Root lesion nematode (*Pratylenchus penetrans*) mitigation through application of oligo-chitin and *Ascophyllum nodosum* extract in the soil prior to seeding red clover (*Trifolium pratense L.*) and birdsfoot trefoil (*Lotus corniculatus L.*).

By
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Root lesion nematode (*Pratylenchus penetrans*) mitigation through application of oligo-chitin and *Ascophyllum nodosum* extract in the soil prior to seeding red clover (*Trifolium pratense L.*) and birdsfoot trefoil (*Lotus corniculatus L.*).

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Abstract

Root lesion nematodes (RLN) are a major pest in agriculture with no effective mitigation strategy. This research assessed the effect of a soil drench with chitin or *Ascophyllum nodosum* extract (ANE) on RLN infection and compared RLN abundance among varieties of birdsfoot trefoil (*Lotus corniculatus L.*) and red clover (*Trifolium pratense L.*). ANE and chitin treatments both resulted in lower populations of RLN/g dry root than the control; ANE had the lowest RLN population at 5185 RLN/g dry root, 30% less than the control. Red clover varieties (3877/g) had lower RLN abundance than birdsfoot trefoil cultivars (11276/g). TRC12-156, a red clover bred to be high in isoflavones, had the least RLN (2088RLN/g); 63% less than TRC12-157, a low isoflavone red clover variety. These results indicate the potential for new RLN mitigation strategies through application of novel soil treatments and selection of forage species and cultivar.

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Chapter 1: Introduction

Soil organisms are important contributors to agricultural systems. Among these organisms are nematodes, commonly referred to as roundworms. They are the members of phylum Nematoda. These organisms are found throughout a wide range of environments (Reece et al. 2011). In order to survive, certain nematodes require a host from which they can draw nutrients. These nematodes are referred to as parasitic nematodes and are a major concern within the agriculture industry (Davies and Curtis 2011). Plant-parasitic nematodes can reduce crop yield by over 15% (Stirling 2014), while animal-parasitic nematodes have negative impacts on animal welfare and productivity as well (Holden-Dye and Walker 2014).

Farmers have been managing parasitic nematodes for generations. Traditional mitigation of parasitic nematodes in plant agriculture includes the application of crop rotation and crop nematicides. However, there are concerns surrounding both of these methods. For example, the use of nematicides has lost popularity due to harsh environmental impacts and reduced efficacy (Duncan and Moens 2013); while crop rotations are often difficult as the host range of many parasitic nematodes remains unknown. In addition to a lack of effective treatments, populations of these parasites are also predicted to increase as a result of climate change (Van Dijk et al. 2010). Shorter, warmer winters, with higher rainfall and fewer ground frosts, will extend the range of the parasites north due to improved overwintering of parasites in fields (Kenyon et al. 2009). In the face of a growing population and demand for food, efficient and sustainable agriculture systems must be developed. One potential element in these systems, which will be addressed in

the following research, is the development of new and effective parasitic nematode mitigation strategies.

1.1 Organisms in agricultural systems

Agricultural systems are made of many complex relationships between organisms. In general, organisms can be classified into three categories: productive biota, resource biota and destructive biota (Altieri 1999; Swift and Anderson 1994). Swift and Anderson (1994) have laid out the frame work through which these classifications are based. The productive biota is the group of organisms chosen by the farmer, such as crop plants and livestock, that are used for the production of food, fibre and other products intended for human use or consumption. These organisms are a major determinant of the complexity and diversity of the organisms within the agriculture ecosystem. The second group are the resource biota. This group of organisms is beneficial to the production system but do not produce a product themselves. Organisms in this group include pollinators, cover crops, decomposers and the predators of pests. The final group of organisms are the destructive biota. They are a detriment to the productivity of the agricultural systems. Weeds, microbial pathogens, insect pests and parasitic nematodes are all considered destructive biota. It is the goal of many agricultural management practices to limit and reduce the latter group of organisms within the ecosystem.

1.1 Soil organisms

Soil organisms can be classified as either resource or destructive biota and include bacteria, fungi and animals, such as nematodes and earthworms. These organisms play a major role in the productivity and sustainability of agricultural systems due to their effects on nutrient availability through organic matter breakdown and as destructive biota (Altieri 1999). Both resource and destructive biota are part of the soil fauna. Management practices of agricultural lands influence the populations and diversity of soil fauna (Swift and Anderson 1994). For example, the use of pesticides in agriculture can result in loss of soil organisms and the input of fertilisers can suppress nitrogen-fixing bacteria and the breakdown of soil organic matter (Thiele-Bruhn et al. 2012).

This research will focus on root lesion nematodes in forage legumes, which is a soil organism and a member of the destructive biota. However, nematodes, in general, are a diverse group of organisms and may be either resource or destructive biota (Smiley 2015). A general overview of nematodes is given in section 1.2 while specific information on root lesion nematodes and the role in agricultural systems will be provided in section 1.2.5.

1.2 Nematodes

Nematodes range in size from less than 0.1mm to over 1m long, such as *Placentonema gigantissima* found in sperm whales which can grow to be over eight meters in size (Gunn 2012). Nematodes are found in a variety of environments, including fresh and saltwater, soil, and throughout the bodies of plants and animals (Reece et al. 2011). Nematodes require moisture for locomotion; therefore, their survival and active life depends on environmental moisture content (Decraemer and Hunt 2013). Nematode bodies are cylindrical, typically come to a tip at the anterior end and are covered by an exoskeleton called the cuticle (Reece et al. 2011). Terrestrial nematodes are the main type found throughout agricultural systems. This group of nematodes tend to be more prolific in sandy soils due to larger pore sizes of the substrate, making it a suitable environment for nematode locomotion and reproduction (Decraemer and Hunt 2013). Estimates show that as many as 3,000,000,000 nematodes may be present in every acre of agricultural soil (Decraemer and Hunt 2013).

Nematodes can be further divided into free-living, such as rhabditids, and parasitic nematodes. While most nematodes are beneficial, contributing to the breakdown of soil organic matter, parasitic nematodes can be devastating to agricultural production systems (Smiley 2015). Parasitic nematodes can be subsequently divided into plant-parasitic, such as *Pratylenchus*, and animal-parasitic nematodes, such as strongylida (Blaxter et al. 1998). Originally, parasitic nematodes were thought to be the more common form, with fewer species of free-living nematodes being found (Filipjev 1934). However, this was largely due to the

focus of nematode research being on important parasitic nematodes, while little was being done in the way of identifying and characterizing the numerous free-living species (Filipjev 1934). It is now theorized that parasitic nematodes developed from their more common free-living counterpart and that this likely occurred several times from different free-living nematodes. However, the origin and closest free-living relatives of many parasitic nematodes are still unknown (Blaxter et al. 1998).

1.2.1 Nematode life cycle

The basic nematode life cycle is common to all forms of nematodes. There are a few variations and extended life cycles, but typically nematode species hatch from eggs then develop through a series of four larval stages before advancing to adult male and female nematodes (Decraemer and Hunt 2013; Stasiuk et al. 2012). The presence of male and female nematodes results in mainly bisexual reproduction (Wallace 1963). Stage one juveniles emerge from their shells and moult to advance to stage two, three and four juveniles. The life cycle of parasitic nematode species includes a juvenile stage that is better adapted to long-term survival against harsh conditions such as cold and desiccation (Decraemer and Hunt 2013). This stage, referred to as the infective stage juvenile, is often where the transition from free-living to living inside a host organism occurs. (Stasiuk et al. 2012). The infective stage larva has a hardened cuticle for protection against environmental stress. Rhoades and Linford (1961) showed that some stage four larva individuals of *Paratylenchus projectus* were able to survive at soil moisture of 2.5% and temperature as low as - 19°C when all other larva stages were no longer

present in the samples. This is likely to be the infective stage larva and is the most numerous in field sites and aged laboratory samples (Rhoades and Linford 1961). Infective stage larva are typically non-feeding and therefore, must be capable of surviving on internal food stores alone (Decraemer and Hunt 2013). Throughout the nematode lifecycle, there are two structures exposed to the environment: the egg shells and the cuticle.

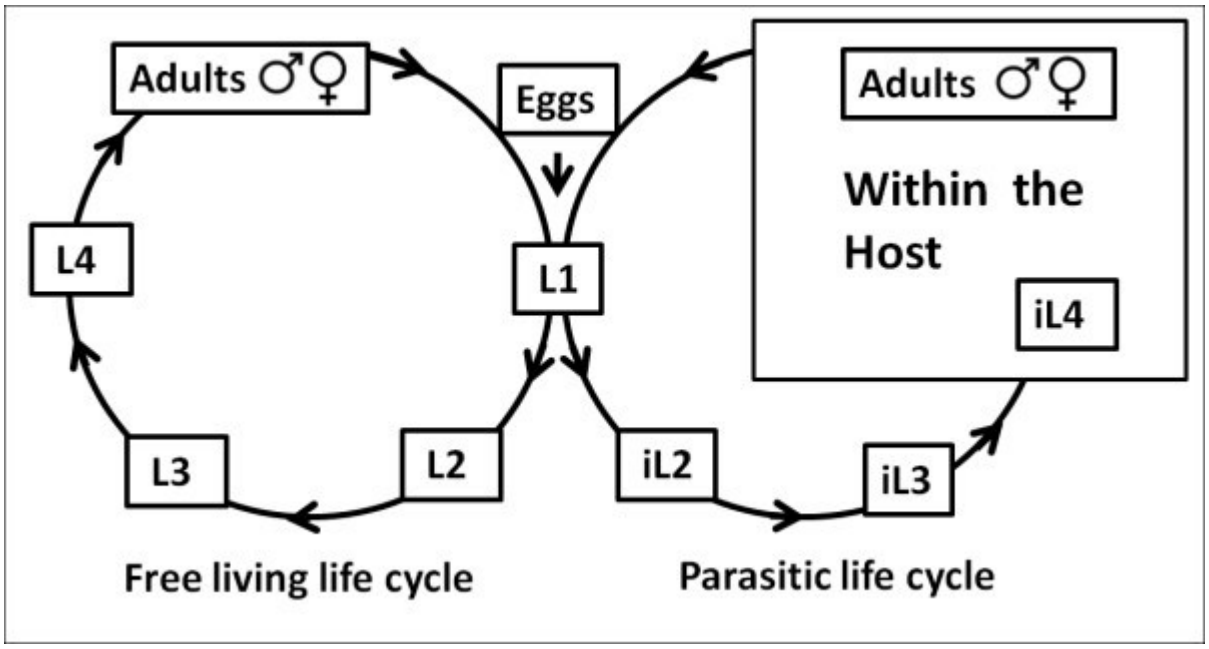

Figure 1-1: Life cycle of *Parastrongyloides trichosuri*, an intestinal nematode species which may develop into either short lived free-living nematodes or into infective larvae (iL3) of parasitic nematodes (Stasiuk et al. 2012).

1.2.2 Nematode egg shells

Egg shells represent one of two structures throughout the nematode life cycle which are directly exposed to the environment. Nematodes may lay their eggs individually or secrete them in groups. Alternatively, the female nematode body may develop into a hardened protective cyst with the eggs inside (Decraemer and Hunt 2013). Nematode egg shells have been said to be one of the strongest biologically produced structures (Wharton 1980). Shells of nematode eggs typically have three layers; an outer lipoprotein vitelline layer, a chitinous layer that provides shape and structure, and the inner lipid layer that controls the permeability of the egg (Decraemer and Hunt 2013; Wharton 1980). In a 1967 study on the egg shells of the cyst nematode *Heterodera rostochiensis*, their egg shells were separated from the cyst wall then further separated from any larva. These shells were then subjected to a series of chemical tests which determined that the primary component was amino acids. The study also found that there was chitin present in the egg shells but not within the larva. The shells also contained lipids, carbohydrates and ash (Clarke et al. 1967). The presence of chitin in the shell was also reported in a review by Wharton (1980). Chitin is produced by chitin synthase, which is controlled through the activation of one gene in nematodes. When this gene is interfered with, reduced larva hatching occurs (Fanelli et al. 2005). The presence of chitin in egg shells is significant, as it creates a vulnerability to egg breakdown via chitinase enzymes produced by microbes, fungi and plants (Sahebani and Hadavi 2008; Sharp 2013).

1.2.3 Nematode cuticle

Nematode bodies are covered in an exoskeleton known as the cuticle, which is present in all larva and in fully developed adults (Reece et al. 2011). After hating from the egg, it is the cuticle that is in contact with the nematodes external environment. The cuticle is shed periodically to allow for nematode growth (Reece et al. 2011). It is secreted by the epidermis and consists of four layers: the epicuticle which is a glycoprotein coat on the outer surface, a cortical zone, a median zone and a basal zone (Decraemer and Hunt 2013). The cuticle has a range of functions including maintaining body form, locomotion, growth, maintaining osmotic pressure and protection from environmental stressors and pathogens (Davies and Curtis 2011). Due to the surface coat of the cuticle being in contact with the environment, it is a critical part of the nematode immune system, primarily through preventing attachment of pathogens (Blaxter et al. 1992). The epicuticle is of even greater importance in parasitic nematodes because it is a dynamic surface capable of adapting to allow the nematode to adhere to host tissues and to avoid detection by the host immune response (Davies and Curtis 2011). For this to be possible, the surface coat of parasitic nematodes must be capable of rapid transformations. In the infective stage larva, it is presumed that in order to make the transition from the free-living environment to the host body, the surface coat must be changed completely to allow survival in the host (Blaxter et al. 1992). The adaptability of the cuticle is key to the success of parasitic nematodes.

1.2.4 Plant parasitic nematodes in agriculture

Parasitic nematodes are a major concern in agriculture, causing losses in both plant and animal agricultural systems. Losses are the result of the feeding habits of parasitic nematodes. Nematode losses in agricultural crops are commonly thought to be substantial. In 1965, the United States Department of Agriculture estimated crop losses due to nematodes to be $388 million (USDA 1965). However, estimates of global crop and economic losses are difficult to predict because there are many limiting factors contributing to crop yield loss (Turner and Subbotin 2013; Wallace 1963); although, estimates of smaller production regions have been made. Root parasitic nematodes are particularly devastating because as they attack the plant's root system, they limit total nutrient uptake, resulting in the suppression of yield potential (Karssen et al. 2013). Yield losses due to cyst nematodes have been documented in a variety of crops, such as potatoes. For example, in Europe, losses due to potato cyst nematodes are estimated to be around nine percent, while some estimates of losses due to the cereal cyst nematode, *Heterodera averae*, are over 90% in heavily nematode-infested wheat crops (Turner and Subbotin 2013).

As of 2013, 4000 plant parasitic nematodes had been identified (Decraemer and Hunt 2013). Plant nematodes may be ectoparasitic, feeding on the exterior of the plant, or endoparasitic, entering the host plant tissue for all or part of the life cycle (Wallace 1963). This thesis will deal with the root lesion nematodes (RLN), which are considered the number one parasite of the Maritime potato industry

(Kimpinski 1979) and are also considered the most economically important nematode in the American corn-belt (Decraemer and Hunt 2013).

1.2.5 Root lesion nematode

Root lesion nematodes (*Pratylenchus*) are migratory endoparasites and the third most damaging plant parasitic nematode behind root knot and root cyst nematodes. There are more than 70 identified species of *Pratylenchus*. They are considered medium sized nematodes with body lengths typically being less than 0.9mm (Duncan and Moens 2013). They are distributed throughout the world in all environments and soil types and are thought to have the largest host range of any plant parasitic nematode including fruit crops, potato, vegetables, cereals, forage crops, coffee and soybean. (Duncan and Moens 2013). In a study by Kimpinski (1979), root lesion nematodes were determined to be the most common plant-parasitic nematode in both potato roots and soil on Prince Edward Island, Canada. They also found large population of root lesion nematodes in forage legumes and grasses grown in the recommended potato crop rotation (Kimpinski 1979; Townshend and Potter 1976). The root lesion nematodes are the most common form of parasitic nematode associated with forage legumes in Nova Scotia (Willis et al. 1971).

Root lesion nematodes typically reproduce through parthenogenesis, a form of asexual reproduction, although sexual reproduction has been observed in some species on occasion (Duncan and Moens 2013). Eggs are typically laid within the root tissue between cortical cells and on the root surface. Females typically lay one to two eggs per day (Zunke 1990). Multiplication of root nematodes is slow

relative to stem and leaf nematodes. Therefore, to cause significant reductions in crop yield, nematodes must already be present in the soil at harmful densities prior to the production year (Schomaker and Been 2013).Root lesion nematode lifespan is 22-46 days on clover roots but can last as long as 12 weeks in cooler temperate climates (Norton 1978).

Root lesion nematodes penetrate the roots from the surrounding soil by placing lips on the root surface and thrusting through the outer cell wall. It is typical for multiple nematodes to enter through one hole in the root wall. Once inside, nematodes alternate periods of feeding, migration and rest (Zunke 1990). Root lesion nematodes are able to move freely through the root tissue and into the soil throughout the life cycle (Wallace 1963). As a result, all juvenile and adult stages of root lesion nematodes can be observed both in plant roots and in the soil environment (Duncan and Moens 2013). Nematode feeding can be for elongated or brief periods. Cells may survive brief feeding periods; however, after an elongated feeding period, plant cell death is the typical outcome and surrounding cells may also experience cell shrinkage (Zunke 1990). Nematode feeding results in necrotic lesions within the root tissue. These lesions often become infected with a secondary fungal or microbial infection (de la Peña et al. 2008). The feeding habits of root lesion nematodes result in root loss and disease leading to a reduction in overall plant health and yield. In the American Corn Belt, heavily infected fields are thought to experience one tonne per hector in yield losses (Duncan and Moens 2013). This parasite is also a major problem in potatoes, because not only is there a reduction in plant yield, but also a reduction in quality

due to potato surface scabbing caused by the nematode feeding (Duncan and Moens 2013; Kimpinski 1979).

1.2.5.1 Current mitigation techniques

Strategies currently available to mitigate threats from plant parasitic nematodes are breeding for resistance, soil cultivation and, most commonly, the use of nematicides and crop rotation. Any one of these strategies alone is unlikely to eliminate the nematode population, thus diverse strategies must be developed (Norton 1978). The level of success of any mitigation strategies is dependent on the target nematode species.

Techniques specific to managing RLN are limited. Common techniques included crop rotation and nematicides. Due to the diversity of root lesion nematodes and their ability to infect multiple hosts, crop rotation is difficult and often ineffective (Marks and Townshend 1973). Nematicides have proven too costly and can have harsh environmental effects (Duncan and Moens 2013; Holden-Dye and Walker 2014; Kerry 1990; Kimpinski 1979; Kimpinski et al. 1999). Environmental concerns are centred around the risk for contamination of groundwater, soils and food products (Kimpinski et al. 1999), as well as the threat to both non-target nematode species and safety of human applicators (Stirling 1991). In spite of the drawbacks to both techniques, these are still the only strategies recommended in root lesion mitigation associated with potato crops by both the Ontario and New Brunswick provincial governments (Government of New Brunswick 2017; Government of Ontario 2009). Due to the shortcomings of the current parasitic nematode mitigation strategies, research is required to continue

developing new alternative mitigation strategies that consider both their effectiveness and sustainability for the future of agriculture. Through the following section, potential avenues for natural alternatives using the plant's system and bio-stimulant applications will be explored.

1.2.5.2 Breeding for root lesion nematode resistance

Breeding for resistance to root knot nematodes has proven successful in the past (Hedin et al. 1984). However, due to differences in the host-parasite relationship, the same success has not been enjoyed in efforts to breed for resistance to root lesion nematodes (Christie and Townshend 1992; Kimpinski et al. 1999). Previous studies have identified potential for resistance or tolerance to root lesion nematode infections (Kimpinski et al. 1999; Papadopoulos et al. 2003; Papadopoulos et al. 2002; Potter et al. 1984). In a study of peach root stock by Potter et al. (1984), it was determined that different genetic groups seemed to be more able to tolerate infection of root lesion nematodes. They determined that two varieties of peaches, Tzim Pee Tao and Rutgers Red Leaf, had reduced response to nematode invasion. The progeny of these varieties also showed a favourable response to nematode invasions. This is interpreted as potential to improve tolerance through plant breeding (Potter et al. 1984), a potential that was further supported by work with forage crops (Christie and Townshend 1992; Kimpinski et al. 1999; Papadopoulos et al. 2002).

Resistance to root lesion nematodes has been found in alfalfa and resistant germplasms are available (Christie and Townshend 1992). Red clover is thought to be a preferred host of nematodes (Townshend and Potter 1976). In a one year

study of 18 red clover cultivars, no cultivar was determined to be immune to root lesion nematodes. However, there were some breeding lines that displayed partial resistance to invasion of root lesion nematodes (Papadopoulos et al. 2002). In a similar study of red clover, variations between cultivars were again observed (Papadopoulos et al. 2003). However, there were discrepancies between the two studies. For example, in 2002, AC Christie was found to be susceptible to root lesion (Papadopoulos et al. 2002) nematodes; while in 2003, the same cultivar displayed partial resistance in both the greenhouse and field trials (Papadopoulos et al. 2003). Inoculation rates from the two studies varied by only 0.5 nematodes per gram of soil; therefore, inoculation rate is unlikely to be the reason for variations in nematode invasion. The variation may be partly explained by genetic differences between the two groups of root lesion nematodes, demonstrating the challenges of breeding resistance to this pest. The work of Kimpinski et al. (1999) found variations in the incidence of root lesion nematode invasion in birdsfoot trefoil, a forage legume; however, they also found these results to be inconsistent from year to year, possibly due to the genetic diversity of both the trefoil and the nematodes themselves.

1.3 Isoflavone

There is a possible alternative to chemical control of nematodes within the plant defence response. One of the groups of compounds produced by plants through the defence response is isoflavones. These are a group of diphenolic plant secondary metabolites, derived from flavonoids as a result of the movement of an aromatic ring from C-2 to C-3 (Baber 2013). Secondary metabolites are those which are non-essential to the plant basic survival (Weston and Mathesius 2013). Plants capable of producing these compounds are predominantly from the subfamily Papilionideae of the Fabaceae family, which includes common forage legumes such as red clover, alfalfa and birdsfoot trefoil (Bucar 2013). They are synthesised throughout the plant in the phenylpropanoid pathway (Bucar 2013; Du et al. 2010). Distribution of isoflavones within the plant is uneven and changes throughout the plant life cycle. However, greater concentrations seem to be located in the root tips of plants (Bucar 2013). Isoflavones were originally thought of as plant waste products stored in a vacuole until disposal; however, it has become more evident in recent years that isoflavones have a diverse range of functions including UV protection, pollinator attraction, establishment of symbioses with nitrogen fixing bacteria, and a role in antimicrobial defence (Dixon and Pasinetti 2010; Du et al. 2010; Ohri and Pannu 2010; Rasmussen 2008).

1.3.1 Effects of isoflavones on nematodes

Plant isoflavone content seems to play a role in resistance to nematode invasion (Cesco et al. 2012; Cook et al. 1995; Ohri and Pannu 2010; Valette et al. 1998). Production of enzymes in the phenylpropanoid pathway is induced by the invasion of nematodes (Edwards et al. 1995; Klink et al. 2009; Wuyts et al. 2006). Through this pathway, isoflavonoids are produced. Legumes have been shown to accumulate isoflavones in response to nematode infections, with the greatest buildup of these compounds occurring in the roots (Cook et al. 1995; Edwards et al. 1995; Valette et al. 1998) In a study by Wuyts et al. (2006), phenylpropanoids in general were shown to reduce the hatch rate of plant parasitic nematodes, while daidzein, an isoflavone produced in the phenylpropanoid pathway, acted as both a hatch rate inhibitor and a nematode repellent. Resistant plants have been shown to have greater concentrations of mRNA required for the enzymes of isoflavonoid phytoalexins synthesis, which are known to have a role in resistance against nematodes (Ohri and Pannu 2010). Resistant plants also have elevated concentrations of isoflavones and activity levels of phenylalanine ammonialyase (Edwards et al. 1995). This agrees with the finding of Klink et al. (2009) in soybeans. In a study by Baldridge et al. (1998), they determined that the root tissues of alfalfa with resistance to the root lesion nematode, *Pratylenchus penetrans*, had a higher content of the isoflavone derivative medicarpin, which hindered the nematodes motility and reduced the scope of damage (Baldridge et al. 1998). Due to these effects, breeding forages for increased isoflavone content could give plants increased nematode resistance, an alternative to chemical

nematicides. At the same time, it should be noted that in a study by Cook et al. (1995), when white clover was infected with a stem nematode, resistant and susceptible plants yielded the same concentrations and varieties of isoflavones, suggesting that resistance was being obtained through other mechanisms.

1.4 Tannins

Tannins are another group of naturally produced plant compounds that have a negative effect on nematodes. They are water-soluble polyphenols that are able to form a precipitate with proteins; however, not all tannins react with all proteins (Niezen et al. 1998a; Van Soest 1994). The ability of tannins to precipitate protein is capable of preventing frothy bloat, commonly associated with the grazing of legumes by ruminants (Broadhurst and Jones 1978; Chiquette et al. 1988). Therefore, increasing the tannin content became a selection goal of many legume breeders. However, it became evident that elevated tannin content decreases the palatability of forage, leading to reduced animal intake (Broadhurst and Jones 1978), meaning, tannin content must be balanced to achieve optimal results (Hoste et al. 2006).

Tannins traditionally have been used in leather making processes due to their antibacterial and antifungal properties (Seigler 2012). These antimicrobial properties allow tannins to function as a form of protection for plants from herbivores, fungi, bacteria and nematodes (Lewis and Yamamoto 1989; Ohri and Pannu 2010). Tannins are produced throughout the plant and have a wide range of functions and structures but can be classified into two general categories: hydrolyzable and condensed tannins (Hoste et al. 2006; Seigler 2012; Van Soest

1994). Hydrolysable tannins are derived in the shikimate pathway and deter large herbivores and insects. Condensed tannins are derived from flavonoid precursors and are thought to defend against microbes (Seigler 2012). Tannins are also capable of reacting with enzymes to deactivate them; however, the enzymes of many herbivores have adapted to this (Van Soest 1994; Wink and Schimmer 2010).

1.4.1 Effects of tannins on nematodes

Aside from preventing bloat, tannins have displayed some nematicide and anthelmintic properties (Athanasiadou et al. 2001; Molan et al. 1999; Molan et al. 2003; Novobilský et al. 2013; Ohri and Pannu 2010). There are many studies on the effects of tannins on nematodes within animal production systems (Athanasiadou et al. 2001; Marley et al. 2003; Molan et al. 2003). In a review by Hoste et al. (2006), it was noted that the majority of studies conducted on the effect of ruminants consuming tannin rich forages resulted in reduced fecal egg counts, commonly attributed to a reduced nematode reproduction rate. This could be occurring through direct effects on the nematode or through a host reaction to the tannins (Athanasiadou et al. 2001; Marley et al. 2003; Molan et al. 2003; Niezen et al. 1995). This positive host reaction could be a result of tannins increasing the protein available to the animal in the small intestine. The enhanced protein availability would enhance the host immune responses, including response to nematode parasites (Athanasiadou et al. 2001; Marley et al. 2003; Molan et al. 1999). It has also been suggested that changes to the pasture stand's microenvironment due to the addition of legumes, which commonly have elevated

tannin content and are typically more leafy than grasses, could also play a role in reduction of infective stage larva (Hoste et al. 2006; Marley et al. 2006; Molan et al. 1999; Niezen et al. 1998b). These environmental changes could include moisture content, temperature and sunlight exposure; all of which are important factors in larval development (Zajac 2006). In a 2006 study by Marley et al., it was found that 31% fewer gastrointestinal nematode larvae were able to successfully develop on birdsfoot trefoil than rye grass. Considering the antiparasitic properties of tannins and the elevated levels of tannins in birdsfoot trefoil relative to rye grass, it is possible that these tannins contributed to the decrease in larva development (Marley et al. 2003).

While work with tannins on animal parasitic nematodes is more common, there has also been some documentation of tannins having an effect on plant parasitic nematodes (Cesco et al. 2012; Chen et al. 1997; Chitwood 2002; Collingborn et al. 2000). In a study of banana response to the burrowing nematode *Radopholus similis,* it was determined that condensed tannins are produced in response to nematode infection and that resistant cultivars contain elevated levels of condensed tannins before and after nematode infection (Collingborn et al. 2000). A study by Chen et al. (1997) found that low concentrations of tannic acid (39mg/L) resulted in increased root knot nematode hatch rates while high concentrations (10 000mg/L) inhibited nematode hatch rates though it was unclear if the stage two juveniles were killed within the eggs. Tannic acid may be effective at mitigating soil nematodes; however, soil amendments can also result in phototoxic effects to the plants (König et al. 1994; Mian and Rodriguez-Kabana 1982).

The mechanism through which tannins interfere with nematodes is not yet clear. One theory is that the ability of tannins to bind to proteins and change the shape of the protein could play a role in their defence against nematodes. As the nematode cuticle is protein rich, it is possible that upon contact of the nematode with tannins, the integrity of the cuticle could be compromised (Athanasiadou et al. 2001; Hoste et al. 2006). It is also possible that when nematode larvae consume tannins, the tannins bind to the mucosa of the nematode digestive track and cause cells to die in a manner similar to the effects on insect larva (Athanasiadou et al. 2001). Tannins could also bind with the nematode internal and external enzymes and denature them as enzymes are made of protein. The addition of tannin rich plant by-products to the soil has also been shown to reduce plant parasite incidence in squash, although there also appears to be some toxic effects to the plant as reduced plant weights were observed at high concentrations of tannic acid (Mian and Rodriguez-Kabana 1982). In contrast, a study on cotton for resistance to root knot nematodes found that there was no connection between condensed tannin content and resistance. Instead, they propose that resistance was being mediated by terpenoids, an additional compound produced by plants (Hedin et al. 1984).

The effectiveness of tannins on nematodes has been shown to vary depending on both the target nematode parasite and the chemical properties of the different types of tannins present. This has been shown in animal parasitic nematodes by Niezen et al. (1998b). The study showed that different species of nematodes respond to tannins in the diet of lambs. They found that lambs on a diet

which included condensed tannins had fewer female *Ostertagia circumcincta* but that *Trichostrongylus colubriformis* populations were unaffected.

In addition to variation in nematode susceptibility to tannins, chemical structures of tannins play a role in their ability to inhibit nematode development (Hoste et al. 2006). This is supported by de Oliveira et al. (2011), who found that all tested tanniniferous plant extracts were able to cause similar inhibition of *Haemonchus contortus* despite varying amounts of both total phenolics and tannins. Molen et al. (2003) found that the prodelphinidin to procyanidin ratios of tannin extracts affected the inhibition of nematodes. Legume extracts from *Lotus pendunculatus* and sainfoin, composed primarily of prodelphinidin tannins, were more active against *Trichostrongylus colubriformis*, an intestinal nematode, than plants containing mostly procyanidin tannins (Molan et al. 2003). Prodelphinidin tannins contain either gallocatechin or epigallocatechin, while procyanidin tannins contain either catechin or epicatechin (Molan et al. 2003).

1.5 Bio-stimulants

Bio-stimulants could be another alternative to the chemical control of nematodes. They are a group of compounds that, when applied to a plant in small quantities, cause a response in crops that enhances plant growth and development which cannot be attributed to traditional plant nutrients. Bio-stimulants cause plant responses by enhancing the activity of various physiological processes of the plant (Sharma et al. 2014). These compounds can be derived from various sources including macroalgae and the exoskeleton of arthropods. Macroalgae bio-stimulants have been shown to influence plant respiration, photosynthesis, nucleic

acid synthesis, and ion uptake resulting in enhanced nutrient availability, water-holding capacity and metabolism, as well as increased antioxidant and chlorophyll production (Sharma et al. 2014). Among macroalgae bio-stimulants, those derived from brown algae are the most common, notably extracts of *Ascophyllum nodosum* and will be explained in section 1.5.1, while products created from the chitin in exoskeletons of arthropods will be discussed in section 1.5.2.

1.5.1 *Ascophyllum nodosum*

The term seaweed refers to multicellular species of algae. Among these, the largest and most complex are the brown algae (Reece et al. 2011). *Ascophyllum nodosum,* or rockweed, is a species of brown algae. *A. nodosum* is found on the rocky shores of the north Atlantic and has a dominating presence on the coasts of Nova Scotia and New Brunswick (Ugarte et al. 2009). In Atlantic Canada, *A. nodosum* is the most important economic contributor of the seaweed industry due to the demand for fertilizers and animal feed derived from it (Ugarte et al. 2009). *A. nodosum* is known for forming symbiotic relationships with the fungi *Mycosphaerella ascophylli* (Craigie 2011; Xu et al. 2008). As a result, fertilisers developed from *A. nodosum* have interesting features due to the components of the fungi, such as the presence of chitin, which can contribute to the benefits of the product to agriculture.

1.5.1.1 *Ascophyllum nodosum* extract (ANE)

Ascophyllum nodosum extract (ANE) is the most commonly produced and studied of macroalgae extracts (Abetz and Young 1983; Craigie 2011; Verkleij 1992). A worldwide review of seaweed extracts by Sharma et al. (2014), found that of 47 commercially available macroalgae extracts, 28 were produced from *A. nodosum.* This included products produced in Canada.

A. nodosum can be harvested either by hand or machine. Ten to 15 centimetres must be left unharvested to allow for regrowth and rest periods of four to six years are recommended in sustainable production (Sharma et al. 2014). Once harvested, seaweed must then be converted to the extract form. There are a number of extraction methouds such as water extraction, acid and alkaline processing cryo-processing, enzyme extraction, fermentation and cell rupture with high pressure treatment (Goñi et al. 2016). The most common extraction process involves heating an aqueous suspension of milled macroalgae with a potassium carbonate solution in pressurized reaction vessels (Craigie 2011; Sharma et al. 2014; Verkleij 1992). The extract produced contains carbohydrates, proteins, lipids, minerals, hormones and other organic compounds (Abetz and Young 1983; Sharma et al. 2014). Extracts can be applied during seed priming, directly to established plants or to the soil prior to planting (Sharma et al. 2014; Verkleij 1992).

1.5.1.2 ANE as a bio-stimulant

ANE has a range of effects on plants that include: improved germination, yield, root development, mineral absorption, tolerance to biotic and abiotic stress, enhanced shoot growth and photosynthesis (Blunden et al. 1979; Craigie 2011; Paracer et al. 1987; Sharma et al. 2014; Verkleij 1992). These effects combined lead to an increase in profit at the farm level; however, as ideal growth conditions are approached, the benefits are decreased (Craigie 2011). These effects are putatively caused by a number of active components within the extract including alginates, carbohydrates, hormones, growth regulators and signalling molecules, such as chitin (Blunden et al. 1979; Sharma et al. 2014). The hormone fraction of seaweed extracts, particularly cytokinins, has been identified as one of the active components of the extract having a role in increasing yields following application (Abetz and Young 1983; Hanssen et al. 1987; Verkleij 1992). The fungal symbiot of *A. nodosum* gives ANE some unique properties and benefits that are not present in other seaweed extracts. This is supported by Hannssen et al. (1987) who compared ANE to extracts produced from *Laminaria*, an alternative type of brown algae with no symbiont. Even so, it was found the ANE is more effective at increasing the yield of lettuce plants through foliar applications than the *Laminaria* alternative (Hanssen et al. 1987).

1.5.1.3 Effects of ANE on nematodes

ANE has been shown to have direct effects on nematodes (Morgan and Tarjan 1980; Verkleij 1992; Whapham et al. 1994). When plants are treated with ANE, the number of *Meloidogyne javanica*, a root knot nematode, present is significantly reduced and can be further reduced when nematode eggs are exposed to a seaweed extract solution during the hatching process (Radwan et al. 2012; Whapham et al. 1994; Wu et al. 1998). This is supported by the work of Morgan and Tarjan (1980), who found a significant reduction in sting nematode populations after soil treatments with ANE. After this, plants contain elevated levels of phenolic components, flavonoids and antioxidants, which are known deterrents of nematodes in spinach (Craigie 2011). This suggests that the ANE application stimulated the plant's internal defence system (Cramer et al. 1993; Morgan and Tarjan 1980; Wu et al. 1998).

There are several active compounds that have been credited with a role in ANE induced nematode resistance in plants including betaines, auxins and oligo-chitin (Ali et al. 2016; Khan et al. 2009; Wu et al. 1998). It has shown that betaines stop nematode development, in a similar way to many antihelminthics or de-wormers (Peden et al. 2013). Also, the use of oligo-chitin in agriculture has been studied in depth and its effects on nematodes have been well documented (Sharp 2013).

1.5.2 Chitin

Chitin is a known component of ANE thought to originate from the cell walls of the *Mycosphaerella ascophylli,* the fungi found within *Ascophyllum nodosum.* It is the second most abundant polysaccharide on the planet, behind only cellulose (Sharp 2013). Chitin is a structural polysaccharide common to fungal cell walls and the exoskeleton of arthropods (Reece et al. 2011). Similar in structure to cellulose, except, chitin has an amino group attachment on carbon two of the glucose monomer. Pure chitin is leathery and flexible but becomes rigid when combined with calcium carbonate (Reece et al. 2011). As outlined earlier, chitin is also a component of nematode eggs and gut linings (Cronin et al. 1997; Sharp 2013; Spiegel et al. 1986; Veech 1977).

1.5.2.1 Chitin as a bio-stimulant

Chitin can be used as a bio-stimulant. It acts as a signalling molecule for plants through receptors in the cell membrane. These receptors signal to the host plant that a fungal infection is approaching. This is done through the binding of chitin to a plasma membrane receptor, typically a protein with high affinity for it. Using this mechanism, the plant begins to prepare a series of defence responses (Kaku et al. 2006). Chitin can affect many of the physiological pathways of the plant, such as: inducing early and increased flowering, reducing transpiration, increasing yield and improving nutritional quality (Mendoza-Sánchez et al. 2016; Sharp 2013). In spite of the many benefits of chitin treatment, treatment above the optimal chitin level can have detrimental effects on the plants, such as reduced yield, tissue death or organism death (Mian et al. 1982; Sharp 2013; Tian et al.

2000). In a study by Godoy et al. (1983), chitin treatments of 0.4% and above reduced root galling as a result of the root knot nematode *Meloidogyne arenaria*; however, treatments above 0.8%, resulted in phytotoxicity.

1.5.2.2 Effects of chitin on nematodes

Chitin application can have a positive effect on a plants ability to withstand or combat parasitic nematodes. The application of chitin directly to plant tissues signals the plant to activate the internal defence mechanisms. These defence mechanisms include the production of chitinase (Cramer et al. 1993), which breaks down nematode eggs and causes lesions within the gut of plant parasitic nematodes (Gan et al. 2007; Sahebani and Hadavi 2008; Sharp 2013). When eggs of the nematode *Meloidogyne hapla* were incubated with chitinases isolated from *Lecanicillium psalliotae* fungi, hatch rates of the nematode eggs were reduced (Mian et al. 1982). Chitinases are produced in both the apoplast and symplast of the plant (Sharp 2013). Not all chitinase are the same as they are produced for different functions (Li et al. 2006). Therefore, some chitinases may be more effective on different nematode species, as demonstrated by the nematode species *Tylenchorhynchus dubius* being more susceptible to degradation by commercial chitinase *in vitro* than *Pratylenchus penetrans* (Miller and Sands 1977). Chitin also stimulates the production of known nematode deterrents, such as phenolic compounds and isoflavones (Khan et al. 2003). In a study where tomato plants were treated with chitosan, a chitin derivative, it was shown to reduce egg hatching, live larvae and nematode parameters of root knot nematodes in the soil and plant. It also enhances tomato growth parameters and

increased the activity of the antioxidant enzymes peroxidase and phenoloxidase (El-Sayed and Mahdy 2015).

The addition of chitin to soil has effects on the soil environment, in part due to the increase in nitrogen availability. In a 1996 study where the soil was amended to have one percent chitin, ryegrass had elevated yields and decreased root growth, while clover decreased nodulation, evidence of the elevated nitrogen content in chitin amended soil (Sarathchandra et al. 1996). This agrees with the previous studies of Mian et al. (1982) and Miller (1976). Addition of chitin to soils is able to reduce total soil parasitic nematode counts and damage to crops by nematodes (Godoy et al. 1983; Kerry 1990; Mian et al. 1982); however, the mode of action is not entirely clear.

One theory is that the nematicidal effect could be due to growth promotion of chitinolytic microbes and fungi which then feed on nematode eggs (Cronin et al. 1997; Gan et al. 2007; Godoy et al. 1983; Kerry 1990; Mian et al. 1982; Sahebani and Hadavi 2008; Sarathchandra et al. 1996; Sharp 2013). In a 1999 study, chitin amendments of one percent (w/w) were able to reduce damage from *Meloidogyne incognita* in the first planting of cotton and positive effects were carried over to a second cotton planting. This was primarily due to an increase in population size of chitinolytic fungi and bacteria compared to populations in non-amended soils (Hallmann et al. 1999). A second theory is that breakdown of chitin in the soil produces ammonia, which at a high level, would have nematicidal effects (Godoy et al. 1983; Kerry 1990; Rodriguez-Kabana et al. 1987; Sharp 2013; Spiegel et al. 1987). In a study by Tian et al. (2000), there was no consistent effect of chitin additions below one percent (w/w) on the reduction of hatch rate in the absence of

chitinolytic microbes. However, after the addition of chitinolytic microbes, Tian et al. were able to isolate five bacteria isolates capable of consistently reducing *Heterodera glycines,*(Tian et al. 2000). Rodriguez-Kabana et al. (1987) also found that chitinolytic microbes reduced *Meloidogyne arenaria* in tomatoes when soil was amended with chitin supplementation below one percent.

1.6 Conclusion

Parasitic nematodes are a large source of agricultural losses. Root lesion nematodes, a crop parasite, are responsible for substantial losses annually, and are considered the most common parasite of the maritime potato industry. Current mitigation recommendations of the combination of crop rotation and nematicides for the treatment of root lesion nematodes are currently unable to adequately combat this growing pest problem. Alternatives could be investigated through plant isoflavones and tannins. Both have been identified as components of the plant defence response to nematodes. These compounds exist naturally at varying levels in all plants. Additionally, bio-stimulants, such as ANE and chitin, have proven to be effective in the mitigation of nematodes through the stimulation of the plant defence system. In this study, we will evaluate the effectiveness of forage with elevated levels of isoflavones and different tannin profiles, coupled with the application of ANE and chitin as alternatives to nematicides in the control of root lesion nematodes in forage legumes.

1.6.1 Objectives

1. Compare incidence of root lesion nematodes in two birdsfoot trefoil and two red clover varieties.

2. Assess the effects of treatment with oligo-chitin and ANE on root lesion nematode infection in red clover and birdsfoot trefoil.

Chapter 2: Materials and methods

2.1 Experimental site description

All experiments were carried out at the Kentville Research and Development Centre, Agriculture and Agri-Food Canada, Kentville, Nova Scotia, Canada (lat. 45.1 Long. −64.5).

2.1.1 Growth chamber description

Growth chambers used in this study were large walk-in chambers with 120” x 120” of floor space, allowing for ten rows of six trays. Trays contained ten plants of the same cultivar and treatment group, each in their own individual four-inch pot (Figure 2-1). Trays were placed directly on the floor of the growth chamber. The chambers were maintained at a photoperiod of 16 hours of daylight (425 µmol m^{-2} s^{-1}) at 21 ± 2ºC and eight hours of dark at 16 ± 2ºC.

Figure 2-1: Replicates three and four in one half of a walk-in growth chamber at the Kentville Research and Development Centre, Agriculture Agri-foods Canada.

2.2 Soil

Soil with a high root lesion nematode, *Pratylenchus penetrans* (Cobb) Filip & Schur. Stek., population of 19/g dry soil was obtained from a soybean field at the Harrington Research Farm, Agriculture and Agri-Food Canada, Crops and Livestock Research Centre, Charlottetown (lat. 46.2 long. −63.1). The soil at this site is a Charlottetown fine sandy loam (70% sand, 20% silt, 10 % clay, 2.7% organic matter; pH 5.9). It was determined to be free of both root knot and clover cyst nematodes to prevent suppression of root lesion nematodes. The soil was collected in December of 2015 using a round mouth shovel to remove only the top layer of soil. It was placed into plastic shipping containers then transported by truck to Kentville Research and Development Centre, Agriculture and Agri-Food

Canada, Kentville, Nova Scotia, Canada. Soil has been stored in the plastic shipping containers at environmental temperatures and approximately 60% soil moisture until being used in August 2016.

2.3 Plant material

Two species of legume from the family Fabaceae were used, birdsfoot trefoil (*Lotus corniculatus L.)* and red clover *(Trifolium pratense L.)*. Two varieties of each species were used for a total of four forage varieties. Two experimental varieties of red clover were used; TRC12-156 (Papadopoulos, unpublished data) and TRC12-157 (Papadopoulos, unpublished data), respectively selected for high or low above ground plant tissues isoflavones. Two commercially available birdsfoot trefoil cultivars, AC Langille (Papadopoulos et al. 1997) and Leo (Bubar 1964), were used.

2.4 Plant growth protocol

Experiment was carried out in four sequential replicates and included plant growth at three stages and in three types of environmental conditions across a period of 5 months (August 2016 – January 2017).

2.4.1 Germination

Seeds of the four varieties were allowed to germinate for one week in separate Petri dishes. Seeds of both cultivars of birdsfoot trefoil had been pretreated with bacterial inoculum, while seeds of TRC 12-157 were scarified and scraped, using a nail file to improve poor germination rates. Scarifying was not required for the TRC12-156. Seeds were then placed on filter papers moistened

with water and kept damp but not submerged for the entire germination process. At the end of germination, seedlings possessed green cotyledons which are the first embryonic leaves (Figure 2-2).

Figure 2-2: Red clover seedlings with green cotyledons prior to transplant into rootainers of soil infected with RLN.

2.4.2 Establishment

Emerged seedlings were then transplanted into rootainers containing approximately 110mL of the infected soil and 15mL of the designated treatment (Figure 2-3). Treatments are described in section 2.5. Transplanting was completed by forming a shallow hole in the surface of the soil placing the young root of the seedling in the hole using forceps. Soil was gently packed around the root, ensuring the cotyledons remained above the soil surface. This occurred on August 9, 2016 (Reps 1 & 2) and August 16, 2016 (Reps 3 & 4). Plants were

allowed to establish and grow for six weeks in the rootainers on a greenhouse bench.

Figure 2-3: Plants TRC 12-156 treated with chitin and ANE establishing on a greenhouse bench in rootainers filled with RLN infected soil.

2.4.3 Transplanting

Six-week-old seedlings were removed from the rootainers, roots were gently loosed by hand and placed in standard four-inch round pots (Figure 2-4). Pots were then filled with the infected PEI soil to approximately 0.5” from the top, leaving room for watering. All root materials were placed below the soil surface while the green plant material was left above the soil surface. The soil was lightly packed and the pots were then transferred in trays of ten pots per tray to the growth chamber. This occurred on September 12, 2016 (reps 1 & 2) and September 19, 2016 (reps 3 & 4).

Figure 2-4: Transplanting of six-week-old red clover seedling to four inch pot to be filled with soil infected with RLN.

2.5 Treatments

This experiment included two treatment groups and one control; all treatments were administered as a liquid root drench.

2.5.1 Treatment preparation

2.5.1.1 Chitin

Treatment one was oligo-chitin prepared from powdered oligo-chitin. This powder was obtained from Dr. Yuguang Du of the Institute of Process Engineering, Chinese Academy of Sciences, Beijing, China. The chitin was extracted from lobster shells in the form of oligo-chitin. The chain length of oligo-chitin is 2-10 N-acetylglucosamine monomers with an average of four polymerizations and the powder is no less than 70% oligo-chitin (Wang 2016). Chitin powder was diluted

with distilled water directly from powder to a concentration of 0.36g/L oligo-chitin solution (Y. Du [Institute of Process Engineering, Chinese Academy of Science. Beijing], personal communication) to be used in the root drench.

2.5.1.2 Ascophyllum nodosum extract

Treatment two using ANE treatment was prepared from Acadian® 100% liquid seaweed concentrates, this is a commercial seaweed extract available from Acadian Seaplants Ltd., and is guaranteed to contain 0.1% total nitrogen and 5% total potash. Pure ANE extract was diluted with distilled water directly to 3g/L (Alghamdi 2017; Wang 2016). Vigorous mixing was required to completely dissolve the viscous ANE into the solution.

2.5.2 Treatment calibration

The volume of soil per individual rootainer was determined to be approximately 110 mL. Using trial and error the volume of water required to reach approximate field capacity was determined. Known volumes of water were poured over 110 mL of lightly packed soil in a sealed bottom container and allowed to percolate through the soil. Once the water had moved through the soil in its entirety water content was estimated. It was determined that 15 mL of liquid is required to reach field water capacity.

2.5.3 Treatment application

Treatments were applied directly to the soil in rootainers prior to transfer of established seedlings from petri dish. Fifteen millilitres of treatment was applied slowly to the top of the rootainers using a syringe in three intervals to allow time for the treatment to seep into the soil without overflowing the top of the rootainers

(Figure 2-5). Treatment was applied no more than 12 hours ahead of transplanting one week old seedlings.

Figure 2-5: Example of rootainer soil treatment drench of water control with syringe.

2.6 Harvest I

Each replicate was harvested on separate days to allow adequate time for nematode enumeration (Section 2.8) and the Christmas vacation of lab staff. The first harvest of rep one was completed on November 7, 2016 and harvest of replications were completed on November 14, 2016 (Rep 2), December 5, 2016 (Rep 3) and December 13, 2016 (Rep 4).

2.6.2 Vigour score

Vigour scores were used to assess the health of the above ground plant matter. Plants were scored on a scale of one to ten, one being dead and ten being very vigorous (Figures 2-6 - 2-8). Criteria taken into consideration were height, number of leaves and shoots, colour, bloom stage and signs of disease such as wilting and leaf spots.

Figure 2-6: A) Example of vigour score of nine and B) example of vigour score of eight for red clover plants at the first harvest.

Figure 2-7: Example of vigour score of A) seven, B) six and C) five of red clover plants at the first harvest.

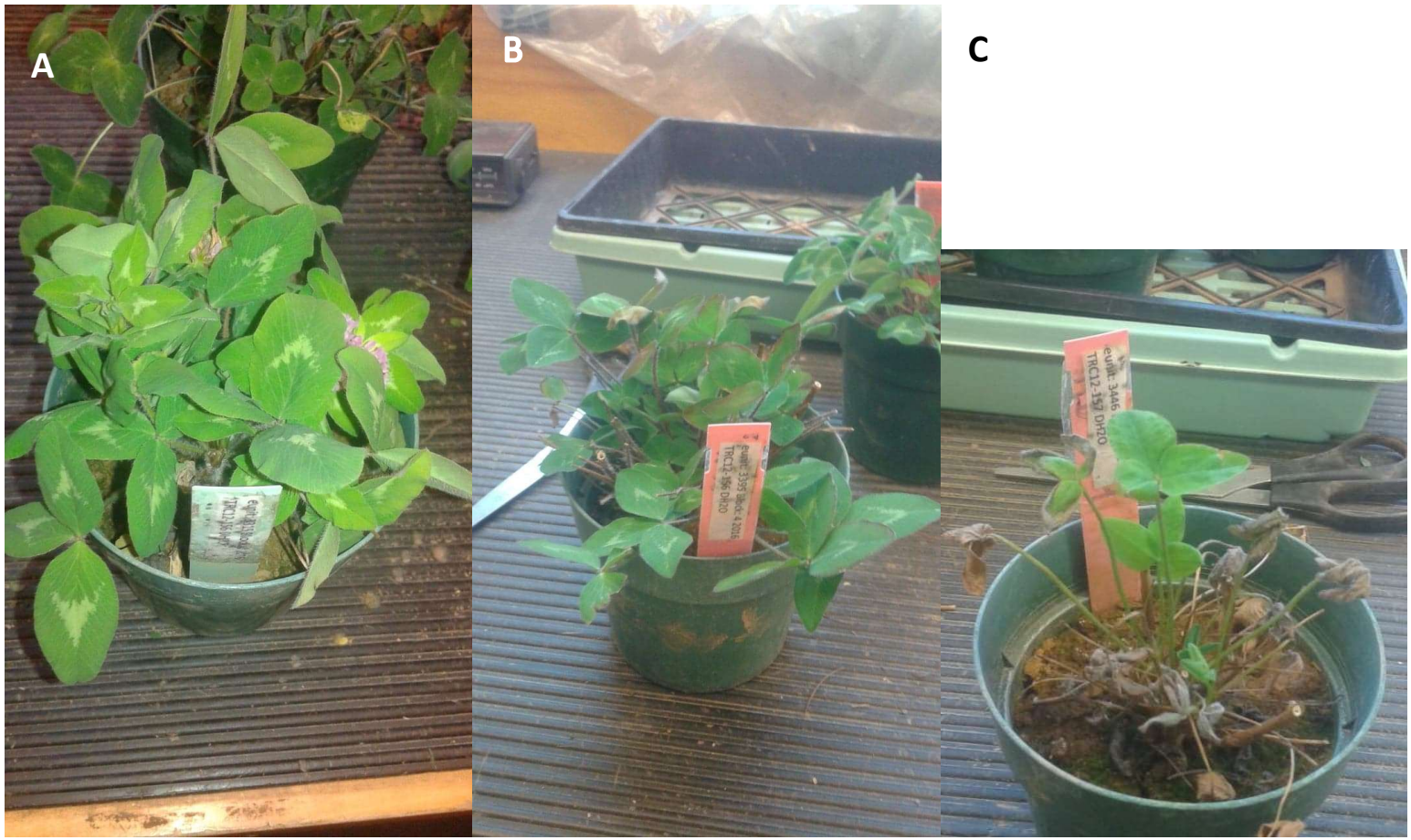

Figure 2-8: Example of vigour score of A) four, B) three and C) two of red clover plants at the second harvest.

2.6.1 Yield

Plants were then trimmed with scissors to five centimetres in height, while root material was left in pots to regrow for the second harvest date described in section 2.7. Harvested shoot material from replicates one and two were placed in individual paper bags and dried in a large drying oven at 70°C for at least one week. The mass of shoot material was taken immediately following removal from the oven (Figure 2-9A). Shoots from replications three and four were placed in whirl packs and plant material was freeze dried in a small Edwards freeze drier (Figure 2-9B). Freeze drying was performed to prevent denaturing of plant phytochemicals for analysis at a later date. After freeze drying, bags were sealed and placed in a minus 80°C freezer and the mass was taken at a later date.

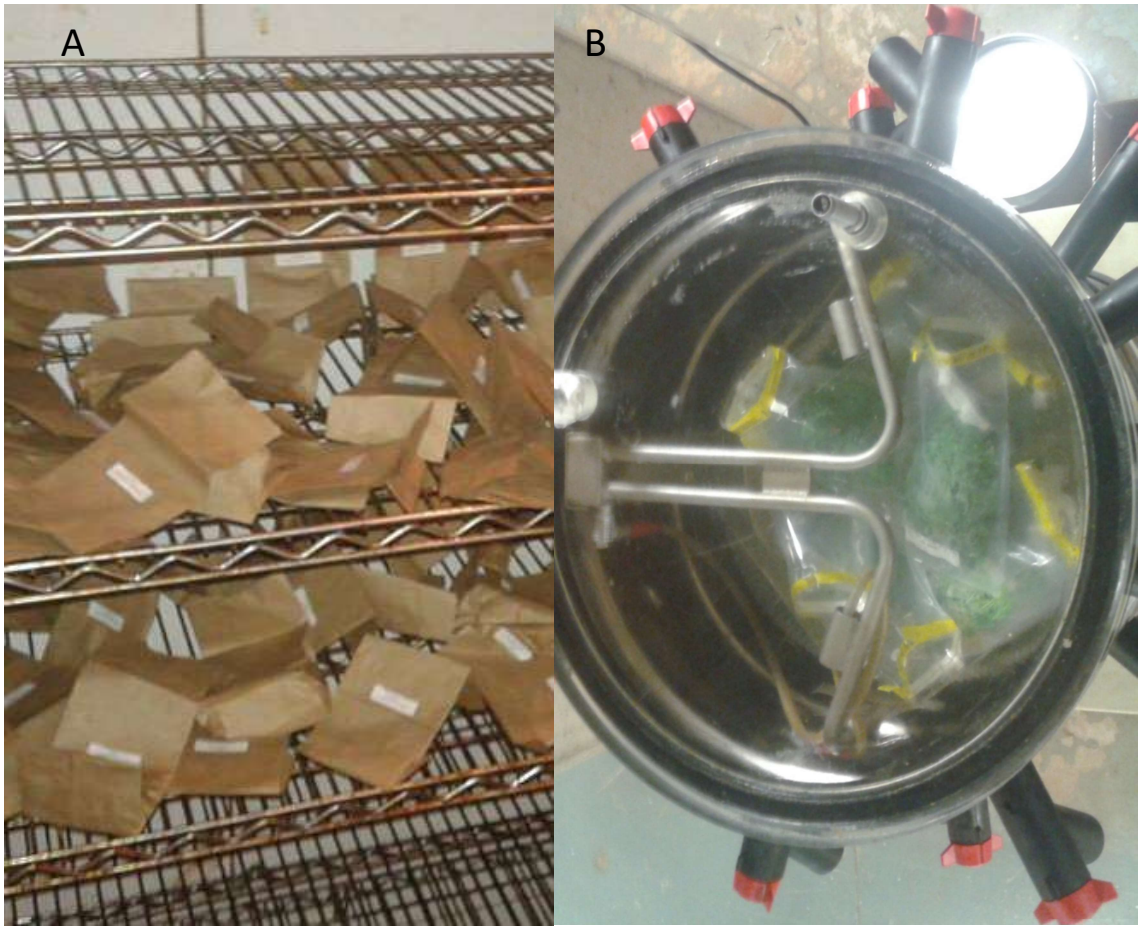

Figure 2-9: A) First cut forage legume samples in paper bags in the drying oven and B) first cut forage legumes samples in whirl packs in Edwards freeze drier at the Kentville Research and Development Centre, Agriculture Agri-foods Canada.

2.7 Harvest II

Final harvest began on December 5, 2016 (Rep 1), and repeated starting on December 13, 2016 (Rep 2), January 2, 2017 (Rep 3) and January 9, 2017 (Rep 4).

2.7.1 Plant materials

Vigour scores were repeated in the same manner as harvest I. Yield measurements were repeated with above ground plant material being trimmed to two centimetres. Above ground plant material from all replications was placed in paper bags, dried at 70°C in a large drying oven for at least one week and massed immediately after being removed from the oven.

2.7.2 Root material

Pots containing root material were left to dry overnight (approximately 18 hours) after the removal of shoots. The following day root samples were removed from the pot (Figure 2-10A) and soil was loosened from the roots by hand, removing as much soil as possible without losing major portions of the root system (Figure 2-10B). The remaining above ground plant material was then removed with scissors leaving only soil and roots. The roots were then inserted into a Gillison's Variety Fabrication root washer for seven minutes (Figure 2-11). Using water pressure, the root washer creates a whirlpool, which gently removes the dirt from roots while causing minimal damage to the root system (Figure 2-10C). When roots were removed from the washer, they were checked and any remaining soil was removed by hand with a water hose. Excess water was then removed from the root with the combination of air drying and patting dry with paper towels. Roots were then packaged individually in plastic bags and placed on ice for shipment to Prince Edward Island Potato Quality Institute, Charlottetown, Prince Edward Island, Canada.

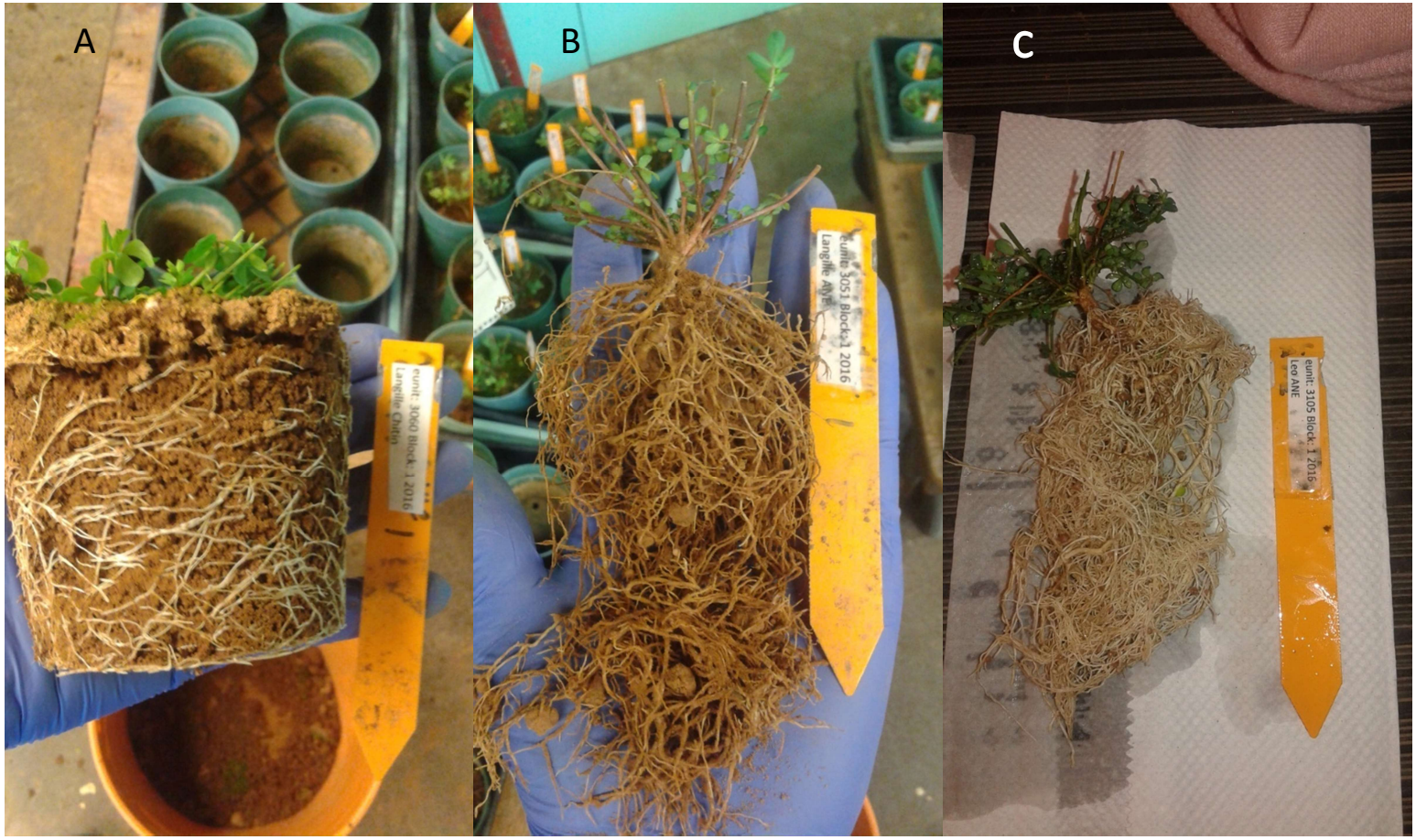

Figure 2-10: Birdsfoot trefoil root sample A) immediately after coming out of the pot, B) after hand removal of dirt and C) after seven minutes in the root washer.

Figure 2-11: Gillison's Variety Fabrication root washer at the Kentville Research and Development Centre Agriculture Agri-foods Canada.

2.8 Nematode extraction and enumeration

Nematode extractions were carried out at the Prince Edward Island Potato Quality Institute Charlottetown, Prince Edward Island, Canada, by Claude Gallant. Nematodes were extracted from clean root material using the modified Baermann funnel method (Kimpinski 1993). Roots were trimmed from the primary tap root then cut laterally into smaller pieces. Root systems were then placed on screens with 80mL of water. Pans containing screens, roots and water were then stacked in sets of 20 and placed in plastic bags to incubate for seven days. Incubation temperature ranged from 21-24°C. After a seven-day incubation period, extraction suspensions were collected into 100mL test tubes and root material was dried and weighed. Suspensions were allowed to settle, with nematode fractions settling to the bottom. Samples were then reduced in volume to 20mL. This was done by syphoning water from the top of the solution. The remaining sample was then re-suspended with a vortex. A five millilitre subsample was then pipetted onto a grid-lined counting petri dish and observed with a stereomicroscope for the purpose of nematode identification and enumeration. Data was collected with a data reduction computer program.

2.9 Statistical Analysis

The experimental design was a latinized nested design. The main plots were forage varieties, and the sub-plots were bio-stimulant treatment groups, resulting in a total of 12 experimental groups. The experiment was carried out in four replicates with ten plants per group for a total of 480 plants. Data analysis was completed in GenStat®. Analysis of variance (ANOVA) was used to determine standard errors of the mean and compared at a probability level of $p < 0.05$. Before the final ANOVA, large residuals were removed to meet the conditions of normality required for the ANOVA test. A $\log_{10}$ transformation was applied to the root lesions, rhabditid and other nematode populations, again to meet the normal distributions. $\text{Log}_{10}(x+75)$, as used in Papadopoulos et al. (2003), was used for this transformation as the +75 provides a means of dealing with zeros in the data set. Seventy five was selected based on an approximation of the number of nematodes if the sample was re-read or re-extracted and resulted in the presence of a single nematode. Transformation was not required for stunt or spiral nematodes as the data was already normally distributed. Orthogonal contrasts were used to evaluate the differences between both chemicals and varieties tested. After statistical analysis, means were de-transformed from the $\log_{10}(x+75)$ scale. Cluster analysis was preformed to compare the differences between populations. Figures and tables were produced in Microsoft® Excel 2016 and SigmaPlot® 13.0.

Chapter 3: Results

The results of the ANOVA test (Sections 3.1-3.3) show that both forage variety ($p = <0.001$) and treatment ($p = 0.025$) are affecting the population densities of root lesion nematodes as well as dry matter yield. There were no significant interaction effects. ANOVA tables can be found in Appendix E.

3.1 Forage varieties

To demonstrate the main effects of forage variety on the measured parameters, means across the three treatment groups are displayed for each of the four forage varieties in figure 3-1 to 3-3. Cluster analysis (section 3.4) reviled three clusters forming based on forage variety.

3.1.1 Root lesion nematode populations

Means of root lesion nematodes extracted from plant roots on the $\log_{10}(x+75)$ scale for the four forage varieties are displayed in Figure 3-1. The RLN counts ranged from 12370 RLN/g (AC Langille) to 2088 RLN/g (TRC12-156) (Figure 3-2). The tested trefoil had significantly ($p = <0.001$) higher populations of root lesion nematodes at 11276 RLN/g root dry matter where red clover contained 65.6% fewer root lesion nematodes with an average of only 3876 RLN/g. TRC12-156, the high isoflavone red clover, had the lowest population of root lesion nematodes; 63.2% less then TRC12-157 (5666 RLN/g), the low isoflavone variety ($p = 0.001$). There were no significant differences in root lesion nematode population between the birdsfoot trefoil cultivars.

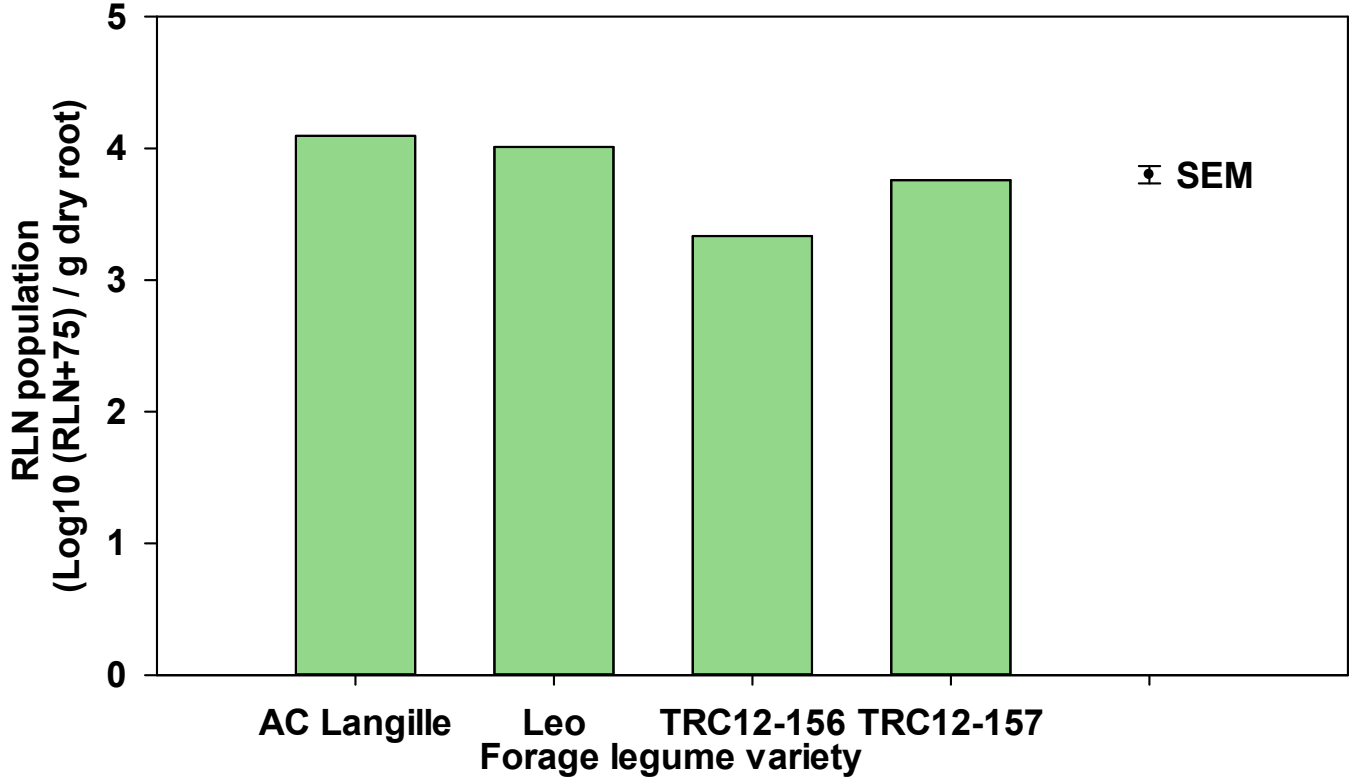

Figure 3-1: Number of root lesion nematode (RLN) per gram root dry matter of four forage legume varieties on a $Log_{10}(x+75)$ scale. Forage was grown in growth chambers and soil infected with 19 RLN per gram dry soil.

Note: SEM = two times standard error of the mean.

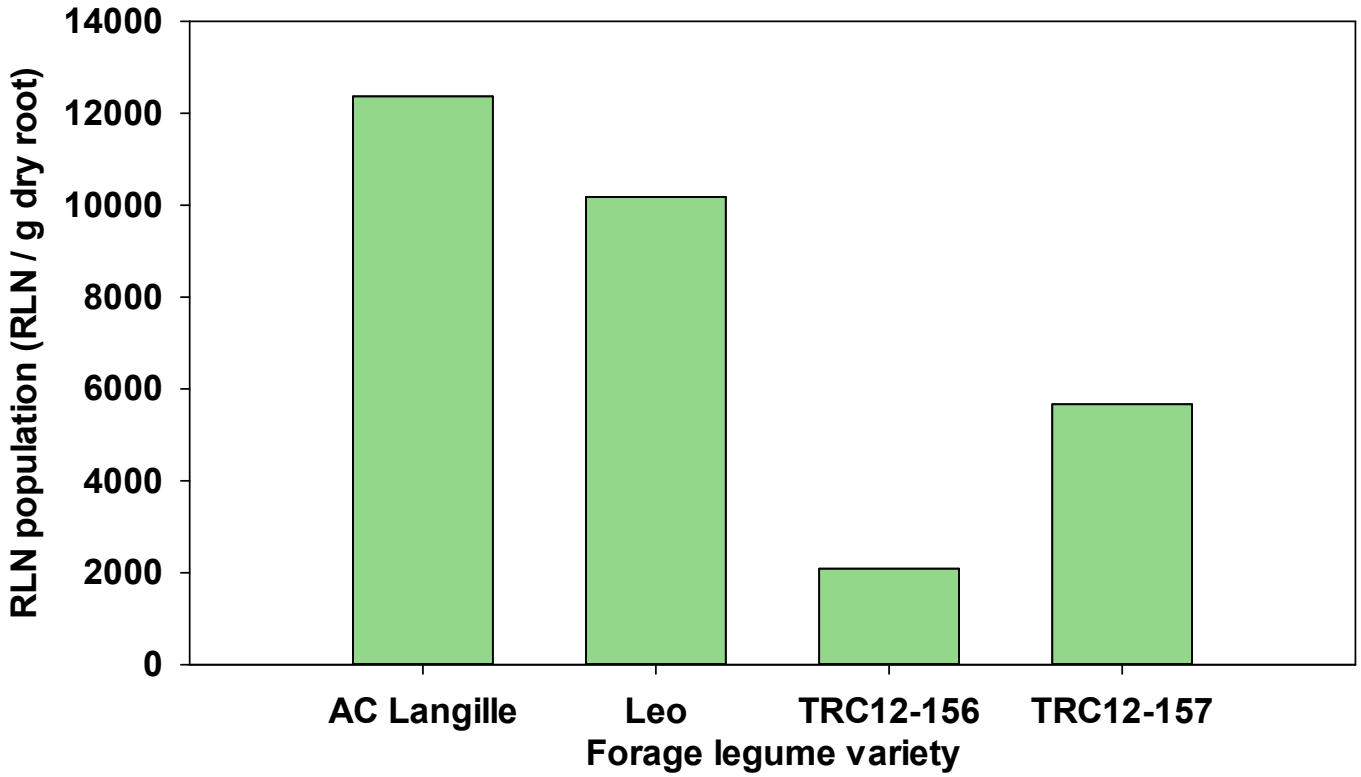

Figure 3-2: Detransformation of means from $Log_{10}(x+75)$ of the population density of root lesion nematodes (RLN) per gram root dry matter of four forage legume varieties. Legumes were grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil.

3.1.2 Regrowth dry matter yield

Forage regrowth dry matter yield from the second plant harvest can be observed in figure 3-3. Yield ranged from 1.525g (TRC12-156) to 1.026g (AC Langille). Red clover varieties had significantly ($p = 0.006$) higher regrowth yield with 1.413g, while the birdsfoot trefoil produced only 1.108g of regrowth. TRC12-156 had a greater yield than TRC12-157 (1.301g), this is not a significant difference but can still be considered a trend ($p = 0.099$)).

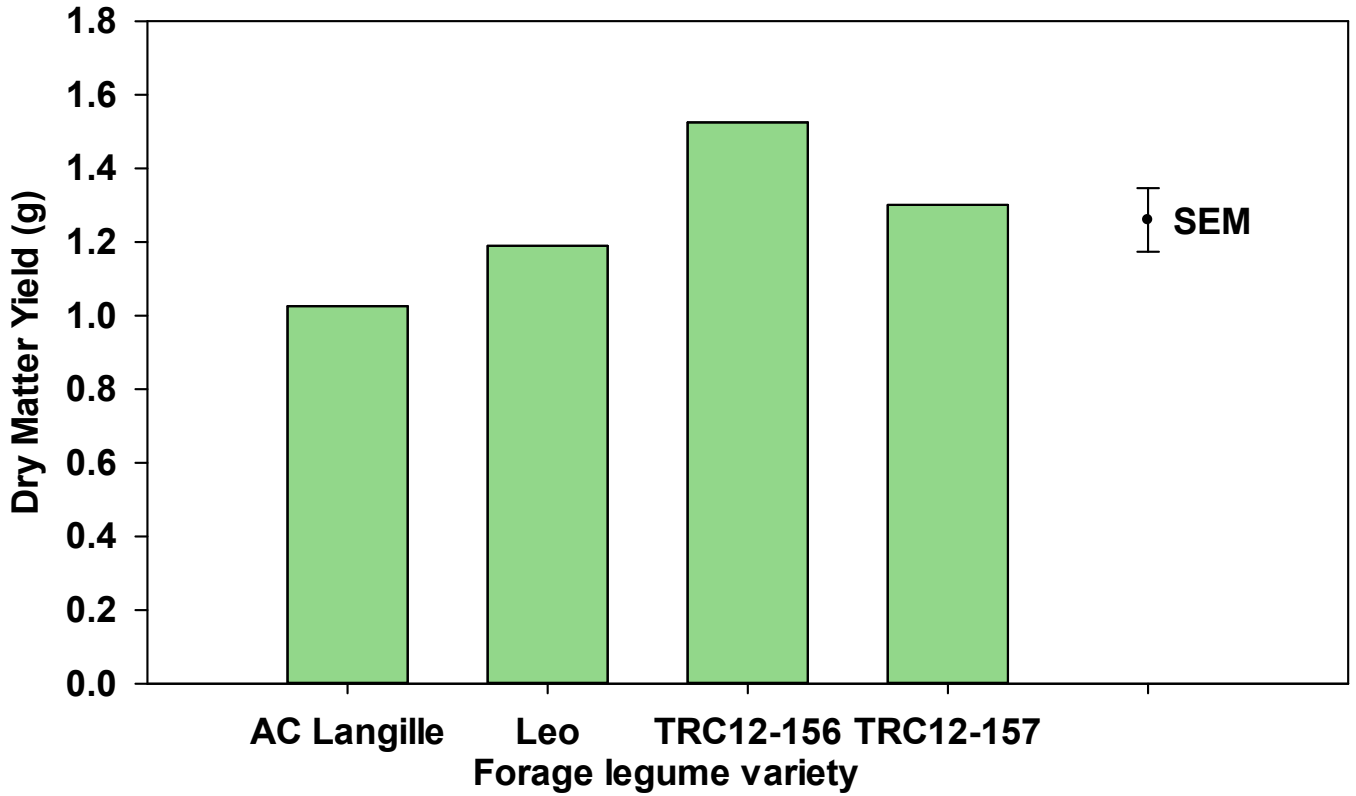

Figure 3-3: Mean dry matter yield (g) of forage regrowth from legume varieties grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

3.1.3 Other nematode populations

Rhabditis showed similar trends to the root lesion nematodes. Their population size ranged from 1198 *Rhabditis*/g root dry matter (AC Langille and Leo) to 309 *Rhabditis*/g root dry matter (TRC12-156). The red clover had significantly ($p = <0.001$) lower populations of *Rhabditis* at 337 *Rhabditis*/g root dry matter, whereas birdsfoot trefoil cultivars contained 71.9% greater populations of *Rhabditis* with 1198 *Rhabditis*/g root dry matter. Spiral and stunt nematodes were found in the samples at abundances of less than 40 per gram root dry matter. Data for all other nematodes is available in Appendix A.

3.2 Soil treatments

To demonstrate the main effects of treatment on the measured parameters, means across the four forage varieties are displayed for each of the three treatment groups in figure 3-4 to 3-6.

3.2.1. Root lesion nematode populations

The $\log_{10}(x+75)$ transformation of mean abundances of root lesion nematodes per gram of dry root in each treatment group are displayed in figure 3-4. The RLN counts ranged from 7424 RLN/g root dry matter (control) to 5185 RLN/g root dry matter (ANE) (figure 3-5). There were significant ($p = 0.02$) differences between the control and the oligo-chitin and ANE treatments. Plants treated with ANE had 30% fewer RLN/g root dry matter than the control. Oligo-chitin treatment (6278/g root dry matter) also caused a 15% reduction in root lesion nematodes. There were no significant differences between the ANE and oligo-chitin treatments.

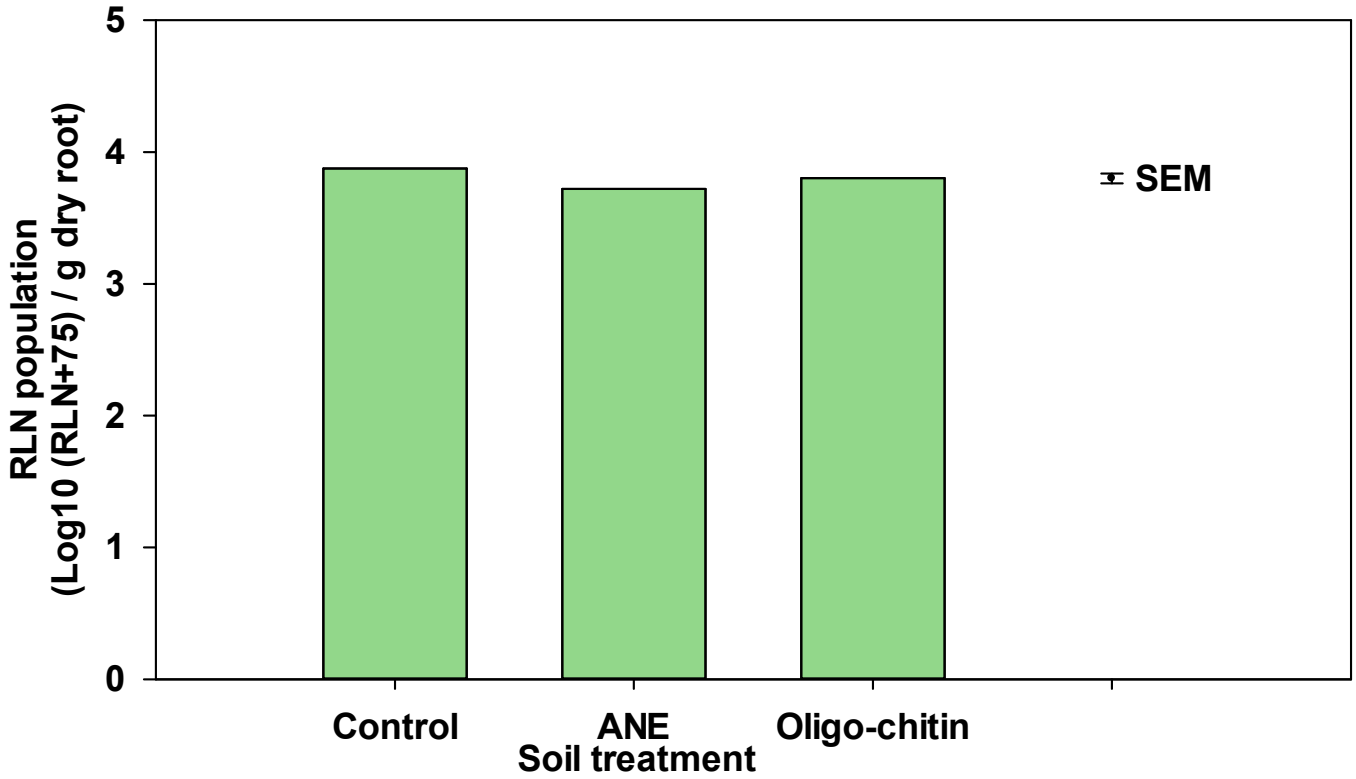

Figure 3-4: Abundance of root lesion nematodes (RLN) per gram root dry matter of three treatment groups applied to four forage legume varieties displayed on a $Log_{10}(x+75)$ scale. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

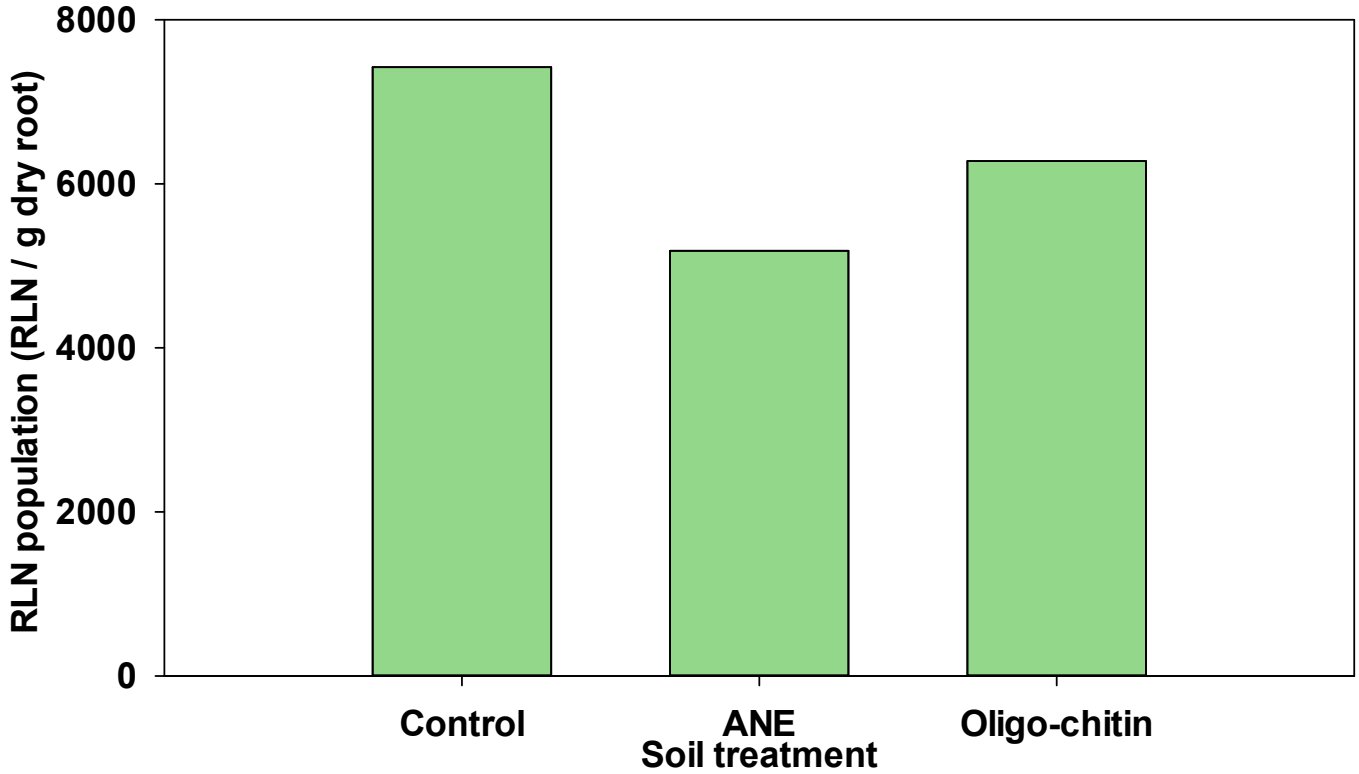

Figure 3-5: Detransformation of means from $Log_{10}(x+75)$ of the root lesion nematodes (RLN) population density per gram root dry matter of three treatment groups applied to four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil.

3.2.2 Regrowth dry matter yield

The mean dry matter yields of individual plants in each of the three treatment groups are displayed in figure 3-6. Dry matter yields ranged from 1.345g (ANE) to 1.189g (Chitin). The ANOVA revealed that there is a trend in treatments ($p = 0.093$), with ANE having a greater yield than chitin with an increase of 11.6% ($p = 0.033$). There were no significant differences between the control (1.274g) and either treatment.

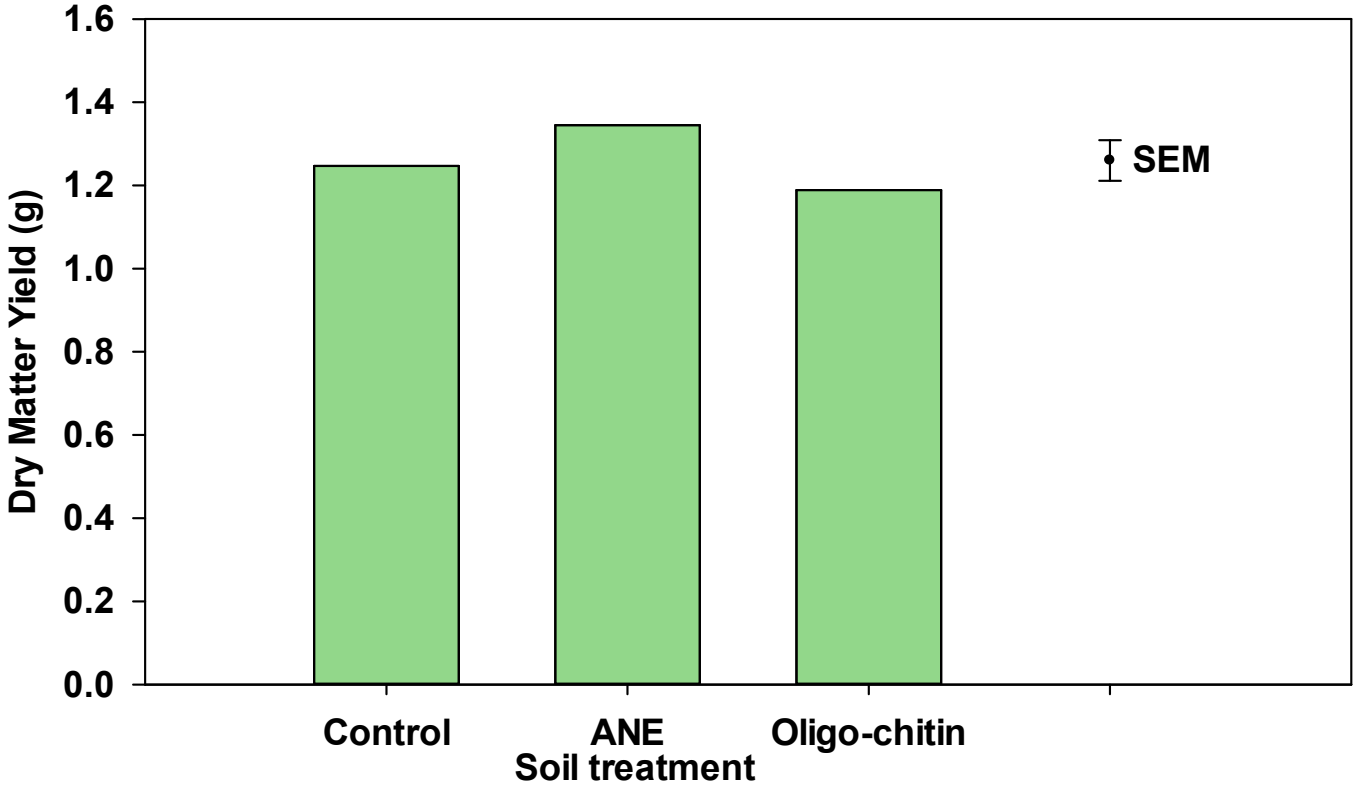

Figure 3-6: Mean dry matter yield (g) of forage regrowth of three treatment groups averaged from four legume varieties grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

3.2.3 Other nematode populations

There were no significant differences for any other nematode populations found in the samples with regards to the treatments applied. Results for these populations can be seen in Appendix A.

3.3 Interactions

There were no significant interactions between forage variety and the treatment applied in this experiment on any of the tested parameters. Forage variety by treatment interaction data can be found in Appendix C.

3.3.1. Root lesion nematode populations

The root lesion nematode abundances per gram root dry matter for each of the possible 12 forage treatment combinations are displayed in the $\log_{10}(x+75)$ scale in figure 3-7. The range in abundance was 16106 RLN/g root dry matter (AC Langille x oligo-chitin) to 1745 RLN/g root dry matter (TRC12-156 x ANE) (figure 3-8). The treatment-forage variety combination with the lowest population of root lesion nematodes occurred in TRC12-156 and ANE with 39% less RLN/g root dry matter than the TRC12-156 control (2856 RLN/g root dry matter). The second most effective combination was TRC12-156 and oligo-chitin (1822 RLN/g root dry matter), which had 36% less than the control. The TRC12-156 control was the third most effective combination. The combination with the greatest population of root lesion nematodes was AC Langille and oligo-chitin, showing a 29% increase relative to the AC Langille control (12456 RLN/g root dry matter). The AC Langille control was the second highest combination. The combination that had the third

highest number of RLN per gram was in Leo and oligo-chitin (12003 RLN/g root dry matter), with a 10% higher population than the Leo control (10915 RLN/g root dry matter).

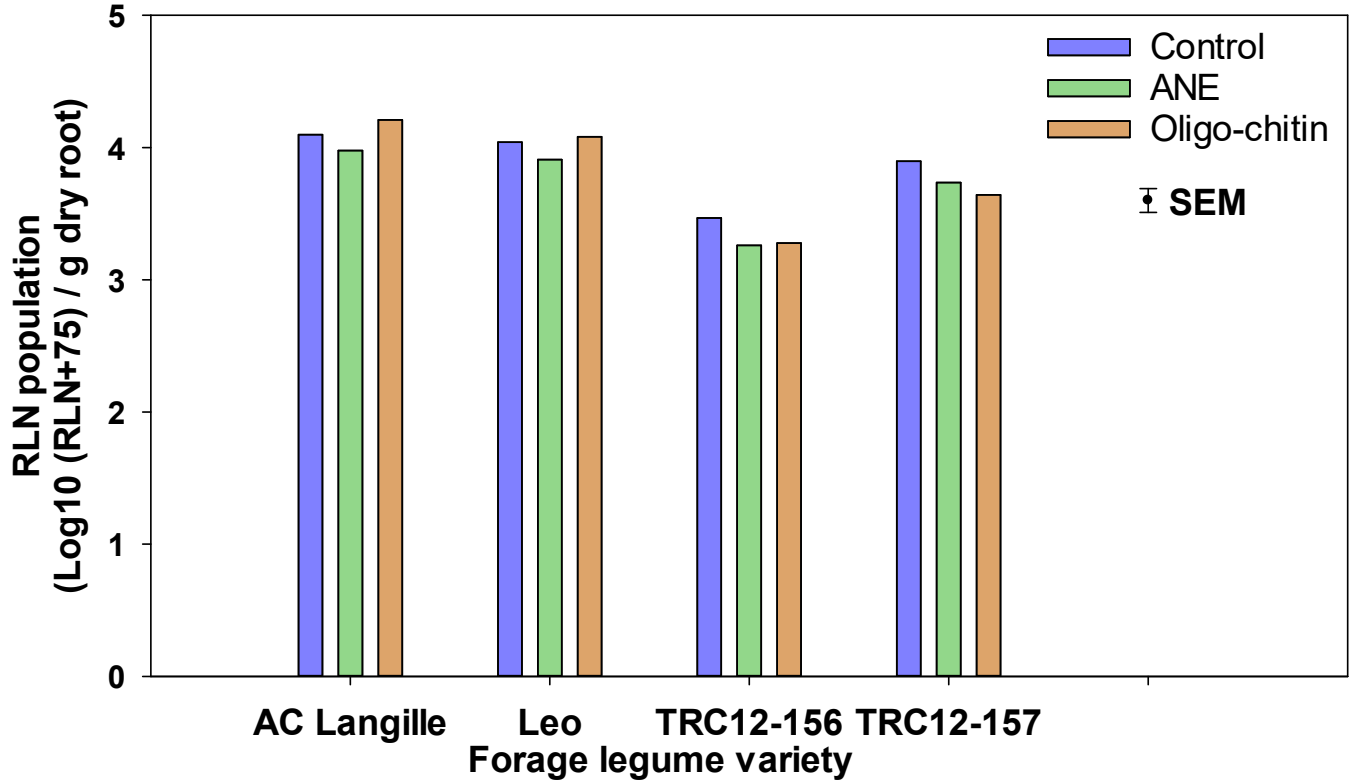

Figure 3-7: Abundance of root lesion nematodes (RLN) per gram root dry matter of three treatment groups applied to four forage legume varieties on a $Log_{10}(x+75)$ scale. Samples were grown in growth chambers in soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

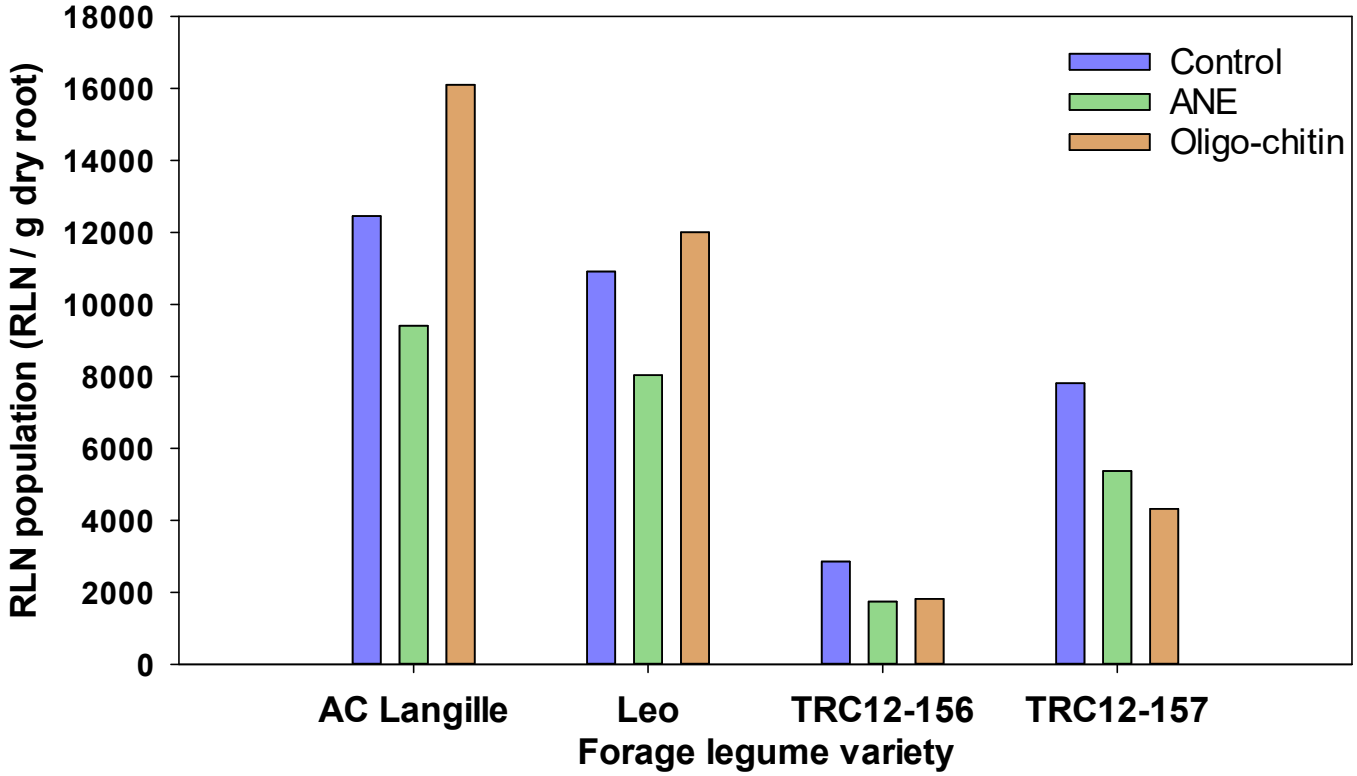

Figure 3-8: Detransformation of means from $Log_{10}(x+75)$ of the number of root lesion nematodes (RLN) per gram root dry matter of three treatment groups applied to four forage legume varieties. Samples were grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil.

3.4 Cluster analysis of root lesion nematode populations

Cluster analysis was performed on mean root lesion nematode counts of the 12 treatment forage variety combinations. The result of this analysis is displayed in a dendrogram in figure 3-18. The cluster analysis revealed three groups which are related by forage variety. The first group includes the red clover, TRC12-156 under all three treatment groups. This group is different from all other conditions, represented by a separate branch on the dendrogram. The second branching separates TRC12-157 treated with chitin and ANE from the remaining treatment conditions. The final group includes all Birdsfoot trefoil and TRC12-157 control (DH2O). The second and third group are more closely related to each other than to the first group. There are some other minor separations; however, these are not strong enough to require further divisions of the main clusters. It should be noted that there is some separation between the Birdsfoot trefoil treated with ANE and the control and chitin treatments. Cluster analysis on all other parameters are available in appendix B.

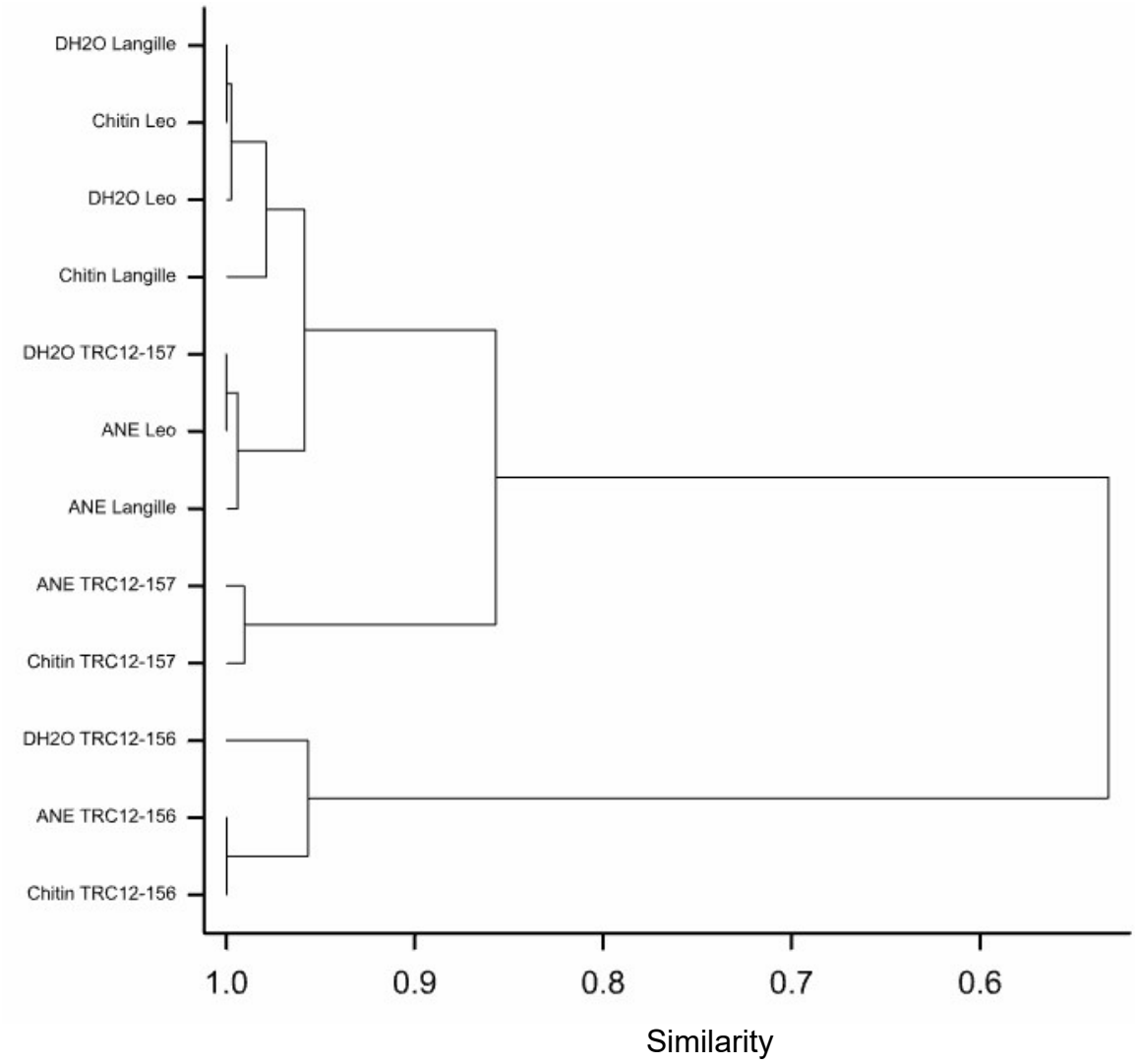

Figure 3-9: Dendrogram of the results of a cluster analysis, performed on the mean abundance of root lesion nematodes (RLN) per gram root dry matter, of three treatment groups applied to four forage legume varieties on a $Log_{10}(x+75)$ scale. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In the figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

Chapter 4: Discussion

Plant-parasitic nematodes can reduce crop yield by over 15% (Stirling 2014). The root lesion nematode is the third most damaging plant parasitic nematode globally (Duncan and Moens 2013) and the most common parasite of the maritime potato industry (Kimpinski 1979). Populations of many parasitic nematodes are also predicted to increase as a result of mild, short winters with higher rainfall improving winter survival (Kenyon et al. 2009). Mitigation strategies for these nematodes are limited and often ineffective. For example, the use of nematicides is associated with harsh environmental impacts and low efficacy (Duncan and Moens 2013). While crop rotations are typically ineffective due to the wide host range of root lesion nematodes. The objective of this research was to examine alternative mitigation strategies through plant isoflavones and tannins and the application of ANE and oligo-chitin.

The results of this study indicate that there are no interaction effects, meaning that differences in the data are explained by the main effects of forage variety and treatment applied. The lack of interaction can be interpreted as all forage varieties having the same response to the treatment application. Furthermore, the lack of difference between the oligo-chitin and ANE treatments indicates that the two treatments have the same effect. Among the cultivars examined, TRC12-156 consistently had the lowest root lesion nematode populations under all treatment conditions.

4.1 Forage varieties

Four forage varieties were tested; two red clovers, TRC12-156 and TRC12-157, as well as two birdsfoot trefoils, AC Langille and Leo. Differences were found between forage varieties in the root lesion nematode and shoot yield.

4.1.1 Root lesion nematodes

Of the four forage varieties tested in this study, the two red clover varieties contained 34% fewer root lesion nematodes per gram dry root than the birdsfoot trefoil cultivars (11276 RLN/g root dry matter). This difference keeps consistent when the research of Papadopoulos et al. (2002) and Kimpinski (1999) are compared. In a study by Papadopoulos et al. (2002), red clover varieties were tested for resistance to root lesion nematodes, resulting in nematode abundances from 750 to 9800 RLN/g root dry matter and an average population of 3200 RLN/g root dry matter. These populations are much lower than those found in a similar study of birdsfoot trefoil varieties by Kimpinski et al. (1999), where the population of root lesion nematodes ranged from 7940 to 26920 RLN/g root dry matter. There are a number of possible explanations for these differences including genetic variation in the nematode inoculum, differences in the procedure of the experiments as well as differences in the in the composition of the two species themselves.

One possible explanation for the reduced RLN counts in red clover could be a superior nematode response system relative to the trefoil. Red clover is traditionally high in phenolic compounds, including isoflavones. These concentrations are high even compared to soybean, which is commonly used in

human health supplements due to its high level of isoflavones (Kroyer 2004; Papadopoulos et al. 2006; Tsao et al. 2006). Phenolics in general, but especially isoflavones, have been linked to plant response to nematodes, leading to plant resistance (Ohri and Pannu 2010). This could enable red clover to react more efficiently and quickly to a nematode invasion. Isoflavones, in particular, have been linked to phytochemical nematode defence, which is the production of chemicals by the plant to protect it from nematodes. Isoflavones are produced in response to nematode invasion, with build-up of isoflavones occurring in roots (Cook et al. 1995; Edwards et al. 1995; Ohri and Pannu 2010; Valette et al. 1998). Nematode resistant plants in previous studies have shown to have elevated levels of the enzymes within the phenylpropanoid pathway, phenylpropanoids and their derivatives, such as isoflavones (Baldridge et al. 1998; Edwards et al. 1995; Klink et al. 2009; Ohri and Pannu 2010). Wuyts et al. (2006) found that phenylpropanoids, in general, have reduced the hatch rate of plant parasitic nematodes, while daidzein, an isoflavone produced in the phenylpropanoid pathway, acted as both a hatch rate inhibitor and a nematode repellent. A study by Tsao et al. (2006) reported the presence of isoflavones and daidzein in the above ground plant material of 13 red clover cultivars.

The association of isoflavones with superior nematode response systems is further supported by the low abundance of root lesion nematodes in TRC12-156 (2088/g root dry matter), a high isoflavone red clover variety. The two varieties of red clover used in this research were developed through a breeding program with the purpose of producing a high isoflavone line (TRC12-156) and a low isoflavone (TRC1-157) red clover. TRC12-156 had the lowest population of root lesion

nematodes (2088 RLN/g root dry matter) in the present research; 63.2% less then TRC12-157 (5666 RLN/g root dry matter). The work of Papadopoulos et al. (2006) show that breeding for isoflavone content is possible. They found that total isoflavone content among 13 red clover varieties had a broad sense heritability of 80%; daidzein specifically was 40%. Thus, indicting the potential to improve the content of both total isoflavone and daidzein content through plant breeding. However, a small gene pool related to daidzein among the tested germplasm in the study could have affected heritability found for daidzein (Papadopoulos et al. 2006).

The separation of TRC12-156 from all the other cultivars was further confirmed through cluster analysis. A cluster was identified containing all three treatment groups of TRC12-156 and was the least similar to all remaining clusters containing the both TRC12-157 and birdsfoot trefoil cultivars. The increased isoflavone content could have contributed to the superior response to nematodes of TRC12-156. The mechanisms behind this reduction in nematodes are still unclear.

The other forage species tested in the research, birdsfoot trefoil, was chosen as a test cultivar because of its relatively high tannin content. Tannins are often associated with anthelmintic effects in the diets of ruminants (Hoste et al. 2006) and have also been linked to plant resistance to plant parasitic nematodes (Chitwood 2002). In this study, the elevated tannin content does not seem to play a role in the root lesion nematode resistance, as both cultivars of birdsfoot trefoil had high numbers of root lesion nematodes per gram of root material. Furthermore, there were no differences identified between the two cultivars of birdsfoot trefoil. It

is assumed that the tannin profiles of the two tested cultivars are different. Not all tannins act on nematodes the same. Molen et al. (2003) found that a high prodelphinidin to procyanidin ratio of tannin extracts is more effective against *Trichostrongylus colubriformis.* While the tannin profiles of AC Langille and Leo are not yet defined, it is unlikely that these profiles are having an effect in this research.

4.1.2 Other nematodes

Since the soil was collected from a field, the presence of other nematode populations was expected. The primary purpose of evaluating other nematodes in the soil was to ensure that no nematodes were present in a great enough abundance to have interfered with the RLN populations in the soil. This was confirmed by all other nematodes occurring at relatively low abundances in comparison to the root lesion nematodes.

The nematodes with the second highest population densities belong to the genus *Rhabditis* in the family Rhabditidae, which include both free-living and parasitic nematodes (Norton 1978). It is worth noting that, while relatively low in total abundance in the samples, the trends observed in RLN were also observed in the *Rhabditis.* There were lower counts in the red clover (337 *Rhabditis*/g root dry matter) than the birdsfoot trefoil cultivars (1198 *Rhabditis* /g root dry matter) which had a 71.9% greater population. This could be an indication that birdsfoot trefoil is more susceptible to nematode invasion in general, not just the root lesion nematodes. Also present in the samples were stunt, sprila and other nematodes. None of these populations were present at high magnitudes and had no impact on attributes recorded in plant performance.

4.1.3 Regrowth yield

The clover varieties in this study also had higher shoot yields than birdsfoot trefoil. Root lesion infection could be playing a role in the differences in yield. Willis and Thompson (1969) found that root lesion nematodes caused yield losses in forage crops in greenhouse conditions. They also determined that birdsfoot trefoil is more susceptible to yield loss than red clover. In addition, they found that birdsfoot trefoil had a lower yield than red clover in soil containing no RLN. This suggests that the high infection of RLN in birdsfoot trefoil could have resulted in yield loses but that existing differences between the two species is likely playing a major role as well.

4.2 Treatments

Although selecting the correct forage variety could reduce root lesion nematodes by as much as 60%, the addition of treatment could offer an additional 30% reduction. Treatments were applied to act as elicitors in an attempt to amplify the natural resistance to root lesion nematodes in the forage. To amplify the natural defence response, plant elicitors can be used to induce plant stress, therefore triggering the plant defence system (Khan et al. 2003). In the present study, ANE and oligo-chitin were used as plant elicitors in an attempt to improve the plant's response to nematodes. Effects of treatment were observed in both root lesion nematode abundance and plant yield.

4.2.1 Root lesion nematodes

Overall, across the four forage varieties, both the ANE treatments and the oligo-chitin treatments successfully reduced RLN abundance. ANE had the lowest population of root lesion nematodes with 5185/g root dry matter, a 30% decrease relative to the control. The reduction in population was expected based on the reduction of other parasites through ANE treatment in previous studies. Research on the use of ANE to mitigate root lesion nematodes has been limited; however, ANE treatment has successfully reduced populations and hatch rates of *Meloidogyne javanica,* a type of root knot nematode (Radwan et al. 2012; Whapham et al. 1994; Wu et al. 1998). ANE is able to stimulate the plant's physiological pathways, including those involved in the plant defence systems, through several active ingredients such as betaines, auxins and oligo-saccharides, like oligo-chitin (Ali et al. 2016; Khan et al. 2009; Wu et al. 1998). After treatment with ANE, plants contain elevated levels of phenolic compounds, flavonoids and antioxidants, produced as a part of plant defence responses (Craigie 2011).

The fungal components of ANE derived from *Mycosphaerella ascophylli,* a symbiote within *Ascophyllum nodosum,* may be among the active ingredients in ANE. When comparing plant performance in response to treatment, *A. nodosum* produced superior performance than treatment with *Fucus vesiculosus* and *Laminaria.* Both *F. vesiculosus* and *Laminaria* are species of brown algae that lack fungal symbionts (Hanssen et al. 1987; Van de Reep 2015). One of the structural polysaccharides found in the cell wall of fungus is chitin. The ANE extraction process includes exposure to high temperatures and pH, which results in the

conversion of chitin to oligo-chitin and oligo-chitosan. Both of these chitin derivatives are capable of acting as plant elicitors though receptors on the cell membrane (Yin et al. 2016). When comparing the transcriptome profiles of soybeans twelve hours after treatment with ANE and serval oligo-saccharide components, it was found that oligo-chitin and oligo-chitosan have the capacity to partially mimic the effects of ANE. Oligo- alginate, the oligosaccharide form of alginate and a structural component of algae cell walls, did not have the capacity to achieve similar transcriptomes to ANE (Wang 2016).

The lack of significant difference between the ANE and oligo-chitin treatments in the present study further supports the findings of Wang (2016), that oligo-chitin was capable of mimicking the effects of ANE. Oligo-chitin resulted in 15% reduction in root lesion nematodes (6278/g root dry matter) across all forage varieties relative to the water control. A reduction in nematode population as a result of oligo-chitin treatment was anticipated due to evidence within the literature (Khan et al. 2003; Sharp 2013; Wang 2016). In previous studies, treatment with chitin is able to reduce nematode populations through stimulating the production of chitinase in the plant. Chitinase is an enzyme that can of breakdown the eggshells of nematodes (Sahebani and Hadavi 2008; Sharp 2013). Chitin also stimulates the production of known nematode deterrents, such as phenolic compounds and isoflavones (Khan et al. 2003).

In the cluster analysis, cluster formation seems to be more dependent on forage variety than treatment, indicating that, in this experiment, forage variety has a greater influence on the root lesion nematode population. This is to be expected as the forage variety controls provide the base susceptibility which may then be

improved by the treatments; yet, it would be unlikely that treatment application would lower nematode populations in all plant species to the same final population.

4.2.2 Regrowth Yield

Treatments had effects on the yield of forages. In this study, oligo-chitin treated plants had the lowest yield among treatment groups (1.189g). While nematode load could be contributing to the yield losses, it is unlikely the main factor as the control had higher yields (1.274g) and nematode load (7424 RLN/g root dry matter) than the oligo-chitin group (6278 RLN/g root dry matter). This could be interpreted as a possible chitin toxicity, reducing the growth rate of some plants in the experiment (Godoy et al. 1983; Sharp 2013). As a result, there was a trend observed that ANE treated plants (1.345g) had higher yields than oligo-chitin treated plants. The difference of 0.156g, while small, results in an 11.6% difference between the two treatments. This level of crop loss, plus the expenses of treatment with oligo-chitin, would be substantial to producers' profit and further highlights the need to avoid chitin concentrations that result in phytotoxic effects. The increase in plant yield as a result of ANE application can also be attributed to ANE's ability to influence the systems of the plant. Yield increases as a result of ANE application have been well-documented in a variety of crops, such as lettuce, tomatoes and barley (Craigie 2011; Hanssen et al. 1987; Khan et al. 2009).

4.3 Interaction

Although there were no significant interaction effects in this experiment, there were some points of interest in the data set that should be highlighted as they may be considered in further research.

4.3.1 Root lesion nematodes

In this study, the two plant species, red clover and birdsfoot trefoil, may have reacted differently to the treatments. Both oligo-chitin and ANE were able to reduce the root lesion nematode counts in red clover relative to water. However, this was not the case in the birdsfoot trefoil. ANE treatment still leads to a reduction in the number of root lesion nematodes, but, the oligo-chitin treatment caused increases in RLN populations relative to the water treatments. An increase in RLN per gram root dry matter of 29% was observed in the AC Langille relative to the control while a 10% increase was noted in Leo. This could be an indication that birdsfoot trefoil is more susceptible to chitin phytotoxicity. Treatment above optimal chitin levels can reduce germination, yields and cause tissue death (Sharp 2013; Tian et al. 2000). The decrease in overall plant health due to chitin phytotoxicity could have contributed to the increase in nematodes per gram root dry matter, as plants would have fewer resources available to combat the nematodes. However, in previous studies, damage attributed to cyst nematodes continued to decrease in response to increasing soil chitin amendments in spite of a decrease in the overall plant health and productivity (Tian et al. 2000). Chitin used in these experiments were crustacean chitin flakes, which could have had a slower release than chitin solution in this study.

The combination with the lowest population of root lesion nematodes is the combination of TRC12-156 and ANE (1745 RLN/g root dry matter), followed by, the combination of TRC12-156 and oligo-chitin (1822 RLN/g root dry matter). Treatment resulted in a 39% and 36% reduction in nematodes, respectively, relative to the TRC12-156 water control (2856 RLN/g root dry matter). These reductions, due to treatment, were also displayed by the low isoflavone red clover TRC12-157. The combination with the greatest population of root lesion nematodes was AC Langille and oligo-chitin (16 106 RLN/g root dry matter), followed by AC Langille untreated (12 456 RLN/g root dry matter) and Leo oligo-chitin (12 003 RLN/g root dry matter). Although these results were not significant, they do further support the trends that TRC12-156 consistently had the lowest nematode counts and the oligo-chitin had adverse effects when paired with birdsfoot trefoil.

Chapter 5: Conclusion

Root lesion nematodes continue to be a major pest in agriculture. The objectives of this research were to assess the effect of a soil drench with oligo-chitin or *Ascophyllum nodosum* extract on RLN infection and, also, to compare levels of RLN infection among varieties of birdsfoot trefoil and red clover. This research showed that, of the tested forage varieties, red clover contained lower numbers of root lesion nematodes per gram root than the tested birdsfoot trefoil cultivars. It was also found that red clover high in plant above ground isoflavones (TRC12-156) had the lowest RLN abundance of all four of the tested forage

varieties. The TRC12-156 had 63% fewer RLN per gram root dry matter compared with the low isoflavone red clover (TRC12-157).

This experiment treatment testing revealed that both ANE and oligo-chitin were able to reduce root lesion nematode populations in the forage legumes in this study. The reductions were 30% and 15%, respectively. Although, differences between the two treatments were not significant. Compiling the two results means that, in accordance with this experiment, the most effective way to reduce root lesion nematodes is to plant TRC12-156, a high isoflavone clover variety, and to treat the soil with either ANE or oligo-chitin prior to planting.

5.1 Future consideration

More research is required before the results of this study may be applied in an agricultural setting. One area of future work that should be considered is field testing. It is reasonable to expect nematode survival, populations and rate of infection to vary between the field and the greenhouse, as they have in previous work. In a study of vegetable crops in Spain, it was determined that root knot nematodes tended to have elevated survival rates in greenhouses despite lower total populations in the soil (Ornat et al. 1999). In a 1979 study by Kimpinski, the number of nematodes removed from an 11-week-old superior potato plant was greater in the greenhouse than from samples taken in the field, in spite of the fact that there were similar numbers of nematodes in the soil of both settings (Kimpinski 1979). In an evaluation of peach rootstocks for root lesion nematode resistance, Culver et al. (1989) found that the resulting rootstock in pots created more rootlets and the optimal temperatures and moisture created the ideal conditions for root

lesion nematode invasion. This resulted in greater amounts of nematodes per gram of root in the greenhouse than in their field trials of the same genotypes (Culver et al. 1989). Taking these three studies into consideration, which all found that the greenhouse supported higher population and better survival of root parasitic nematodes, it is reasonable to expect that if this experiment were repeated in a field setting, root lesion nematode counts could be further reduced from the results of the present study. It should also be noted that a 2003 study on red clover cultivars found that greenhouse plants had lower root lesion abundance than plants that were placed in a field setting. However, it is unclear if there were differences in the population of root lesion nematodes in the two settings as soil RLN counts for the field trial is not given (Papadopoulos et al. 2003).

Before any recommendations to producers can be made from the results of this study, repeats over many years will be required, which should include different population groups of root lesion nematodes. In previous work, variations from year to year in the level of resistance and sensitivity of forage crops to root lesion nematodes have been noted. Comparing a 2003 and 2002 study on red clover populations, cultivars identified to be susceptible in 2002 were deemed resistant in the following year (Papadopoulos et al. 2003; Papadopoulos et al. 2002). While in birdsfoot trefoil, Kimpinski et al. (1999) found that RLN populations in birdsfoot trefoil roots changed between years of observation, citing both genetic differences in the forage and the nematodes as the likely cause.

The final recommendation for future investigation is to look deeper into the effects of isoflavones on nematodes. This study suggests that isoflavone content may be connected to plant resistance to nematodes due to the superior

performance of an experimental red clover variety, which has been bred to be high in above ground plant isoflavone content. However, this study makes no attempt to quantify or qualify these isoflavones and the exact mechanisms behind the reduction of RLN in these plants are still unclear. Future studies should consider the effects of different isoflavone species and how they reduce the incidence of not only root lesion nematodes, but also other species of plant-parasitic nematodes. There is evidence to suggest that not all isoflavones use the same mechanisms to reduce nematode damage, for example, daidzein, an isoflavone produced in the phenylpropanoid pathway, inhibits hatch rate and repels nematodes (Wuyts et al. 2006), while medicarpin, an isoflavone derivative, hinders nematode motility and therefore, reduces the extent of damage (Baldridge et al. 1998).

5.2 Potential application

Through further development of this research and field trials, new mitigation strategies for root lesion nematodes could be developed through the use of new cultivars and treatment with ANE or chitin as a part of agriculture systems. The development of an effective method for reducing nematodes in forage crops is important for the use of forage in crop rotation with cash crops, such as corn and potatoes, which both suffer from yield loss as a result of RLN (Decraemer and Hunt 2013; Kimpinski 1979). Forage legumes, in particular, are an appealing addition to crop rotation as they reduce erosion, add nitrogen back to the soil through nitrogen fixation and can be ploughed under as a green manure to contribute to the soil's organic matter. However, their susceptibility to parasitic nematodes has caused soil nematode populations to increase (Kimpinski 1979). The two-step strategy laid

out in this study would be 1) select resistant forage varieties, such as TRC12-156, a high isoflavone red clover, and 2) a one-time treatment at seeding with either ANE or chitin. These two steps could be easily incorporated into corn and potato cropping rotations, resulting in the prevention of nematode increase and, in a best-case scenario, reducing the total abundance of soil parasitic nematodes the following year, thereby reducing losses in the cash crop.

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Appendix A: Other nematode data

Table A-1: Figure 3-6: Number of nematodes per gram root dry matter of four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 RLN per gram dry soil.

Name	RLN Log10(x+75) /g dry root (detransformed[a])	*Rhabditis* Log10(x+75) /g dry root (detransformed[a])	Sprial /g dry root	Stunt /g dry root	Other Log10(x+75) /g dry root
Birdsfoot Trefoil	4.053 (11276)	3.105 (1198)	5.114	8.7435	2.54
Langille	4.095 (12370)	3.105 (1198)	7.405	10.55	2.577
Leo	4.011 (10182)	3.105 (1198)	2.823	6.937	2.503
Red Clover	3.547 (3877)	2.614 (337)	24.745	30.09	2.3745
TRC12-156	3.335 (2088)	2.584 (309)	35.06	22.21	2.326
TRC12-157	3.759 (5666)	2.644 (366)	14.43	37.97	2.423
Grand Mean	3.8	2.86	14.93	19.42	2.457
Standard error of the mean	0.06607	0.07331	7.892	5.649	0.08584
p-values					
Variety	<0.001	<0.001	0.074	0.015	ns
… Birdsfoot trefoil vs Red clover	<0.001	<0.001	0.035	0.004	0.085
… AC Langille vs Leo	ns	ns	ns	ns	ns
… TRC12-156 vs TRC12-157	0.001	ns	0.098	0.080	ns

Notes: a De-transformation of means from the log10(x+75) scale.
ns = F probability greater than 0.10.

Table A-2: Abundance of nematodes per gram root dry matter of three treatment groups applied to four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil.

Treatment	**RLN Log10(x+75) /g dry root (detransformed[a])**	***Rhabditis* Log10(x+75) /g dry root (detransformed[a])**	**Sprial /g dry root**	**Stunt /g dry root**	**Other Log10(x+75) /g dry root**
Control	3.875 (7424)	2.874 (673)	20.48	15.31	2.511
ANE	3.721 (5185)	2.788 (539)	6.966	25.42	2.433
Oligo-chitin	3.803 (6278)	2.918 (753)	17.34	17.53	2.428
Grand Mean	3.8	2.86	14.93	19.42	2.457
Standard error of the mean	0.03732	0.04754	5.803	6.58	0.05845
p-value					
Treatment	0.025	ns	ns	ns	ns
.. Control vs ANE, oligo-chitin	0.020	ns	ns	ns	ns
.. ANE vs oligo-chitin	ns	ns	ns	ns	ns

Notes: a De-transformation of means from the log10(x+75) scale.
ns = F probability greater than 0.10, *Ascophyllum nodosum* extract (ANE)

Table A-3: Figure 3-1: Number of nematodes per gram root dry matter of three treatment groups applied to four forage legume varieties. Samples were grown in growth chambers in soil infected with 19 root lesion nematodes per gram dry soil.

Treatment **Forage variety**	**RLN Log10(x+75) /g dry root (detransformed[a])**	***Rhabditis* Log10(x+75) /g dry root (detransformed[a])**	**Sprial /g dry root**	**Stunt /g dry root**	**Other Log10(x+75) /g dry root**
Control					
Langille	4.098 (12456)	3.089 (1152)	8.16	6.271	2.509
Leo	4.041 (10915)	3.174 (1418)	8.467	0.004	2.657
TRC12-156	3.467 (2856)	2.611 (333)	48.48	17.22	2.464
TRC12-157	3.897 (7814)	2.62 (342)	16.82	37.73	2.415
ANE					
Langille	3.977 (9409)	3.037 (1014)	14.05	25.39	2.615
Leo	3.909 (8035)	2.968 (854)	0.00	3.697	2.405
TRC12-156	3.26 (1745)	2.596 (319)	8.347	16.87	2.316
TRC12-157	3.736 (5370)	2.55 (280)	5.463	55.72	2.394
Oligo-chitin					
Langille	4.209 (16106)	3.19 (1474)	0.001967	0.00	2.607
Leo	4.082 (12003)	3.173 (1414)	0.001542	17.11	2.448
TRC12-156	3.278 (1822)	2.547 (277)	48.35	32.55	2.198
TRC12-157	3.643 (4320)	2.762 (503)	21	20.45	2.46
Grand Mean	3.8	2.86	14.93	19.42	2.457
Standard error of the mean	0.08989	0.1068	12.33	12.14	0.1284
p-value					
Treatment x variety	ns	ns	ns	ns	ns

Notes: [a] De-transformation of means from the log10(x+75) scale.
ns = F probability greater than 0.10, *Ascophyllum nodosum* extract (ANE)

Appendix B: Cluster analysis

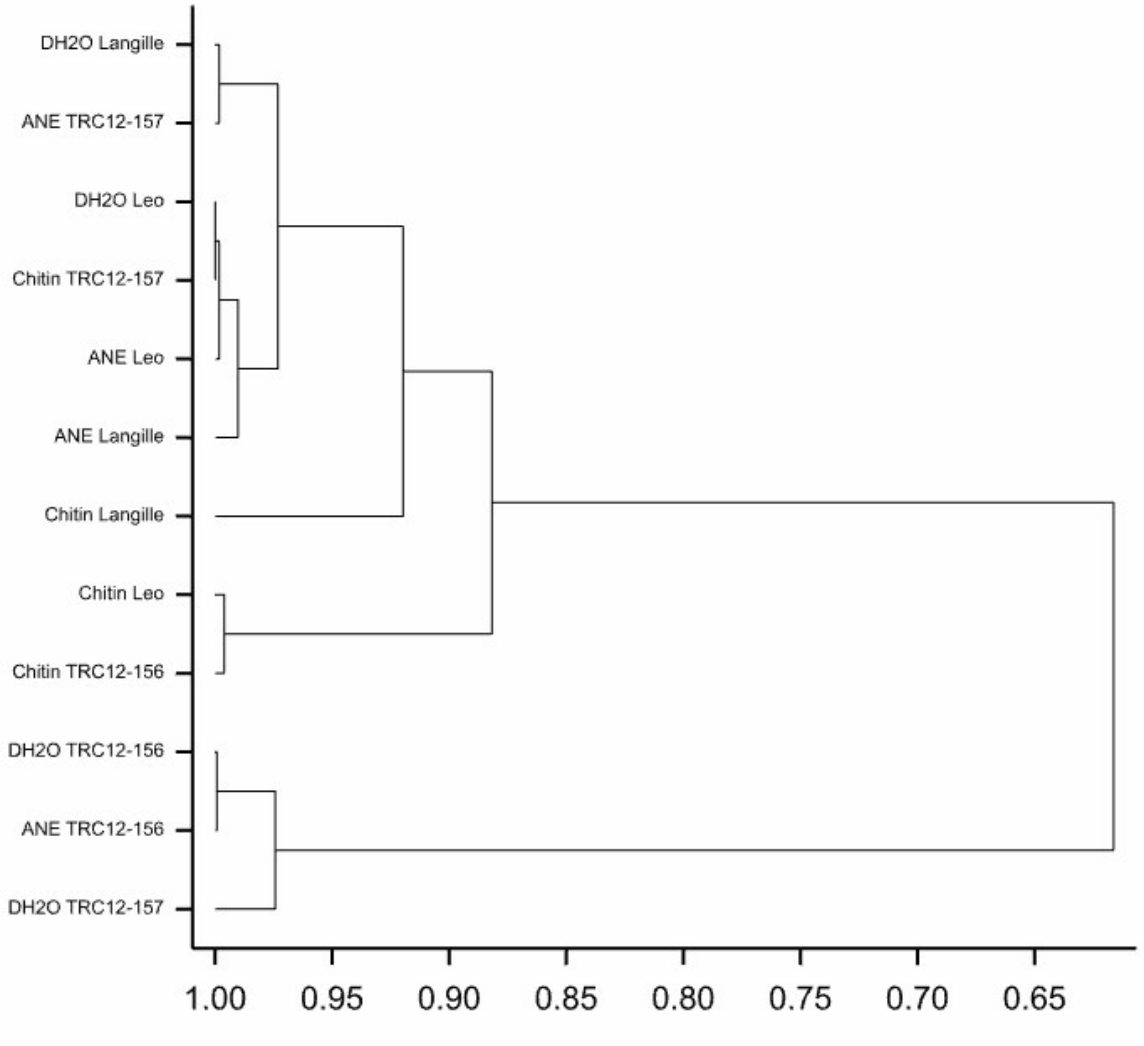

Figure B-1: Dendrogram of the results of a cluster analysis, performed on the mean vigour scores taken prior to the first shoot harvest, of three treatment groups applied to four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In this figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

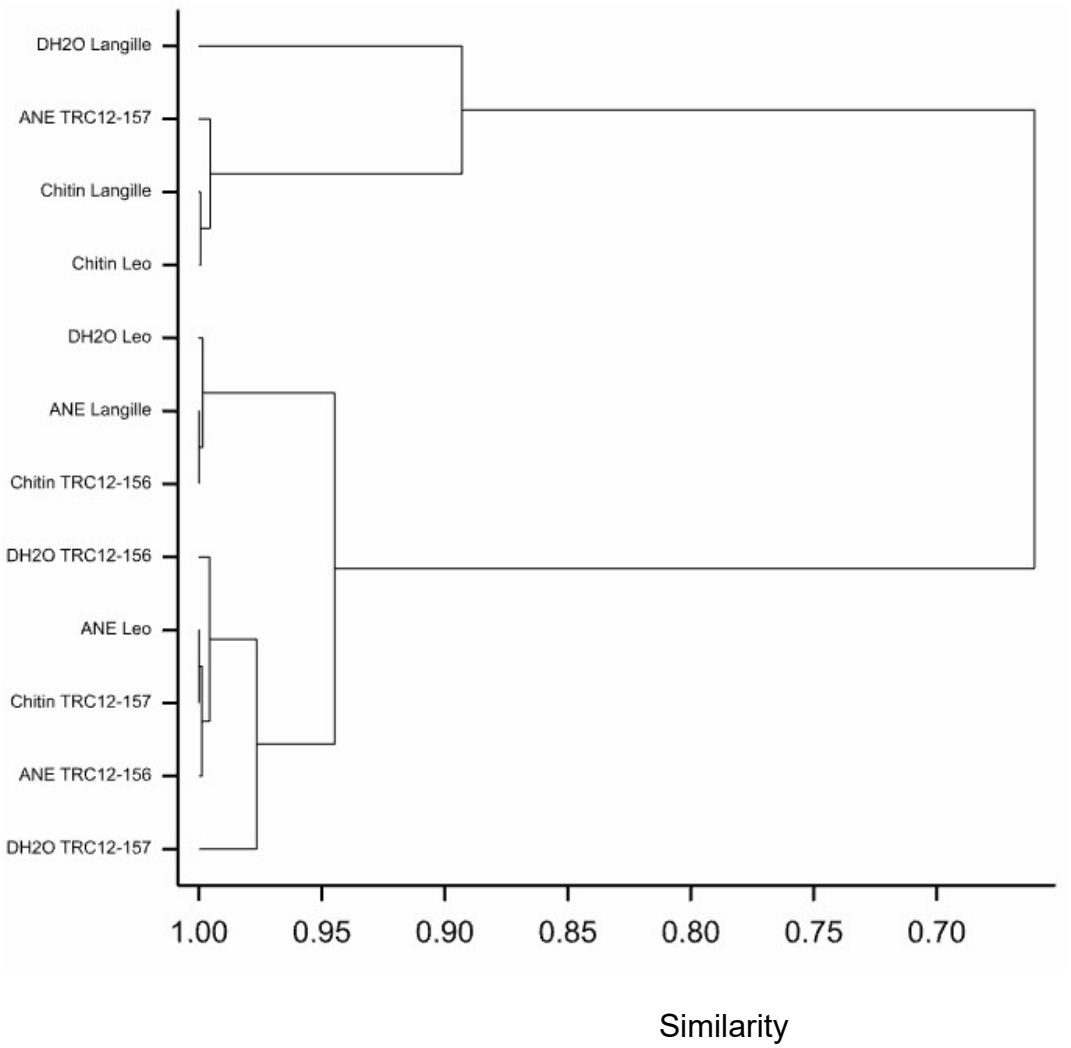

Figure B-2: Dendrogram of the results of a cluster analysis, performed on the mean vigour scores taken prior to the second shoot harvest, of three treatment groups applied to four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In this figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

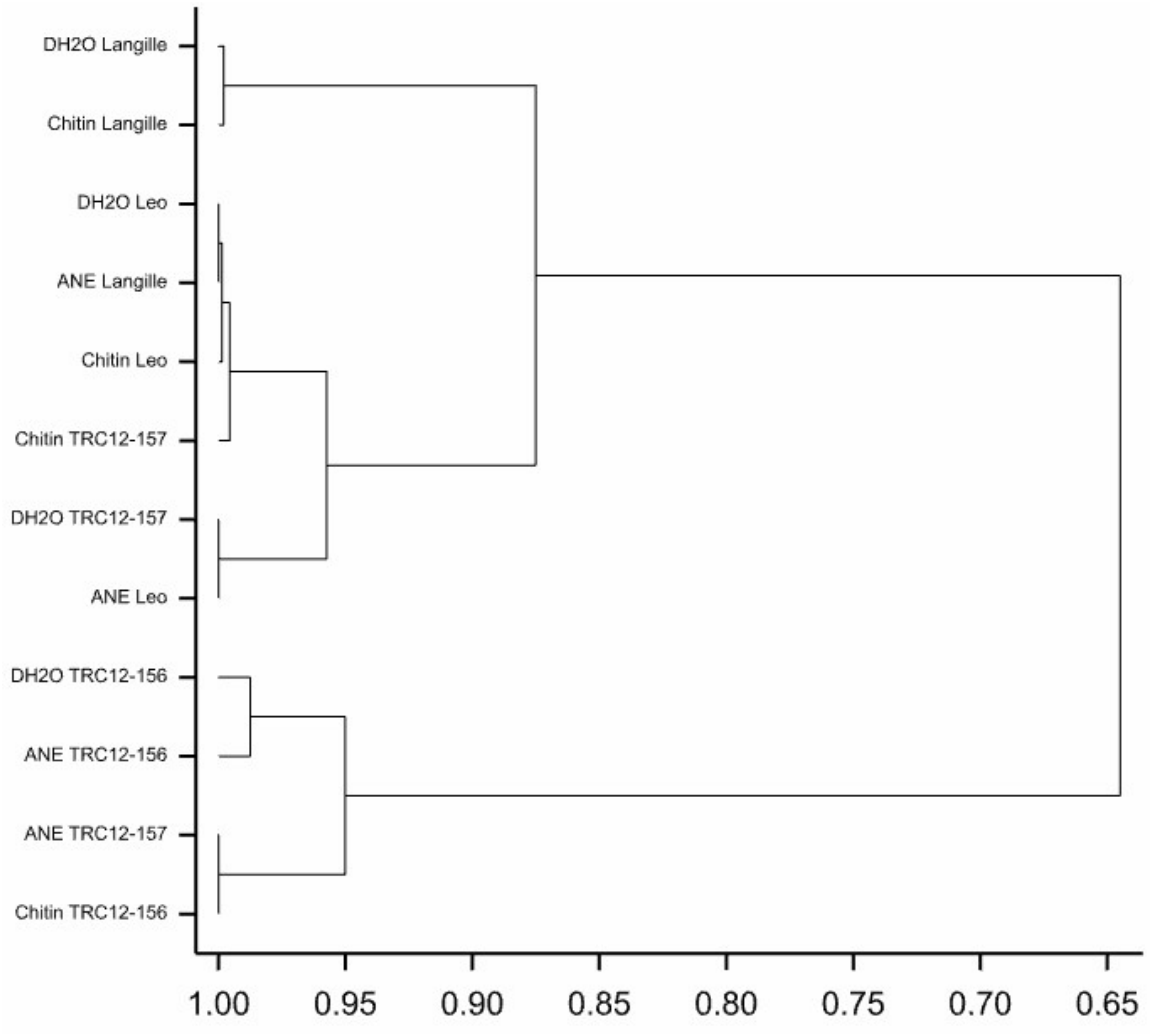

Figure B-3: Dendrogram of the results of a cluster analysis, performed on the mean dry matter yield for shoot regrowth, of three treatment groups applied to four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In this figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

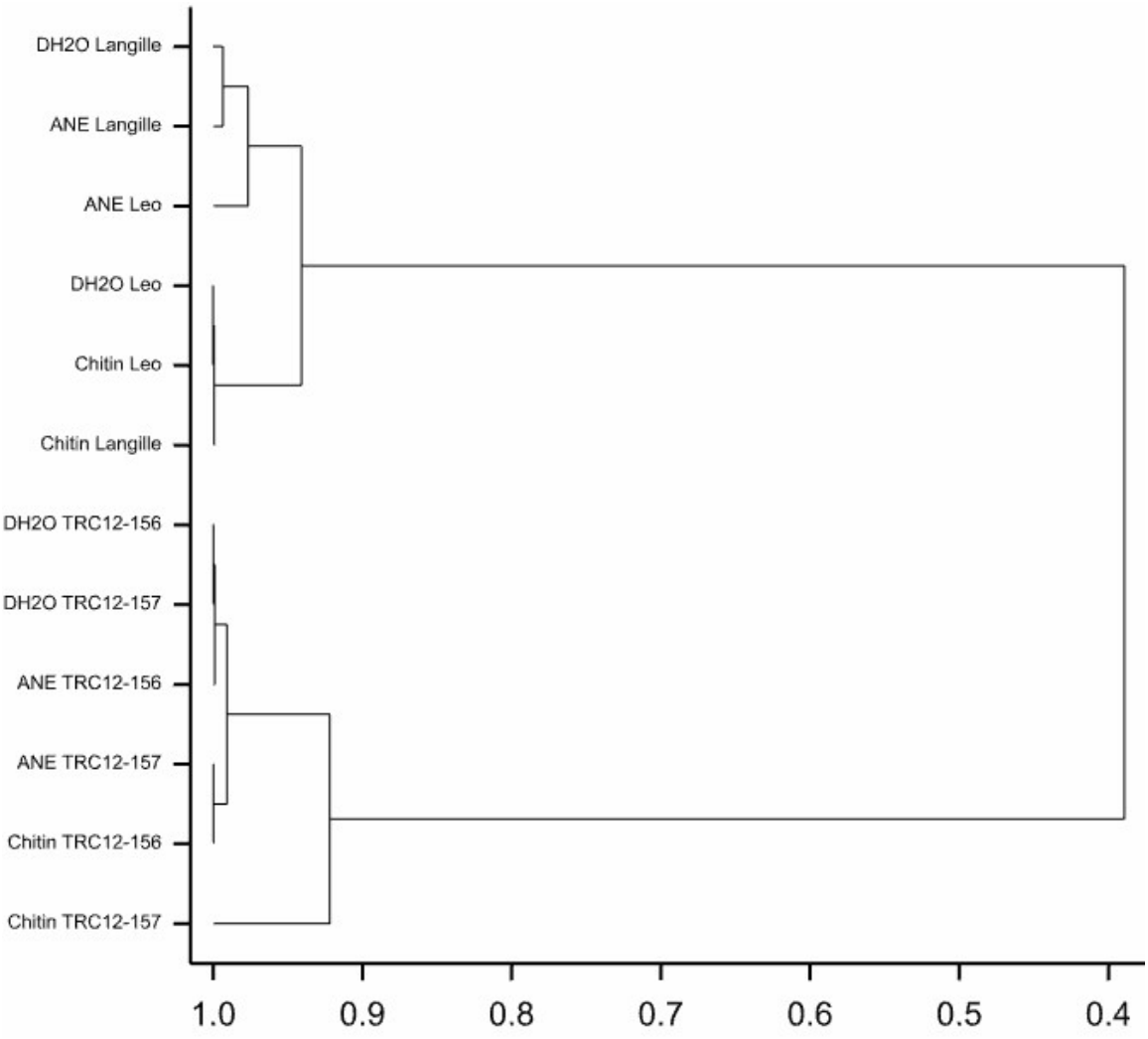

Figure B-4: Dendrogram of the results of a cluster analysis, performed on the mean population of *Rhabditis* nematodes per gram root dry matter, of three treatment groups applied to four forage legume varieties on a $Log_{10}(x+75)$ scale. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In this figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

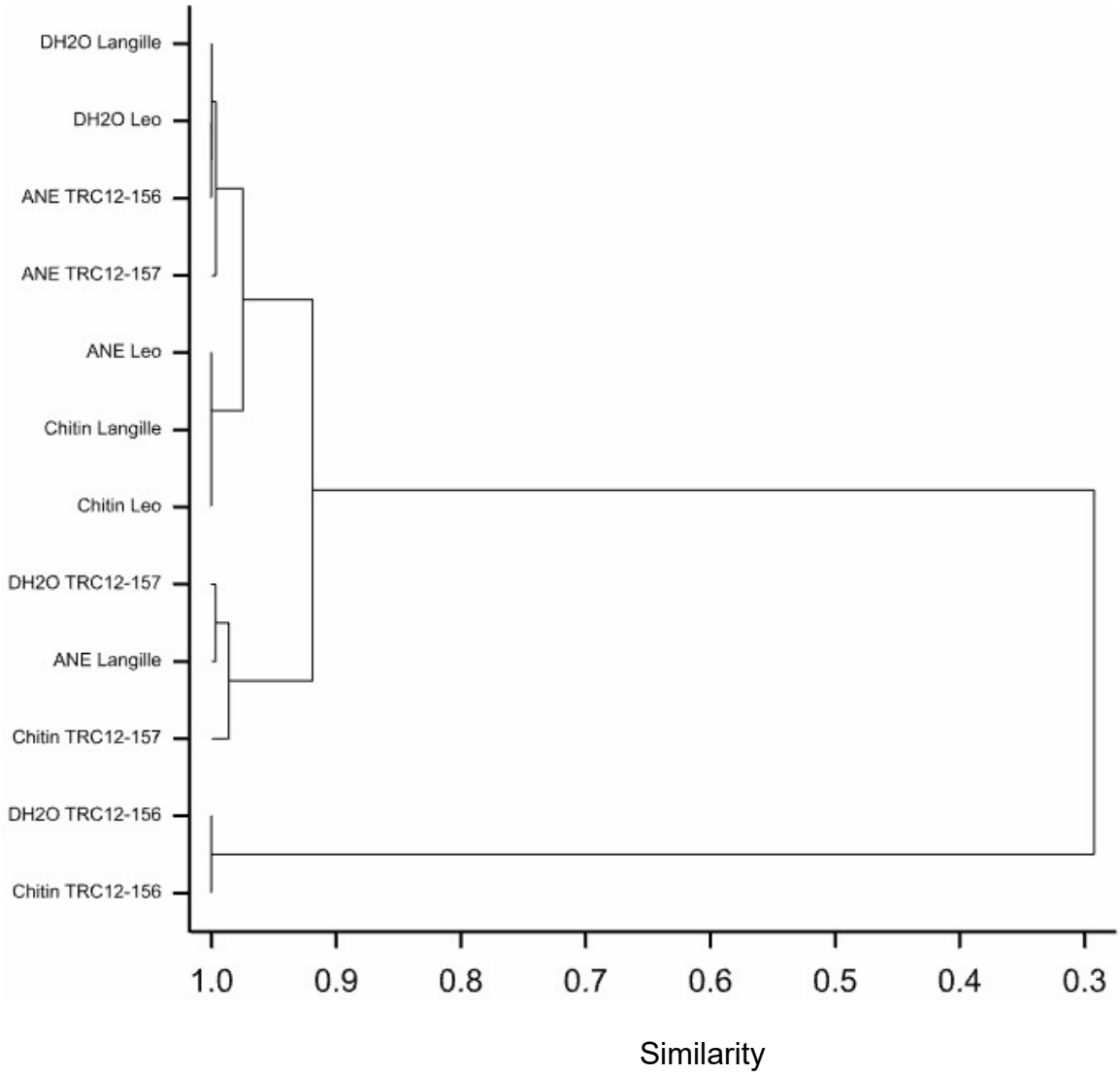

Figure B-5: Dendrogram of the results of a cluster analysis, performed on the mean population of spiral nematodes per gram root dry matter, of three treatment groups applied to four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In this figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

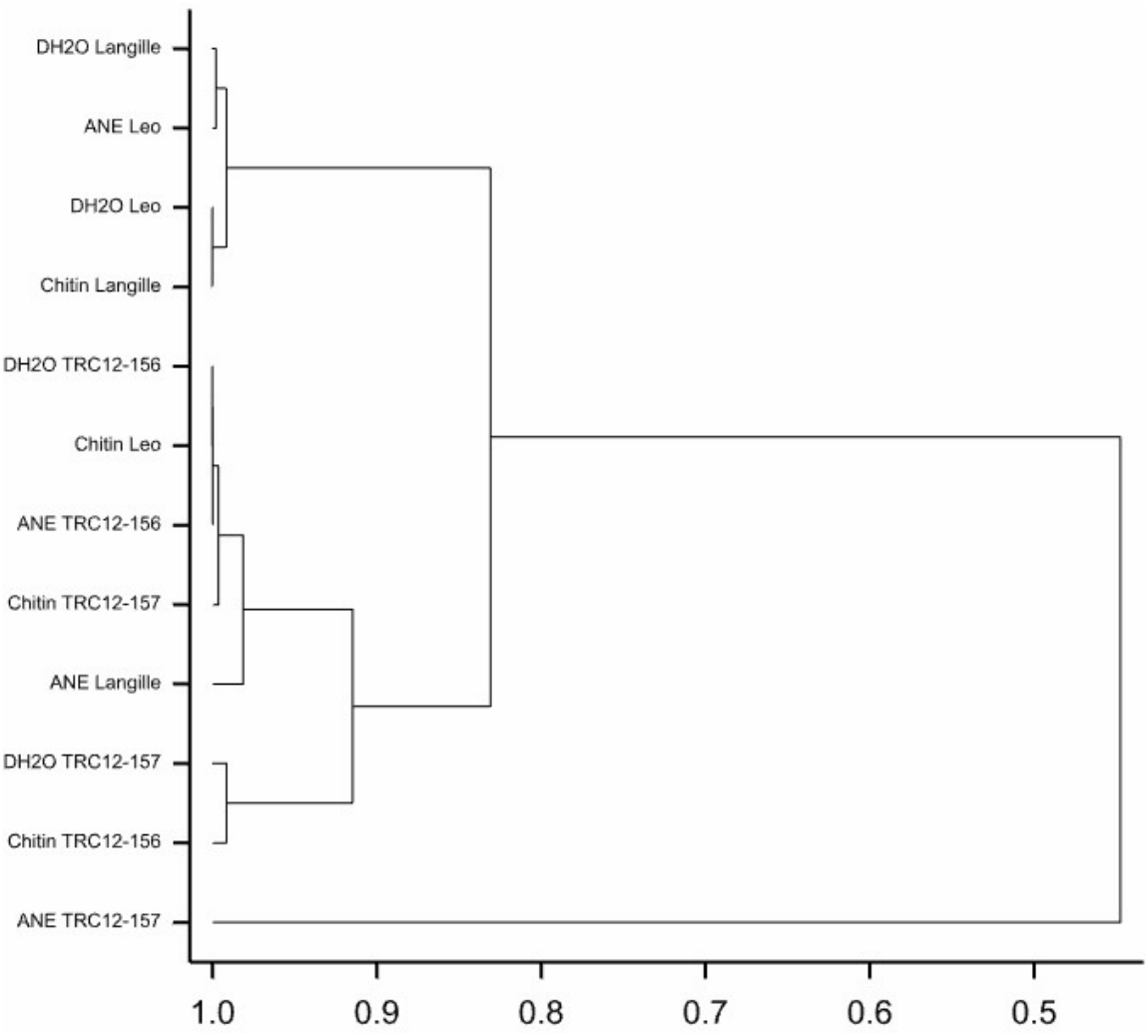

Figure B-4: Dendrogram of the results of a cluster analysis, performed on the mean population of stunt nematodes per gram root dry matter, of three treatment groups applied to four forage legume varieties. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In this figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

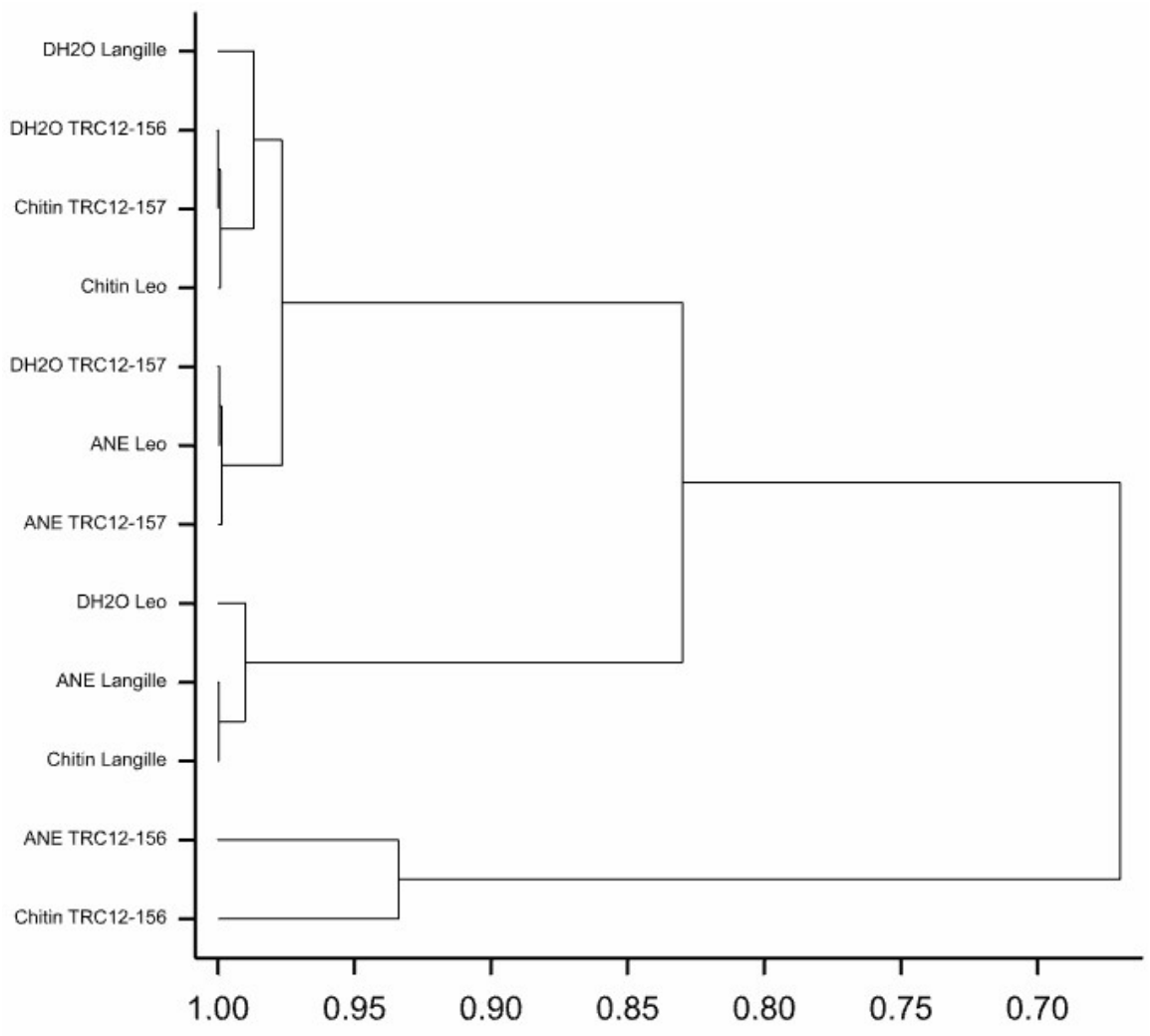

Figure B-7: Dendrogram of the results of a cluster analysis, performed on the mean population of all other nematodes per gram root dry matter, extracted from root samples of three treatment groups applied to four forage legume varieties on a $Log_{10}(x+75)$ scale. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. In this figure, chitin represents the oligo-chitin treatments and DH2O represents the control groups.

Appendix C: Interaction between treatment and forage variety

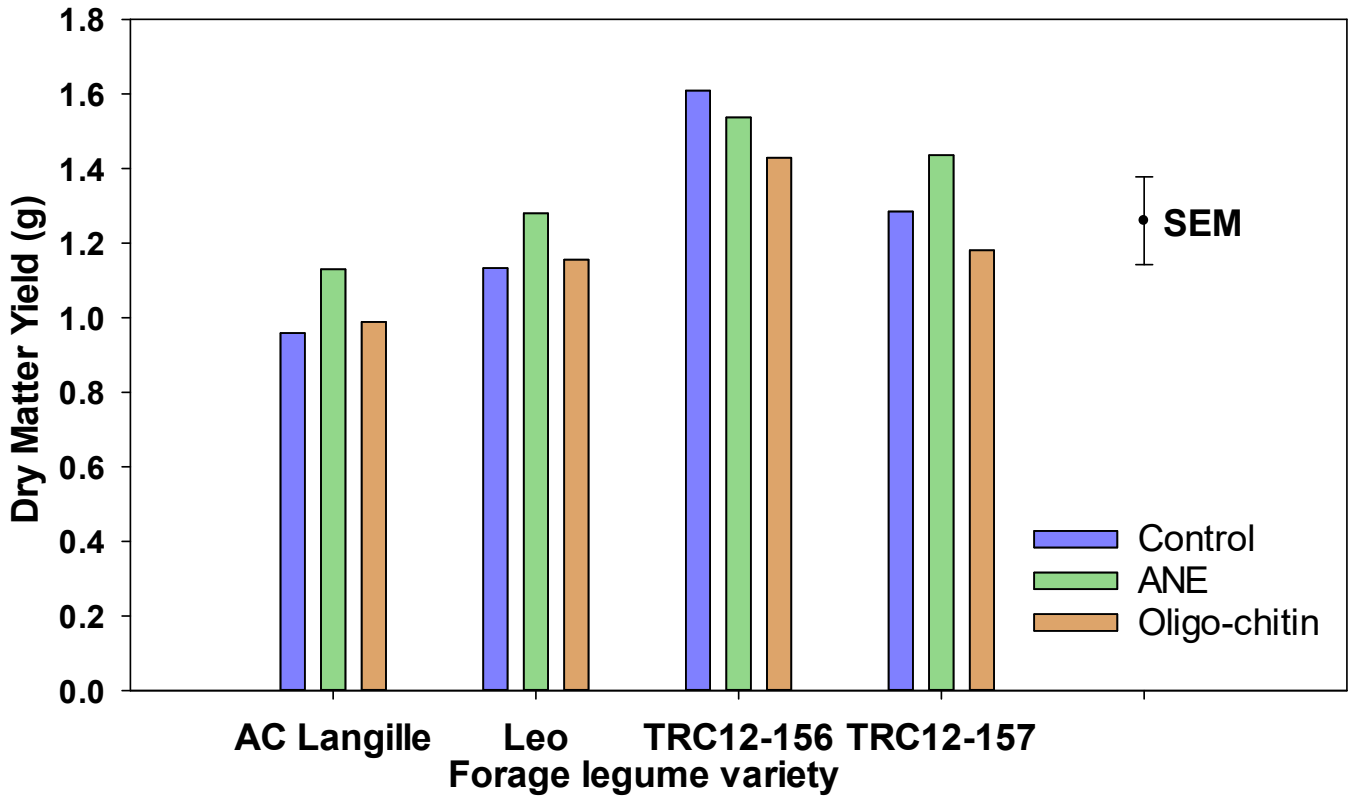

Figure C-1: Mean dry matter yield (g) of forage regrowth of three treatment groups applied to four legume varieties. Legumes were grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

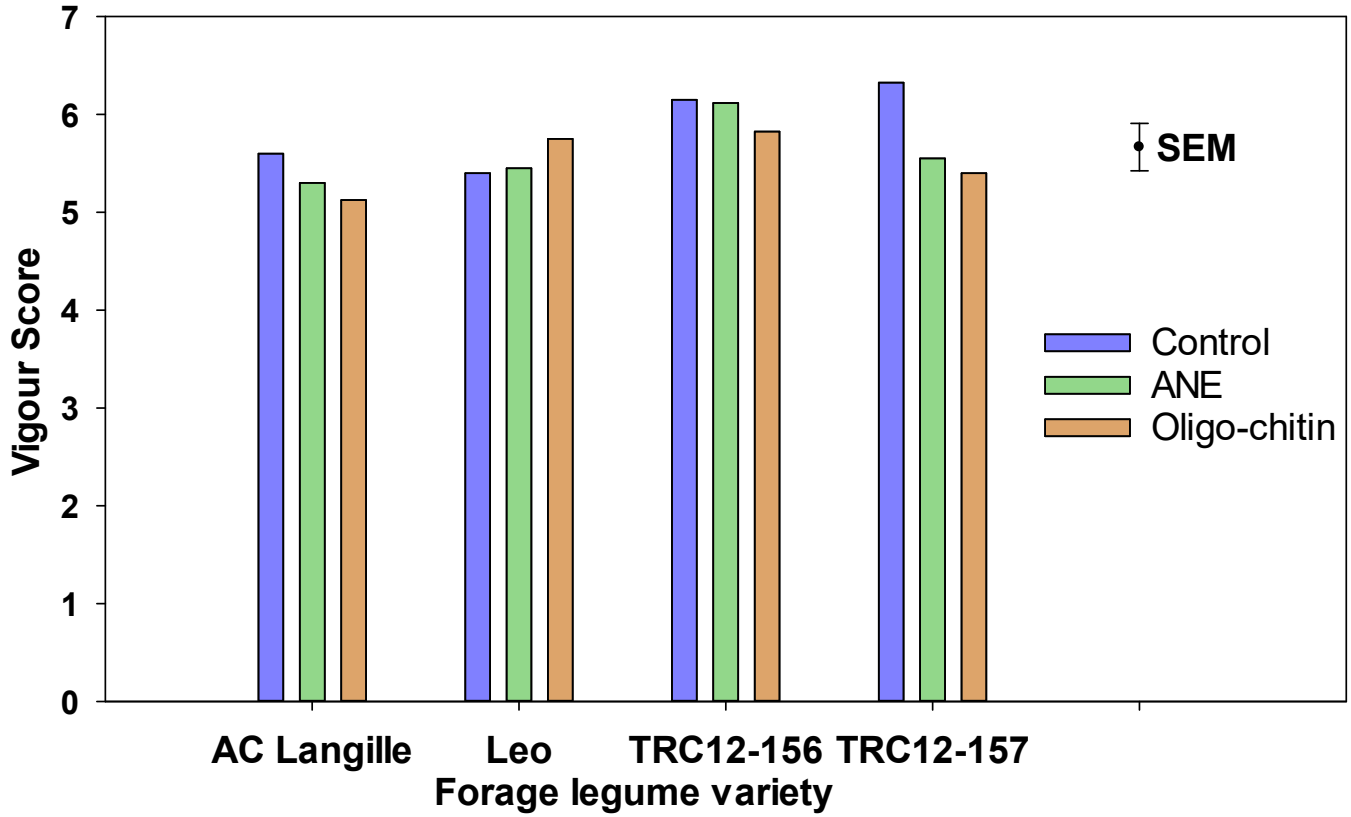

Figure C-2: Mean vigour score of three treatment groups applied to four forage legume varieties. Scores were taken prior to the first harvest of above ground plant material. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

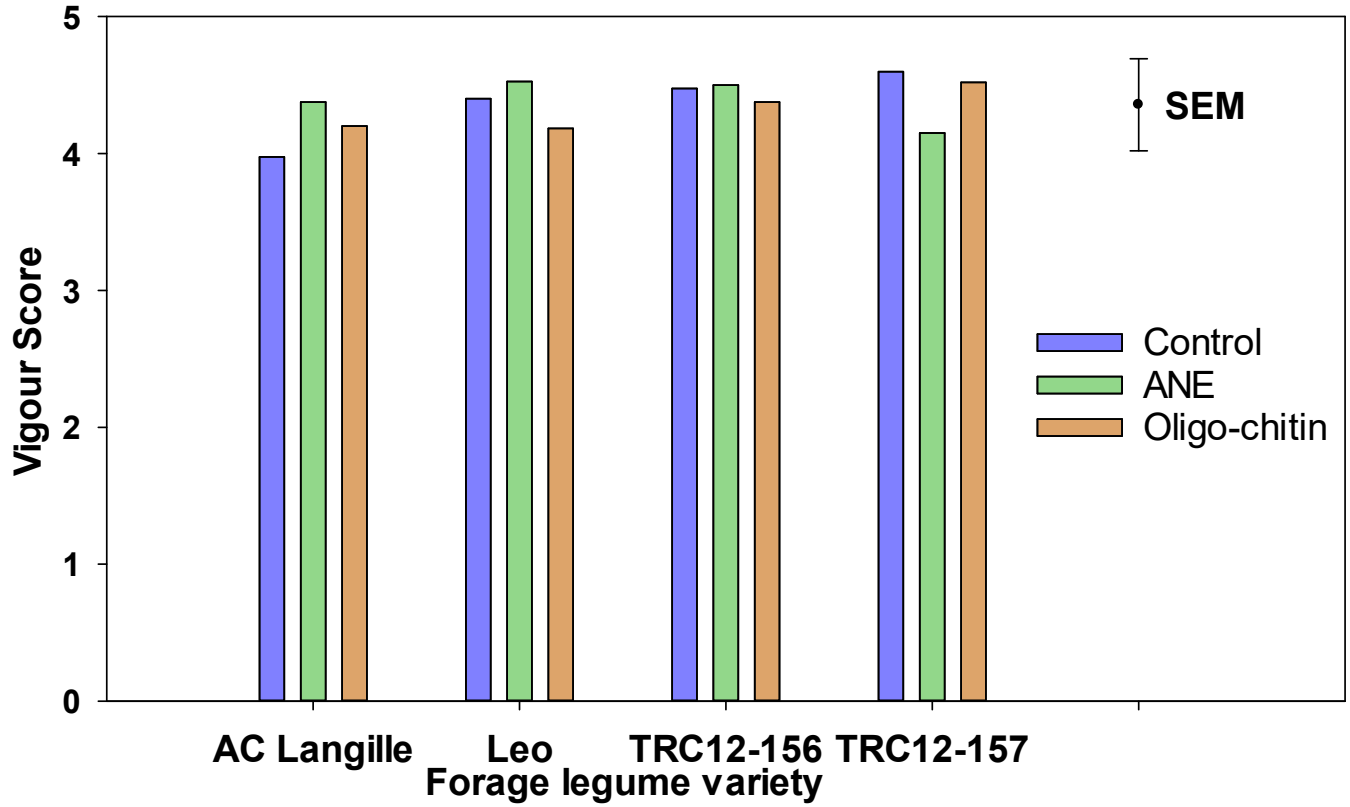

Figure C-3: Mean vigour score of three treatment groups applied to four forage legume varieties prior to the second harvest. Forage was grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

Appendix D: Vigour scores

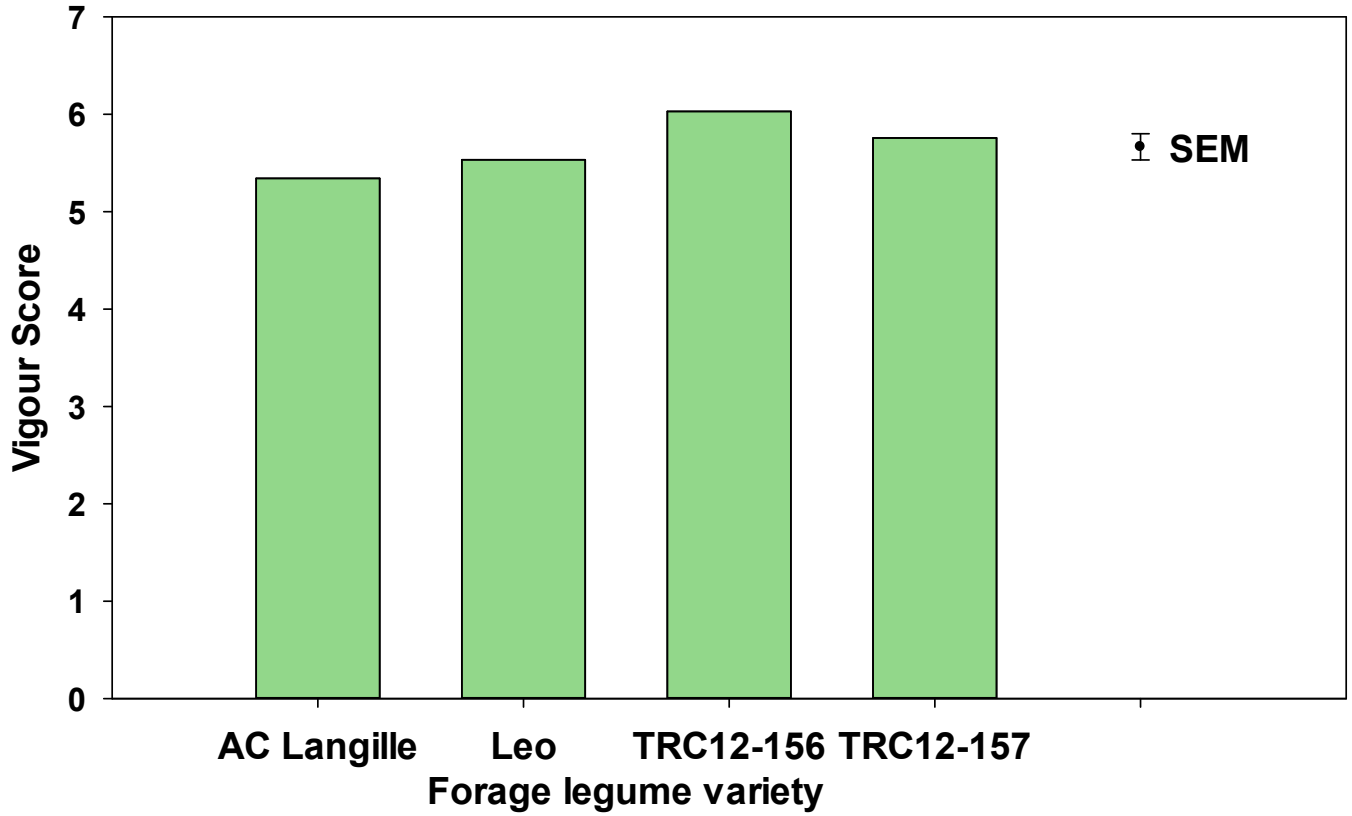

Figure D-1: Mean vigour score of four forage legume varieties prior to the first harvest of above ground plant material. Plants were grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

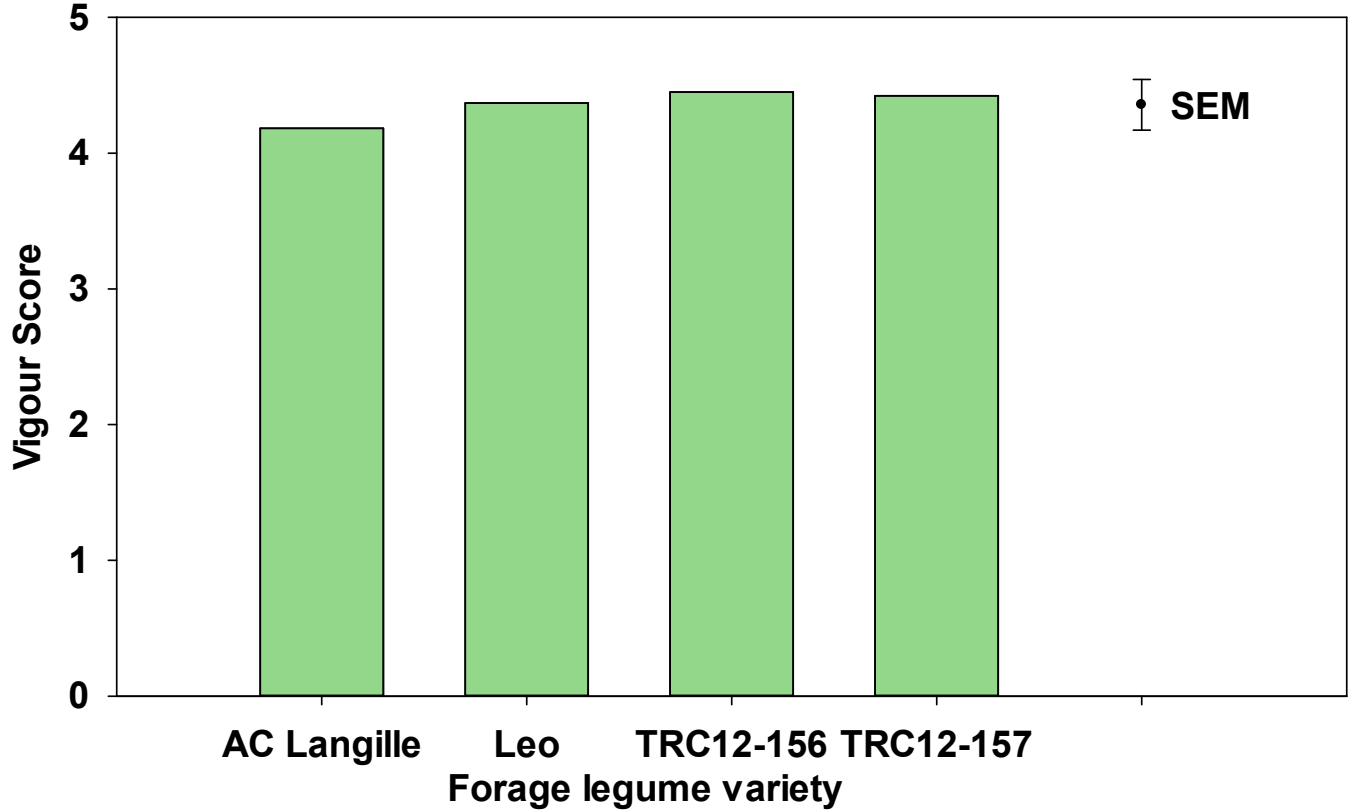

Figure D-2: Mean vigour score of four forage legume varieties prior to the second harvest of above ground plant material. Plants grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

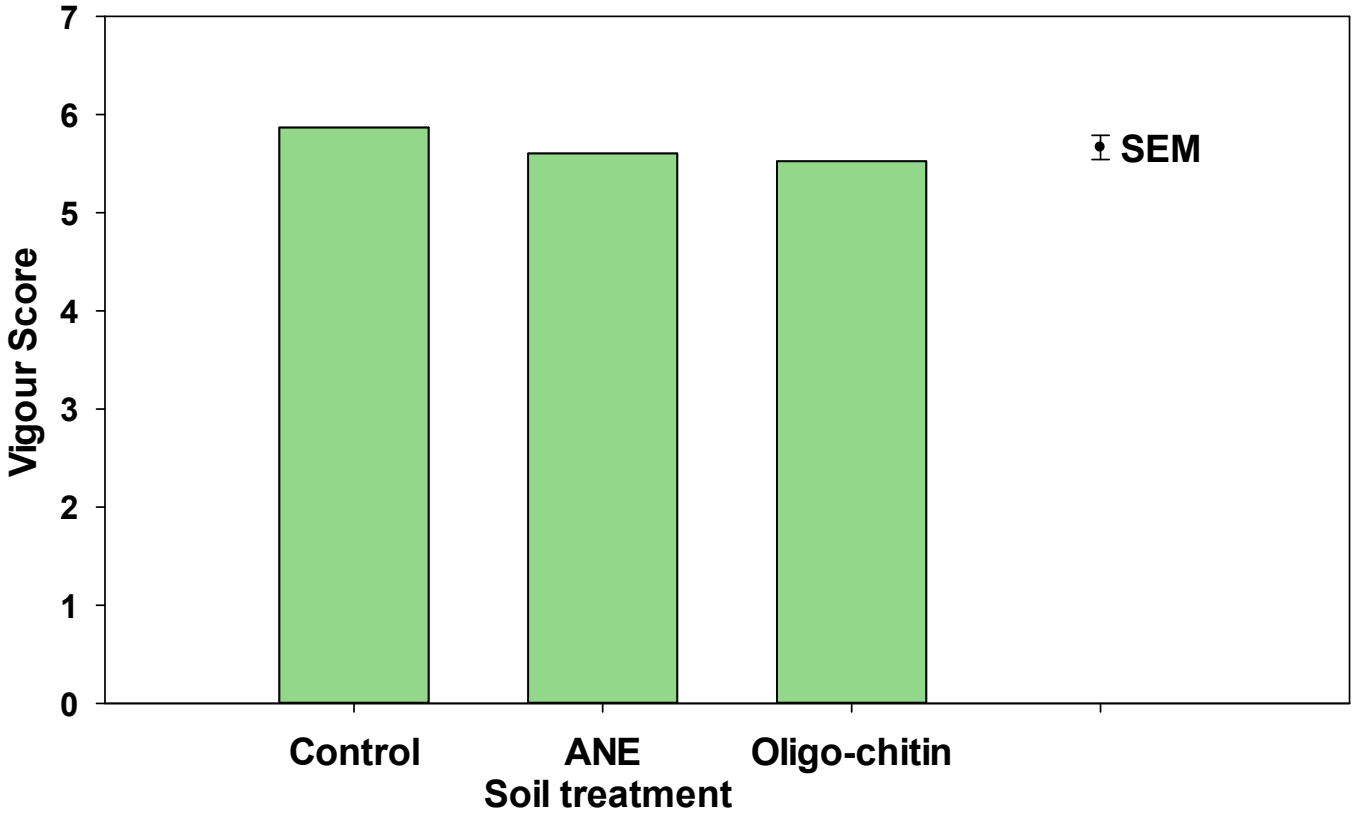

Figure D-3: Mean Vigour score of the treatment groups averaged across four forage legume varieties. Scores were taken prior to the first harvest of above ground plant material. Plants were grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

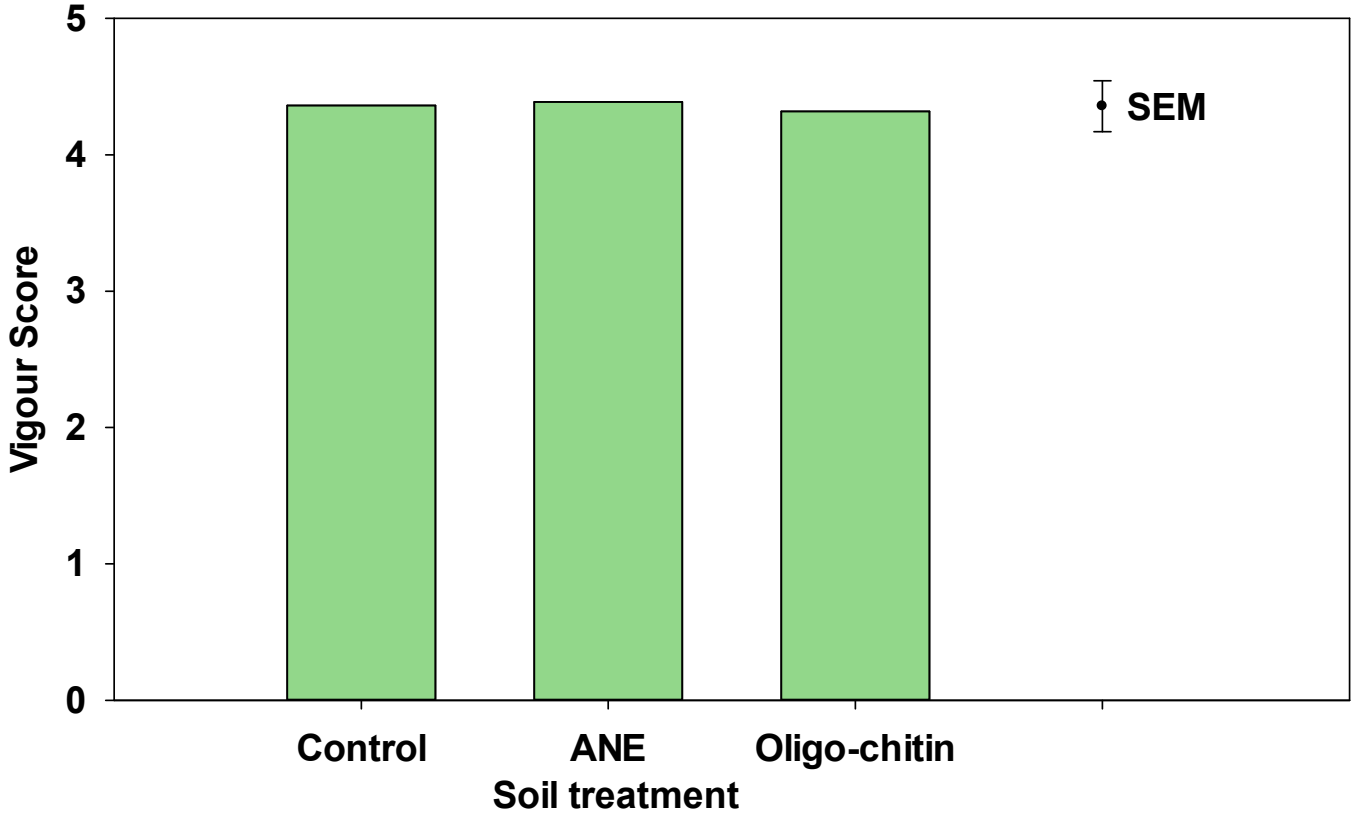

Figure D-4: Mean vigour score of treatment groups across four forage legume varieties. Scores were taken prior to the second harvest of above ground plant material. Plants were grown in growth chambers and soil infected with 19 root lesion nematodes per gram dry soil. Note: SEM = two times standard error of the mean.

Appendix E: ANOVA tables

Table E-1: Analysis of variance for RLN populations (Log_{10}(RLN+75)/ g dry root)

Source of variation	D.F.	Mean squares RLN population
Block stratum	3	0.09997
Block.bCol.bRow stratum		
Variety	3	13.97***
Birdsfoot trefoil vs Red clover	1	30.71***
AC Langille vs Leo	1	0.4246
TRC12-156 vs TRC12-157	1	10.77**
Residual	9	0.5238
Block.bCol.bRow.sRow stratum		
Treatment	2	0.958*
Control vs ANE,Oligo-chitin	1	1.375*
ANE vs Oligo-chitin	1	0.5411
Treatment.Variety	6	0.3633
Control vs ANE,Oligo-chitin.Birdsfoot trefoil vs Red clover	1	0.84
ANE vs Oligo-chitin.Birdsfoot trefoil vs Red clover	1	1.158
Control vs ANE,Oligo-chitin.AC Langille vs Leo	1	0.02207
ANE vs Oligo-chitin.AC Langille vs Leo	1	0.03614
Control vs ANE,Oligo-chitin.TRC12-156 vs TRC12-157	1	0.001172
ANE vs Oligo-chitin.TRC12-156 vs TRC12-157	1	0.1226
Residual	24	0.2229
Block.bCol.bRow.sRow.sCol.sPlant stratum		
Plant	9	0.08356
Treatment.Plant	18	0.09673
Control vs ANE,Oligo-chitin.Plant	9	0.1265
ANE vs Oligo-chitin.Plant	9	0.06695
Variety.Plant	27	0.1465*
Birdsfoot trefoil vs Red clover.Plant	9	0.2648**
AC Langille vs Leo.Plant	9	0.07147
TRC12-156 vs TRC12-157.Plant	9	0.1033
Treatment.Variety Plant	54	0.08383
Control vs ANE,Oligo-chitin. Birdsfoot trefoil vs Red clover.Plant	9	0.08772
ANE vs Chitin.Birdsfoot trefoil vs Red clover.Plant	9	0.02502
Control vs ANE,Oligo-chitin. AC Langille vs Leo.Plant	9	0.2129
ANE vs oligo-chitin.AC Langille vs Leo.Plant	9	0.06923
Control vs ANE,Oligo-chitin.TRC12-156 vs TRC12-157.Plant	9	0.03759
ANE vs oligo-chitin.TRC12-156 vs TRC12-157.Plant	9	0.0705

Residual	323	0.08322
Total	478	

Notes: Degrees of freedom (D.F.), Root lesion nematodes (RLN), *Ascophyllum nodosum* extract (ANE), significance probabilities: *(P < 0.05), **(P < 0.01), ***(P < 0.001).

Table E-2: Analysis of variance for regrowth dry matter yield (g pre plant)

Source of variation	D.F.	Mean squares Dry matter yield
Block stratum	3	15.21
Block.bCol.bRow stratum		
Variety	3	5.267*
Birdsfoot trefoil vs Red clover	1	11.17**
AC Langille vs Leo	1	1.609
TRC12-156 vs TRC12-157	1	3.022
Residual	9	0.8944
Block.bCol.bRow.sRow stratum		
Treatment	2	1.005
Control vs ANE,Oligo-chitin	1	0.04442
ANE vs Oligo-chitin	1	1.966*
Treatment.Variety	6	0.1871
Control vs ANE,Oligo-chitin.Birdsfoot trefoil vs Red clover	1	0.5534
ANE vs Oligo-chitin.Birdsfoot trefoil vs Red clover	1	0.04753
Control vs ANE,Oligo-chitin.AC Langille vs Leo	1	0.003152
ANE vs Oligo-chitin.AC Langille vs Leo	1	0.002806
Control vs ANE,Oligo-chitin.TRC12-156 vs TRC12-157	1	0.299
ANE vs Oligo-chitin.TRC12-156 vs TRC12-157	1	0.2168
Residual	24	0.3824
Block.bCol.bRow.sRow.sCol.sPlant stratum		
Plant	9	0.2257
Treatment.Plant	18	0.1166
Control vs ANE,Oligo-chitin.Plant	9	0.05424
ANE vs Oligo-chitin.Plant	9	0.1791
Variety.Plant	27	0.1428
Birdsfoot trefoil vs Red clover.Plant	9	0.1353
AC Langille vs Leo.Plant	9	0.07849
TRC12-156 vs TRC12-157.Plant	9	0.2146
Treatment.Variety Plant	54	0.1235
Control vs ANE,Oligo-chitin. Birdsfoot trefoil vs Red clover.Plant	9	0.1712
ANE vs Chitin.Birdsfoot trefoil vs Red clover.Plant	9	0.1169
Control vs ANE,Oligo-chitin. AC Langille vs Leo.Plant	9	0.1117
ANE vs oligo-chitin.AC Langille vs Leo.Plant	9	0.06779

Control vs ANE,Oligo-chitin.TRC12-156 vs TRC12-157.Plant	9	0.1271
ANE vs oligo-chitin.TRC12-156 vs TRC12-157.Plant	9	0.1462
Residual	323	0.1466
Total	478	

Notes: Degrees of freedom (D.F.), *Ascophyllum nodosum* extract (ANE), significance probabilities: *(P < 0.05), **(P < 0.01), ***(P < 0.001).