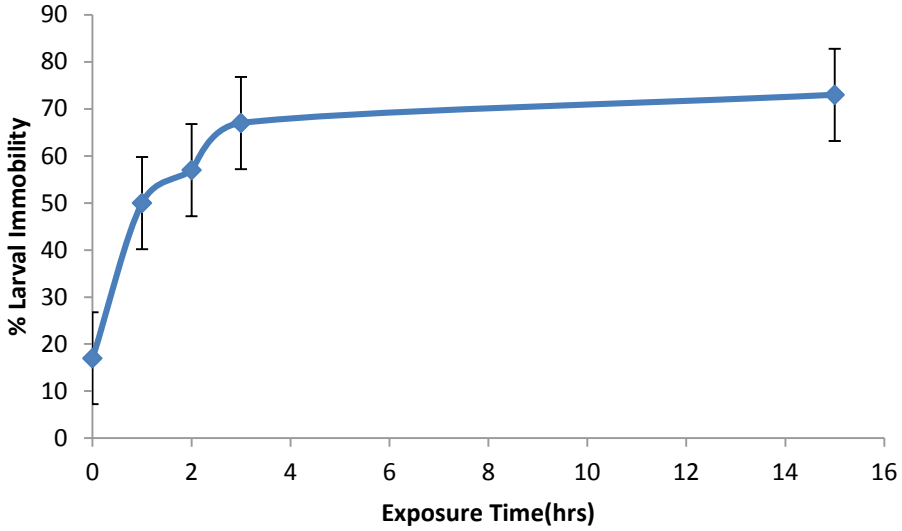


**Figure 22: Percentage of immobile *Teladorsagia circumcincta* L3 larvae at different exposure times when exposed to AR542 seed extract.**

For *T. colubriformis* the mean immobility was 26% at time 0 , 31% at time 1 hr , 49% at time 2 hrs , 67% at 3hrs and 75% at 15 hrs. Percentage immobility of *T. colubriformis* L3 at one AR542 concentration was not significantly different at time 0, 1 hr, and 2 hrs from the previous time but was significantly different after 3 hrs. Percentage immobility was again not significantly different after 15 hrs.

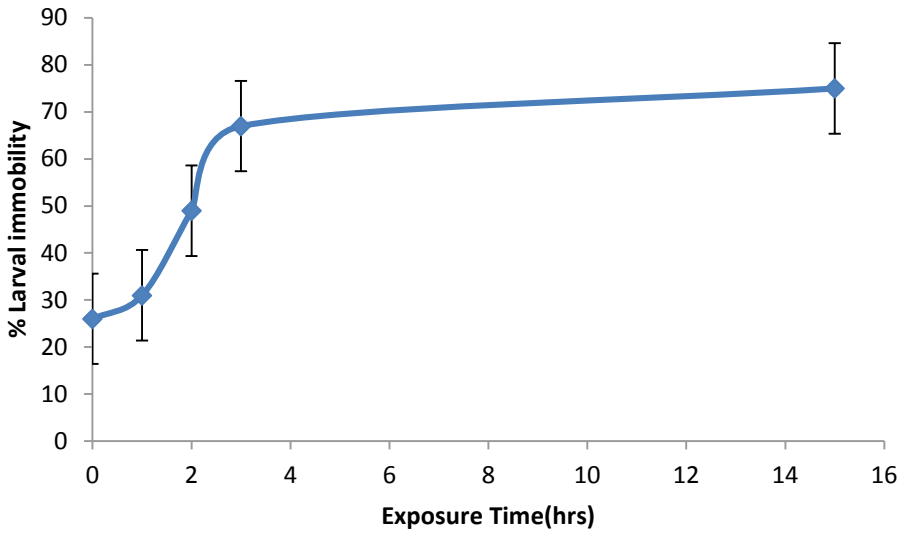


**Figure 23: Percentage of immobile of *Trichostrongylus colubriformis* L3 larvae at different exposure times when exposed to AR542 seed extract.**

### 4.3.3 Effect of re-dilution on immobility

The impact of extract concentration and dilution of extract on larval motility after 15h incubation is shown for *T. circumcincta* and *T. colubriformis* in Figures 24 and 25, respectively. Overall, there was no interaction between nematode species and the response to dilution at different concentrations (P=0.315) with both species responding similarly to dilution. For both species, larval immobility increased with increasing concentration of AR542 extract after 15h incubation (P<0.01). For both nematode species, percent immobility of larvae at the extract concentration at 0.0625 was lowest, being 42 ± 8.3 for *T circumcincta* and 41 ± 17.2 for *T. colubriformis* and was not affected by redilution (P>0.05 for both species). In contrast, redilution of larvae of *T. circumcincta* decreased percent immobility from 50 ± 2 to 21 ± 7.2 at an extract concentration of 0.125 and from 85 ± 15.0 to 20 ± 1.1 at an extract concentration of 0.25 (P<0.001 for both concentrations). Similarly, redilution of *T. colubriformis* larvae decreased percent immobility from 66 ± 15.9 to 35 ± 2.9 at an extract concentration of 0.125 and from 83 ± 0 to 29 ± 5.5 at an extract concentration of 0.25 (P<0.001 for both concentrations).

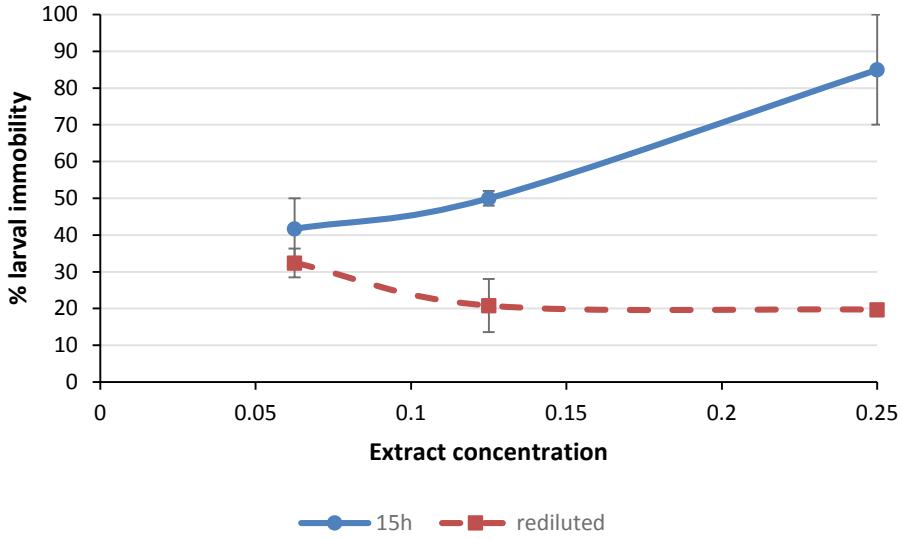


**Figure 24: A comparison of % larval immobility of *Teladorsagia circumcincta* when exposed to AR542 seed extract at 15 hrs and after re-dilution**

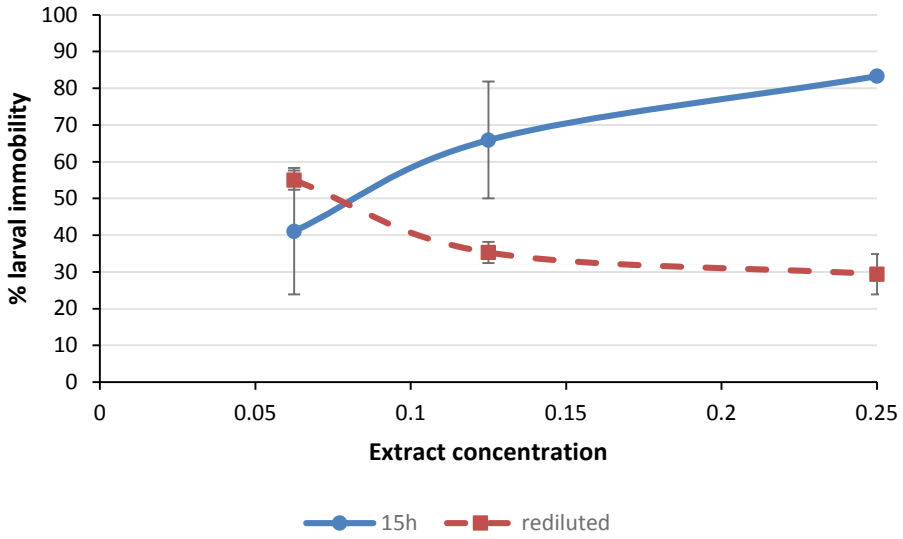


**Figure 25: A comparison of % larval immobility of *Trichostrongylus colubriformis* when exposed toAR542 seed extract at 15 hrs and after re-dilution**

# Chapter 5

# Discussion and Conclusion

The overall objective of the study was to establish the effects of seed extracts of endophyte infected grasses on egg hatching, larval mobility and larval development of the animal nematodes *T. colubriformis* and *T. circumcincta* under *in-vitro* conditions. The eggs and larvae were specifically targeted in these studies as eggs are disseminated into the environment where they hatch and develop to L3 larvae which then infect the hosts. As such, any disturbance on these larval stages is likely to influence the extent these nematodes affect grazing animals.

One of the principal findings of the study was that there were apparent anthelmintic properties for all seed extracts measured by both the egg hatching and larval motility assays of *T. colubriformis* and *T. circumcincta.* The anthelmintic properties were dose dependent with results showing a clear reduction in inhibition rate as the concentration of extracts decreased, with water showing very low or almost nil inhibition and the 1:1 dilution completely lysing eggs and larvae. Such effects appeared independent of seed variety and endophyte type. The fact that high concentration of seed extract can lyse eggs and L1 larvae was also observed by Bizimenyera, Githiori, Eloff, & Swan (2006) when using plant extracts at concentrations of 5 and 25 mg/ml in *Trichostrongylus* experiments. This also followed general trends in chemical properties of other anthelmintic extracts which show greater anthelmintic activity with increasing concentration. For example Shai, Bizimenyera, Bagla, McGaw, & Eloff, (2009) showed increasing inhibition of larval motility and percentage of dead larvae of *T. colubriformis* with increasing concentration from 0.32 to 1000 µg/ml of betulinic acid and lupeol from *Curtisia dentata* (Cornaceae) leaf extracts. There is a paucity of published information in literature on the direct effect of grass seed extracts on parasitic nematodes; however, the results from this current study are comparable with findings by authors who have reported extracts from plant tissues such as roots, leaves and seeds to have anthelmintic effects on gastro-intestinal nematode egg development. For example work by Molgaard et al., ( 2001), using aqueous extracts, showed that 0.5 mg/ml of leaf and bark and 0.8 mg/ml of root extracts of *Peltophorum africanum* were effective against newly excysted cestodes of the worm *Hymenolepis dimunita* following 24 hrs incubation. Their

work, however, was not extended to cover other classes of helminths. Results from this current study are also similar to earlier studies by Soetan & Lasisi, (2008) in which Pearl millet (*Pennisetum glaucum*) saponin extract was shown to inhibit bovine nematode eggs *in vitro* at concentrations of 25 to 500 µg/ml. Bizimenyera et al., (2006) also reported the *in vitro* activity of *Peltophorum africanum* Sond (Fabaceae) extracts on egg hatching and larval development of *T. colubriformis* and (Bachaya et al., (2009) reported that fruits (pods with seeds) of *Acacia nilotica* had anthelmintic properties against *T. circumcincta*. Eguale, Tilahum, Gidey, & Mekonnen, (2006) also observed anthelmintic effects on *H. contortus* when using aqueous extracts of *Acacia nilotica, Cyperus macrostachyus* and *Eligia capensis.*

Another key finding of this study was that the anthelmintic properties appear to be independent of presence of endophytes or endophyte type. Within the rye grass varieties, no significant difference in the LD50 for *T. colubriformis* and *T. circumcincta* egg hatching was observed between AR1, AR37, wild-type or nil-ryegrass. For loline producing endophytes, the LD50 for *T. circumcincta* egg hatching was greater for AR542 compared with either nil Tall fescue or meadow fescue, the latter two not differing from each other. It is nevertheless difficult to conclude that the lolines were the causes of the inhibition as the other loline producing grass Meadow Fescue had comparable results with the nil endophyte variety. A few studies have been carried out to investigate the effects of alkaloids on animal and plant nematodes and results indicate that a number of alkaloids can have an effect on nematodes. In animals, *in-vitro* and *in-vivo* studies by Al-Qarawi, Mahmoud, Haroun, & Adam, (2001) indicated that alkaloids extracted from both the latex and leaves of C*alotropis procera* (Sodom's apple) were effective in inhibiting the development of L3 larvae of *H. contortus* to L4 larval stage in sheep. Lateef, Iqbal, Khan, Aktar, & Jabbar, (2003) also reported that alkaloids and their glycosides extracted from the roots of *Adhatoda vestica* were effective against mixed gastrointestinal infections in sheep and (Onyeyili, Amin, Gambo, Nwosu, & Jibike, (2001) pointed out that tannins and alkaloids, the active ingredients of *Nauclea latifolia* (Rubiaceae) bark, were effective against mixed infections in sheep. In plant nematode studies (Bacetty et al., 2009) used root extracts with several ergot alkaloids and loline alkaloids on *Pratylenchus scribneri,* a nematode pest of tall fescue. The bioassay results of these authors indicated that root extracts from the endophyte infected grasses were nematicidic. The alkaloid ergovaline was nematicidic even at low concentrations of 5 and 50 µg/ml and α-ergocryptine was more nematicidic at

concentrations above 50 µg/ml. Nevertheless, the alkaloids orgocornine and ergonovine were not nematicidic at most concentrations. Loline (N-formylloline), a pryrrolizidine was nematicidic from 50 to 200ug/ml and more so at 100 and 200µg/ml. Motility in nematodes exposed to ergonovine gradually decreased from the lowest concentration of 5 µg/ml, to the highest, 250 µg/ml with the highest concentration stopping all motility. Panaccione et al., (2006) also carried out studies to determine the effects of ergot alkaloids, ergovaline and setoclavine using *P. scribneri* in perennial ryegrass. The results of these authors showed that ergovaline caused a significant reduction in nematodes numbers again supporting the results by (Bacetty et al., 2009). In the current studies, the possible direct effect of ergovaline was not clear. Although the wild-type endophyte seed extracts, which may be expected to contain ergovaline, showed nematicidic or anthelmintic properties, the effects of this seed extract was not any greater than the remaining seed extracts which would not be expected to contain ergovaline.

The results of the current experiment also suggested that nematicidic activity of the extracts increased with greater level of exposure. This larval immotility increased with increasing concentration of extracts and also increased with greater exposure time. These observations are consistent with those of Bacetty et al. (2009) who reported the importance of exposure time, with nematicidic activity increasing from 24 h exposure to 72 h exposure. 24 hrs. Even at a relatively low concentration of 5 µg/ml, these authors reported ergovaline treatments caused 100% reduction in motility after 72 hrs. At the 72 hr time period, nematode motility ceased in the loline treatment in excess of 5 µg/ml and for α-ergocryptine treatments nematode motility ceased at concentration in excess of 50 µg/ml. Further, in studies aimed at evaluating the effects of 1,2 dehydropyrrolizidine alkaloids (PAs), a group of secondary plant metabolites found in hundreds of plant species throughout the world, on plant-parasitic and free-living nematodes, Thoden, Boppré, & Hallmann, (2009) observed that PAs induced nematicidic, ovicidal and repellent effects on different plant-parasitic and free-living nematodes. The fact that the results in this current study suggested that the inhibition effects of these extracts was independent of the presence of alkaloids, supporting the possibility that similar or related chemicals present in all the extracts may have been responsible.

Despite the apparent inhibitory effect on egg hatching, larval development and larval motility in the current study the mechanism of the inhibitory effect was not able to be

determined. However, it was clear that anthelmintic effects were present against both *Trichostrongylus* and *Teladorsagia* allowing the suggestion that the effects are common for these nematode species. These observations contrast to those with condensed tannins in which a reduction in worm fecundity and worm numbers was reported for intestinal, but not abomasal nematode species (Athanasiadou, Kyriazakis, Jackson, & Coop, 2000a, 2000b). In part, the discrepancy of species-specific effects between the current investigations and those of Athanasiadou et al (2000a, 2000b) may reflect the relative exposure to the extracts as both species in the current study were exposed for the same length of time whereas under *in vivo* conditions the abomasal species may be expected to be exposed to the negative effects of tannins for a shorter time than intestinal nematodes. Similarly, Paolini et al., (2003a) and Paolini, Frayssines, De La Farge, Dorchies, & Hoste, (2003b) observed reductions in worm numbers of 33%, 70% and 66% for *H. contortus, T. circumcincta* and *T. colubriformis* respectively when compared with unsupplemented controls in goats that received condensed tannins before being infected with third-stage larvae. According to Sommerville & Rogers, (1987) the hatching process in nematode eggs is initiated by environmental stimuli which lead to the release of hatching enzymes which include chitinases, proteases beta-glycosidases and leucine amino peptidases. Inhibiting some of these enzymes has been shown to reduce the rate of egg hatching or even abrogate it altogether (Rogers & Brooks, 1977). Similarly, Horigome, Kumar, & Okomoto, (1988) demonstrated that condensed tannins inhibited endogenous enzyme activities, but nevertheless the grasses used in this current study are generally considered to have very low levels of condensed tannins (Hoste et al., 2009). Further, substances such glycoproteins present in plant extracts can bind on larval cuticle and can cause mortality or immobility in larvae (Molan & Faraj, 2010). Niere, Waghorn, Charleston, & Waghorn, (1995) also pointed out that these substances may impair vital processes or bind and disrupt the integrity of the larval cuticle. Alternatively, the effects of the extracts on the eggs or larvae observed in the current study may also be related to the osmotic pressure caused by the different concentrations of extracts. For example, infective larvae of *T. colubriformis* lost water and became inactive in media of high osmotic concentration (Wharton, Perry, & Beare, 1983) but the inactivity could also result from increased ionic stress. Support for this in the current study could be observed from the fact that the inhibition of larval migration was reversible following dilution with water which presumably would be accompanied by a return to physiologically normal osmotic gradient.

It was clear from the present observations that larval motility was regained following redilution hence motility in the current study was temporary, indicating that any disruption to the integrity of the larvae was reversible. The fact that larval immobility can be reversible depending on conditions was observed during migration studies of *H. contortus* and *T. colubriformis* by Paolini, Fouraste and Hoste (2004). L3 larval migration and adult worm motility were stopped when the larvae were exposed to tannin rich sainfoin, hazel tree, and oak extracts. When polyethylene glycol (PEG) an inhibitor of tannins was added into the multi-well plate, larval migration and adult worm motility were restored in all the wells involved. Alonso-Diaz et al., (2008a, 2008b) also observed reversal of larval immobility when they added polyvinyl polypyrrolidone (PVPP) another tannin inhibitor in studies using extracts of Sainfoin (*Onobrychis viciifolia* Scop) on *H. contortus*. Although several possible causes for the inhibitory effect on egg hatching and larval motility can be hypothesised, it is clear that further investigation is required to determine if one or a combination of these are the cause of the apparent *in-vitro* anthelmintic properties of the seed extract observed in this study.

Chemical analysis for compounds other than alkaloids was beyond the scope of this study. In other studies the presence of tannins was suggested as being responsible for the anthelmintic effects. (Molan, Duncan, Barry, & McNabb, 2000a ; Molan, Waghorn, & McNabb, 2002; Molan, Waghorn, Min, & McNabb, 2000b) reported the inhibitory effects of tannins against gastrointestinal nematodes and deer lungworms. Nevertheless condensed tannins cannot be associated with anthelmintic properties of the loline producing varieties such as tall fescue and rye grass varieties as these are said to have low condensed tannin levels (Hoste et al., 2009). Macedo et al., (2010) reported that other compounds such as *Eucalyptus globulus* essential oils inhibited 99.3% egg hatching and 98.7% larval development at concentrations 21.75 and 43.5 mg/ml. Shai et al., (2009) showed that the compounds betulinic acid and lupeol from *Curtisia dentata (Cornaceae*) leaves caused inhibition of larval motility and killed larvae of *T. colubriformis*. (Molan, Waghorn, & McNabb, 2000 ; Molan, Meagher, Spencer, & Sivakumaran, 2003 ; Paolini & Hoste, 2003c) also showed that other extracts such as flavonol, flavonol glycosides and proanthocyanidins showed inhibitory effects on migration of *Haemonchus* L3, further supporting findings in the current studies. In comparison, Kermanshai et al., (2001) identified the benzyl isothiacynate as the main biochemical component responsible for the anthelmintic effects of papaya

seeds. Nevertheless no report has mentioned if any of these compounds are present in the pasture extracts which were used in this study hence further chemical tests are necessary to verify the chemicals responsible for these anthelmintic effects.

*In-vitro* methods are useful as a means to rapidly screen for potential anthelmintic activities of different extracts (Hounzangbe-Adote, Fouraste, Moutairou, & Hoste, 2005; Hounzangbe-Adote, V, Fouraste, Moutairou, & Hoste, 2005) The positive results from this current study warrant further investigation of these extracts using *in-vivo* methods. This is due to the divergence in the conditions encountered in laboratory and actual animals for example the availability and or concentration of any active compound as well as metabolic transformation. This contrast was obtained with condensed tannins for which *in-vitro* and *in-vivo* trials gave different results (Athanasiadou, Kyriazakis, Jackon, & Coop, 2001; Chan, Waghorn, Molan & Brookes, 2011). The relevance of these methods with regards to the anthelmintic activity, is greatly influenced by difference in the physiology and the bioavailability of the plant preparations within the hosts (Githiori, Hoglund, & Waller, 2005) hence the need to look at all these in perspective. *In-vitro* results remain indicative and have to be confirmed through *in-vivo* studies with experimental nematodes infections in target host species (Githiori, Athanasiadou, & Thamsborg 2006). A conclusive answer as to whether plant extracts can affect egg hatching in real production settings, or not, can therefore be established following an appropriate study.

Relating the results of *in vitro* tests to likely observations *in-vivo* can be complicated by the absorption of material through the gastrointestinal tract. Little is known about the fate and metabolism of ingested alkaloids such as lolines in livestock and likewise there is a paucity of information available on the amount and fate of these alkaloids in dung of animals ingesting endophyte infected tall fescue grass. Westendorf et al., (1993) recovered about 70% of ingested ergovaline in the dung of sheep ingesting tall fescue but were unable to detect lolines in dung. When endophyte-infected tall fescue was fed, loline alkaloids were excreted only at low concentrations in the urine of sheep (Westendorf et al., 1993) and horses (Takeda, Susuki, Kamei, & Nakata, 1991) and (Tepaske, Powell, & Petroski, 1993) also found lolines in urine soon after ingestion by cattle. During *in-vitro* studies, N-formyl loline was converted by rumen liquor and gastro intestinal secretions to other loline derivatives (Tepaske et al., 1993 ; Westendorf et al., 1993), but recovery of these broken down

products from the abomasum and faeces of cannulated sheep fed endophyte-infected tall fescue was very low or nil (Westendorf et al., 1993 ; Gooneratne, Patchett, Wellby, & Fletcher, 2012). This was also supported by Patchett, (2007) who recovered only 3.8 % and 6.1 % of loline metabolites in urine and faeces respectively. This demonstrated that generally most of the lolines disappear from digesta as it passes through the gastro-intestinal tract. If the alkaloids cannot pass through the gastrointestinal tract unaffected they might be of limited benefit in interrupting the nematode life cycle. However, there is some evidence that the biological activity of alkaloids can survive passage through the gastrointestinal tract. It has been observed that beef cattle grazing endophyte-infected tall fescue pastures have fewer face flies than cattle grazing endophyte-free pastures, perhaps because of poor survival of larvae related to unfavourable chemical and physical properties of the dung (Dougherty & Knapp, 1994). Further, faeces from animals grazing endophyte containing pastures have been reported to have reduced degradation rates, suggesting a direct effect on the dung micro-fauna (Cripps & Edwards, 2013). Overall, it is clear the fate and activity of alkaloids in the faeces of parasitized animals is an area that requires further investigation and can have severe implications as to the ability of endophyte containing pastures to impact nematode parasite epidemiology. On the one hand, the suggestion by Westendorf et al., (1993) that some alkaloids can pass the gastrointestinal track unaffected and hence can be in contact with nematode eggs and larvae when dropped in grazing lands may provide a positive anthelmintic effect through inhibiting the hatching, growth, development and survival of dung- dwelling larvae. But, on the other hand, such effects may be at least partially mitigated through the extended faecal degradation time, thus providing greater opportunity for larval development within the faecal pat and subsequent migration onto the surrounding pastures.

## 5.1 Conclusion

The apparent anthelmintic properties demonstrated *in-vitro* in these studies indicate that seed extracts, independent of endophyte and alkaloid type can have deleterious effects on nematode viability. Although reversible, the anthelmintic activity exhibited by the extracts studied has a significant importance considering the emergence of anthelmintic resistance worldwide. If the effects shown in these studies could also apply *in-vivo* and results concluded that there are anthelmintic effects, consumption of the studied seeds by animals infected with adult worms could be followed by reduction in both egg and larval viability and

therefore lowered environment or pasture contamination. By itself, a reduction in egg hatching can also help to modulate the risk of parasitism by limiting the infectivity of pastures grazed by ruminants. These extracts are therefore possible candidates for treatment of gastrointestinal larval infections but their effectiveness still remains to be further tested before practical applications in farm conditions.

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