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University of Nebraska-Lincoln

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COMPARISON OF METHODS AND CORN ROOT TYPES FOR EFFICIENT EXTRACTION OF ENDOPARASITIC NEMATODES

by

Jae L. Behn

A THESIS

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COMPARISON OF METHODS AND CORN ROOT TYPES FOR EFFICIENT

EXTRACTION OF ENDOPARASITIC NEMATODES

Jae L. Behn, M. S.

University of Nebraska, 2012

Advisor: Tamra A. Jackson

Endoparasitic nematodes annually reduced the yield of corn in the United States. *Pratylenchus* spp. and *Hoplolaimus* spp. are endoparasitic nematode genera that parasitize corn in the Midwest. Previous research has shown nematode population densities to be highly variable and extraction methods may not provide consistent results. In order to determine more consistent and time efficient results, comparisons were made between four standard nematode extraction techniques for preferences of nematode genera for extraction method, corn root type, and extraction time. The extraction methods evaluated were aeration-incubation, a modified Baermann funnel, Seinhorst's mister, and shaker incubation. Research plots were established at two Nebraska locations in 2009 and 2010 with documented histories of high population densities of these endoparasitic nematode genera. Nematodes were extracted from six root types on four extraction methods for 3, 5 and 7 days in a complete factorial treatment design. In 2009, *Pratylenchus* spp. were best extracted from the first set of anchor roots on the Baermann funnel ($p<0.0001$) or in the aerated incubation. *Hoplolaimus* spp. had the greatest recovery from seminal roots on the Baermann funnel in 2009 ($p<0.0001$). The mist extraction method did not have consistent results in 2009. For 2010, fine feeder roots in mist or aerated incubation had the greatest recovery of both genera of endoparasitic nematodes ($p<0.0001$). In most comparisons, 3-5 days of incubation was sufficient to

extract at least 50% of the 7 day population density. For most root types, extraction timings, and nematode genera, the shaker method yielded the fewest nematodes per gram dried root ($p<0.0001$). Based on these results, it can be concluded that nematode extraction efficiency varies by genus for root type and extraction method.

I would like to dedicate the thesis to my husband, Ryan. He has taught me many things, the most important one being “anything is possible”.

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Corn (*Zea mays* L.), an annual plant from the Gramineae family, is grown around the world (Farnham, et. al., 2003; Kiesselbach, 1999). It is a monoecious plant having both male and female reproductive parts on the same plant (Kiesselbach, 1999). Maize, as it is also commonly known, is also the most commonly produced field crop grown in the United States today comprising 50% of the world's total maize production. Over 81 million acres were harvested in the U.S. in 2010, equating to 12 billion bushels of yield (National Agricultural Statistics Service, 2010). In 2007, corn sales approached $40 billion. Nebraska ranked third in 2010 for maize production in the U.S. growing 1.4 billion bushels (National Agricultural Statistics Service, 2010). Corn is utilized in a variety of facets, not simply as a food source for humans. It is commonly used for fuel production, livestock feed, and sold as an export commodity (Farnham, et. al., 2003; Windham and Edwards, 1999). Every year, demands for corn and other crops steadily increase as the world population increases. Producers need to increase yields without an increase of area in production (Farnham, et. al., 2003). Acquiring the greatest yield from each acre is becoming more important as these demands increase. Proper management of soil, water, diseases, weeds, insects, and nutrients are all methods used to achieve higher yields. However, concerns with toxicity and pest resistance from chemical usage limits the options available for producers to maintain high yielding crops. Every input and management technique needs to be scrutinized for sufficient efficacy to ensure the highest potential yield and economic gain. Part of the solution requires greater accuracy in diagnosis and management of diseases.

PLANT PARASITIC NEMATODES

Agricultural crops have a variety of pathogens that diminish yield potentials every season. Of those, nematodes are some of the least understood by producers. Approximately 4,100 species of plant parasitic nematodes have been described world-wide (Decraemer and Hunt, 2006). Since most nematodes cannot be positively identified by the unaided eye, producers face the challenge of recognizing the source of their problems. Symptoms of nematode infection are vague and can often be mistaken for numerous other pathogens as well as abiotic factors (Castillo and Vovlas, 2007; Windham and Edwards, 1999), making accurate diagnosis a more involved process. The above ground visual symptoms are usually easiest to view early in plant growth. Later in the growing season, plants can overcome the initial damages sustained from nematode feeding. Visual differences above the soil and delayed plant development are no longer detectable until yields are assessed (Schomaker and Been, 2006). Accurate diagnosis of plant parasitic nematodes can only be conducted for samples submitted to a qualified laboratory for nematode testing.

Most plant parasitic nematodes feed on the root system although a few nematodes can feed on the leaves and stems of host plants. The three genera having the greatest economic impact are, in order of importance, cyst (*Heterodera* spp.), root-knot (*Meloidogyne* spp.), and root-lesion (*Pratylenchus* spp.) (Sasser and Freckman, 1987). It is no surprise that these highly detrimental pathogens are all endoparasites. Endoparasitic nematodes can enter the root and feed on the inside whereas ectoparasites feed only by stylet insertion while the nematode body remains outside the root (Decraemer and Hunt,

2006). The entry and internal migration of the endoparasitic nematode can cause significant physical damage to the root system, therefore reducing yield potentials very early in the infection process (Hussey and Williamson, 1998). Openings in the root tissue made by nematodes allow secondary pathogens access to the damaged tissue, further decreasing the plant's ability for growth, production, and in some cases, survival (Duncan and Moens, 2006; Krall, 1978; Windham and Edwards, 1999). Such nematode-microbe interactions constitute disease complexes. These relationships have been observed with various fungal and bacterial pathogens including, but not limited to, *Fusarium moniliforme*, *F. oxysporum*, *Gibberella zeae*, *Helminthosporium pedicellatum*, *H. sativum*, *Rhizoctonia fragariae*, *R. solani*, and *Verticillium dahliae* (Duncan and Moens, 2006, Windham, 1998). In 1994, the estimated loss of corn yield in Nebraska due to phytoparasitic nematodes was 0-1% (Koenning, et al., 1999). However, this equated to over $2 billion dollars lost. This illustrates how relatively small levels of nematode damage, even as little as 1%, can greatly impact commodity production on a much larger scale.

Many plant parasitic nematodes have several key morphological features in common. They are microscopic, transparent, worm-like animals with un-segmented, bilaterally symmetrical bodies (Decraemer and Hunt, 2006; Ferris and Ferris, 1998; Windham and Edwards, 1999). Their bodies can be described as a tube within a tube; the outer tube being the body wall, or cuticle, and the inner tube containing the reproductive system (Decraemer and Hunt, 2006). Most nematodes spend all or part of their life in a vermiform body shape. Sexual dimorphism is usually only apparent on adults and varies

by genus. The head region contains cephalic framework that can be heavily sclerotized (Decraemer and Hunt, 2006).

Life cycles of most nematodes have six stages, beginning with an embryo followed by four juvenile stages, then to an adult (Decraemer and Hunt, 2006). Eggs can be laid in soil or root tissue (Ferris and Ferris, 1998). A newly laid egg contains a nematode in the first juvenile stage, also known as the J1 stage. While inside the egg, most genera will molt into a second juvenile stage, the J2 stage. The J2 hatches from the egg using its stylet to pierce the shell (Khan, 2008). At this growth stage, most nematode genera can begin feeding on a suitable host. These juveniles will go through three additional molts, finally reaching adult hood (Windham and Edwards, 1999). On average, life cycles range from 2 to 6 weeks depending on species and environmental factors (Agrios, 2008; Castillo and Vovlas, 2007; Khan, 2008; Windham and Edwards, 1999). Sexual identities are established during the last molt into adulthood. Most genera are dioecious, having separate male and female nematodes (Decraemer and Hunt, 2006; Ferris and Ferris, 1998). Reproduction occurs either between mating partners or parthenogenetically, where females bear only female offspring without need of fertilization (Windham and Edwards, 1999). Parthenogenesis is specific by species within genera (Agrios, 2008).

Once the nematode is ready to feed, it begins the search for a suitable host. Nematodes can find host tissues through chemotaxis, chemokinesis, or by random movement within soil (Khan, 2008; Lavallee and Rohde, 1962). By using sensory organs, such as phasmids or amphids, the nematodes detect changes in temperature,

moisture, carbon dioxide, oxygen, and chemical substrates (Khan, 2008; Norton and Niblack, 1991; Robinson and Perry, 2006). This sensory information guides the nematode through the soil profile; the data leads them either to potential hosts or away from harmful environments.

The nematode is attracted to root exudates of host plants (Krall, 1978; Tsai and Van Gundy, 1990). Depending on species migratory tendencies, nematodes may settle on one root or move between several roots for feeding (Huang and Ole Becker, 1997; Todd and Oakley, 1996). Once a host is found, the nematode then searches for a suitable feeding site by touching various areas on the root surface (Khan, 2008; Zunke, 1990). Finding a feeding site, the nematode begins feeding by insertion of its stylet into the root tissue. The stylet is used by plant parasitic nematodes primarily for feeding and is generally a hollow sclerotized tooth-like structure (Agrios, 2008; Ferris and Ferris, 1998). This feeding structure is similar to an insect with piercing-sucking mouth parts. Feeding depth within the root varies by genus (Robinson and Perry, 2006). Most nematodes secrete chemicals to aid in the breakdown of root tissues (Zunke, 1990). An organ within the digestive tract, known as the median bulb, expands and contracts, acting as a pump to aid the nematode in ingesting plant cytoplasm (Khan, 2008). The host plant is commonly fed upon as long as it provides sufficient nutrients to the nematodes. Once the root tissue has ceased activity or the plant has perished, the nematodes may either move on to a new plant specimen or wait in quiescence for another favorable host/environment (Duncan and Moens, 2006).

Most plant parasitic nematodes are obligate parasites, needing live plant material to feed upon (Khan, 2008; Windham and Edwards, 1999); they also cannot reproduce well, or sometimes survive, on non-host plants. However, many species can feed on a variety of plants, so a suitable host is not always a limiting factor for populations. Although a host is needed for adequate sustenance, most nematodes have key behavioral and physiological strategies to endure lack of host or unfavorable environmental conditions, such as quiescence (Agrios, 2008; Castillo and Vovlas, 2007; Ferris and Ferris, 1998; Norton and Niblack, 1991). During quiescence, the nematode is in a reduced metabolic state induced by levels of water, salt concentration, temperature, or oxygen. Survival while in dormancy depends upon a number of factors including, but not limited to, duration, predators, and host availability (Ferris and Ferris, 1998; Norton and Niblack, 1991).

Nathan A. Cobb once discussed the prevalence of all nematode communities stating, “In short, if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable, and if, as disembodied spirits, we could then investigate it, we should find its mountains, hills, vales, rivers, lakes, and oceans represented by a film of nematodes” (Cobb, 1915). Plant parasitic nematodes are no exception as they can be found on every continent in every ecosystem in the world (Castillo and Vovlas, 2007). Since they are obligate parasites, they are concentrated in areas containing suitable host species. Population densities are in a constant flux depending on host availability, crop rotation, and environment, among other things (Ferris and Bernard, 1971a; Ferris and Bernard, 1971b; Norton and Niblack, 1991).

Nematode communities are affected by several biotic and abiotic factors. Soil organisms, parasites, and predators often influence nematode survivability and reproduction (Bilgrami, et. al., 2008; Edmunds and Mai, 1966; Sikora, 1992; Walker, 1969). Soil texture, aeration, temperature, moisture, pH and aeration, and other edaphic factors may also influence nematode life processes (Agrios, 2008; Brodie, 1976; Castillo and Vovlas, 2007; Decraemer and Hunt, 2006; Khan, 2008; Norton, et. al., 1971; Norton and Niblack, 1991; Thomas, 1978).

Nematode spatial distribution is highly aggregated in soils and can be irregularly distributed within fields (Norton and Niblack, 1991). Vertical distribution of nematodes can be temporal and affected by several factors. Soil texture, soil type, moisture, temperature, root distribution and host cultivar/variety can influence the presence and reproduction of phytoparasitic nematodes (Brodie, 1976; Castillo and Vovlas, 2007; Ferris and Bernard, 1971b; Forge et. al., 1998; Kable and Mai, 1968; Kimpinski et. al., 1976; Lindsey and Cairns, 1971; Norton and Niblack, 1991; Nyczepir and Lewis, 1979; Rebois and Huettel, 1986; Taylor and Evans, 1998; Townshend, 1972; Townshend and Webber, 1971; Zirakparvar, et. al., 1980). Nematodes can be classified as migratory or sedentary. Migratory plant parasites move frequently, feeding on several areas of the root system. Sedentary nematodes find a suitable feeding source and remain in that location for the rest of their lifetime or the host's lifetime. Nematodes require a film of water for movement through pore spaces between soil particles (Decraemer and Hunt, 2006). Movement in a season can range from 0.3 to 2 meters in a year (Agrios, 2008; Castillo and Vovlas, 2007; Khan, 2008; Windham and Edwards, 1999). Movement

requires the alternate contraction of muscles within the nematode body, creating undulations in the dorso-ventral plane (Decraemer and Hunt, 2006; Norton and Niblack, 1991). During dry periods, the nematode movement and survival is limited (Agrios, 2008). Optimum temperature for nematode activity is from 16-32 °C (Windham and Edwards, 1999). Of course, ideal temperatures vary by species, environment, and stage of development (Robinson and Perry, 2006; Windham and Edwards, 1999). Since nematodes have little range of dispersal on their own, their long distance transportation is dependent on other means. Nematodes can be carried by water or wind-blown soil particles and plant tissue, but mechanical transfer of infested material is the primary mode of nematode dispersal (Castillo and Vovlas, 2007; Duncan and Moens, 2006; Morgan, et. al., 2002; Norton and Niblack, 1991; Windham and Edwards, 1999). This dissemination can occur locally, within a single field, or globally. The highly inconsistent population distribution within a field causes very high statistical variability when conducting research on nematodes. This leads to challenges for producers to manage the populations accurately, economically and efficiently. Having a precise assessment of nematode populations is imperative for proper management strategies to be implemented (Decraemer and Hunt, 2006). By understanding the fragile and delicate balance of profitability within field crop production, severe economic consequences due to over- or under-management can be avoided.

PLANT PARASITIC NEMATODES OF CORN

More than 60 species of plant parasitic nematodes can feed on corn in North America (Norton, 1983; Windham and Edwards, 1999). All of these species are obligate

parasites of corn and other crops (Windham and Edwards, 1999). Nematodes of corn can cause several visual symptoms, along with yield loss. Symptoms on upper plant parts may include stunting, chlorosis, lodging and wilting (Castillo and Vovlas, 2007; Duncan and Moens, 2006; Griffin, 1964; Norton, 1983; Norton and Hinz, 1976). These symptoms can mimic other known corn problems, such as low fertility, poor drainage, or herbicide injury (Windham and Edwards, 1999). Root systems can exhibit heavily branched root tips, stunted root growth, lack of root hairs, and dark red-brown lesions (Agrios, 2008; Windham and Edwards, 1999). If the infestation is significant enough, the nematode feeding may even cause plant death. However, this occurrence is rare due to the nematodes being obligate parasites. Highly damaging nematode populations appear in a field as round to oval areas of suppressed growth within a planted area (Windham and Edwards, 1999). The only way to correctly identify a nematode population is by analysis of a sample collected from the infested soil and/or root material (Windham and Edwards, 1999).

To feed on corn, nematodes use their stylet to puncture root cell walls and extract cell nutrients. Some genera release or inject enzymes into the plant tissue through their stylet (Duncan and Moens, 2006; Rebois and Huettel, 1986). The enzymes aid in the breakdown of cell wall tissues and digestion of nutrients. Either by the mechanical or chemical injury to root cells, the nematode renders the root less productive in absorbing water and nutrients from the soil. As feeding continues, cortical root tissue begins to breakdown (Windham and Edwards, 1999). During this time, the corn plant produces additional lateral roots in a possible attempt to overcome the damage caused by the

nematode feeding (Ogiga and Estey, 1975). The rate of tissue decay is enhanced as the number of nematodes feeding on the root increases. After the root tissue ceases function, the nematodes may leave the dead area to search for another feeding location or remain stationary in the tissue awaiting another suitable host plant so as to repeat the disease cycle again.

Studies have shown that corn seminal root damage, as early as three week old plants, can reduce yields up to 9% (Kiesselbach, 1999). Understanding how nematode feeding can physically injure root tissues, estimates of yield loss by nematodes could range from 10-26% in maize (Khan, 2008; Windham and Edwards, 1999). The probability of yield loss due to high nematode population densities is an influential consideration in determining a producer's management strategy. However, environmental factors may play an important role in the level of damage nematode populations can have. Water is a serious yield-limiting factor, especially during important crop stages such as flowering and pollination (Farnham, et. al., 2003). Smolik and Evenson observed decreased yield loss from irrigated corn versus rain-fed corn in severely nematode-infested fields (Smolik and Evenson, 1987). Normal plant stresses can add to the degree of damage accrued on nematode parasitized corn. Also, nematodes are usually found in mixed populations, so determining the actual species that caused yield loss may be difficult to assess (Windham and Edwards, 1999).

VARIATIONS IN HOST

In addition to the variability in nematode communities, there is also variability within the host plants. Maize has an extensive fibrous root system that requires abundant moisture throughout the year (Farnham, et. al., 2003). This large root system can mask nematode damage, only becoming evident during periods of environmental stress (Windham and Edwards, 1999). There are two main root types: seminal and nodal (Abendroth, et. al., 2011; Kiesselbach, 1999). From these roots grow many lateral roots and fine root hairs. The radicle is the first root to grow from the seed, followed soon after by several branch or lateral roots (Farnham, et. al., 2003; Kiesselbach, 1999). These roots comprise the seminal root system. Their primary function is water uptake for the first 2-3 weeks after germination, but they have been shown to still function for the duration of the corn plant's life (Abendroth, et. al., 2011; Farnham et. al., 2003; Kiesselbach, 1999). Within this 2-3 week period, the nodal roots begin development. Nodal roots are also known as adventitious, crown, anchor, or brace roots. Each set of nodal roots develops in accordance to a leaf emerging from the stalk; therefore, the age of the roots depends on its location within the root system (Abendroth, et. al., 2011; Kiesselbach, 1999). These roots, once present, are responsible for water and nutrient absorption from the soil (Farnham, et. al., 2003). The nodal roots comprise the majority of total corn roots by the emergence of the sixth leaf and for the remainder of the plant's life (Kiesselbach, 1999). From each nodal root, several lateral roots emerge along with numerous root hairs. Lateral roots aid in water uptake and stability of the plant. Root hairs are single-cell extensions of the root epidermis (Kiesselbach, 1999). Their main

function is to increase the surface area of the root system. Once established, they become the main root tissues used in water and nutrient absorption.

The depth of the root system depends on environmental and soil factors, but generally the root system of corn can be 1-2 meters deep (Abendroth, et. al., 2011; Farnham et. al., 2003; Kiesselbach, 1999; Robertson, et. al., 1979). The plant achieves this depth at maturity, 80-90 days after emergence (Farnham et. al., 2003). Estimates of total root length are approximately 6 miles per plant (Kiesselbach, 1999). Studies show root growth and development are directly correlated to corn developmental stages (Foth, 1962; Kiesselbach, 1999; Mengel and Barber, 1974). During vegetative growth, roots grow diagonally downward from the stalk to a depth of 12-15 inches. By tasseling, the roots in this region of the soil profile have produced numerous lateral roots. After the reproductive stages begin, the roots grow deeper into the soil profile. For the duration of the life cycle of corn, the majority of root tissue is concentrated in the top 12-15 inches of soil (Robertson, et.al., 1979). Similarly, Foth determined the major lateral distribution of root tissue, by weight, was within 5 inches radius of the stalk (Foth, 1962). However, the roots can spread up to eight feet in diameter from the stalk (Kiesselbach, 1999). There is a positive relationship between root density in the upper 15 cm and corn yield (Kuchenbuch and Barber, 1987). Interestingly, MacGuidwin and Stanger found approximately 50% of *Pratylenchus scribneri* populations were also found in the upper 15 cm of corn root systems (MacGuidwin and Stanger, 1991). Contradictory to MacGuidwin and Stanger, others have indicated the highest population densities of *Pratylenchus* spp. to be in a layer of soil 15-30 cm deep, although there were still

significant numbers of the nematodes in the top 15 cm of soil (McSorley and Dickson, 1990; Norton and Edwards, 1988; Pudasaini, et. al., 2006). Corn root growth, distribution, pattern, and timing information may be utilized to locate where nematodes may feed throughout the season. *Pratylenchus* spp. have been shown to migrate vertically depending on where feeding sites may be located (Pudasaini, et. al., 2006; Smiley et. al., 2008; Windham and Edwards, 1999). In addition, maize roots have been shown to influence nematode egg hatch (De Waele, et. al., 1988). Knowing this, it seems reasonable to sample for nematodes where there are the most roots actively growing, assuming to find the most nematode attraction and egg hatch.

The size of roots also varies within the root system. Root diameter increases from root hairs to feeder roots to lateral roots to nodal roots. Seminal roots can vary in diameter but usually fall between feeder roots and lateral roots. There is some degree of debate where nematodes prefer to feed, either on established roots, new roots, or even on root hairs. Zunke studied the feeding habits of *P. penetrans* on various hosts; his results concluded that the majority of nematodes moved directly to the root hair region of each host, regardless of host type or growth stage of nematode (Zunke, 1990). Georgi et. al., found more *Pratylenchus hexincisus* per gram of root in seminal roots, at least for the first 9 weeks of a corn plant's life (Georgi et. al., 1983). Todd and Oakley detected similar results with *P. neglectus* and *P. scribneri*, but also showed negative correlations between test weights of corn and late-season collections of nematodes from adventitious roots (Todd and Oakley, 1996). Kimpinski, et. al., observed more *Pratylenchus minyus* (syn. *P. neglectus*) in the seminal roots of wheat than any other root type; they concluded

this was due to seminal roots of cereals having greater physiological activity than the other root types tested (Kimpinski, et. al., 1976). LaMondia studied strawberry roots and saw that *P. penetrans* inhabited new feeder roots in much greater quantities than older structural roots (LaMondia, 2002). LaMondia's study agrees with earlier work by Zirakparvar on *P. hexincisus* on corn (Zirakparvar, 1979). Although, Zirakparvar termed his root types as "fibrous" and "coarse".

MANAGEMENT OF NEMATODES

A number of cultural, chemical, and biological techniques for nematode management have been utilized over decades of corn production. Many cultural practices have been shown to aid in reducing nematode population densities. Rotation and cover crops using non-host plants have proven very beneficial in reducing plant parasitic nematodes (Ball-Coelho, et. al., 2003; Duncan, 1991; Ferris and Bernard, 1971a; Jackson et. al., 2005; Johnson, et. al., 1975; Koenning, et. al., 1985; Kratochvil et. al., 2004; LaMondia, 2006; McSorley and Gallaher, 1993). However, some nematode species behave differently within a genus. For example, several species of *Pratylenchus* spp. have a broad host range; therefore, rotation is not a viable option for most producers (Barker and Olthof, 1976; Bélair et. al., 2007; Jordaan and De Waele, 1988). Delayed planting dates can influence nematode infection (Koenning et. al., 1985). Leaving land fallow for a growing season is also an effective cultural practice for control of nematodes, however, can be very costly to the producer (Koenning et. al., 1985; Kratochvil, et. al., 2004; Windham, 1998). With no host crop, nematodes do not have a food source and the soil becomes very warm and dry. Tillage practices have demonstrated beneficial effects,

but those results vary by nematode species, soil type, host plant and location (McSorely and Gallaher, 1993; Thomas, 1978; Windham and Edwards, 1999). Soil amendments have also caused reductions in plant parasite populations. These soil amendments include poultry manure, pigeon manure, saw dust and soybean meal (Hassan, et. al., 2009; Kratochvil, et. al., 2004; Walker, 1969). Sanitation is always a good practice for reducing the spread of plant pathogens (Duncan and Moens, 2006). Normal cultural practices, such as adding nitrogen to soil, can also have nematicidal activity. Walker found that application of NKO_2 had a complete reduction of phytoparasitic nematodes in soil after one week (Walker, 1969).

When cultural techniques leave farmers with few desirable options, they turn to more potent chemical alternatives. For decades, fumigation was a popular choice for nematode control with methyl bromide being a very popular choice, particularly in high value crops (Nyczepir and Lewis, 1979; Olthof, 1989; Young, 1964). However, the use of methyl bromide has been severely limited by government regulations so fumigation has proven less economically efficient (McKenry et. al., 1994). McKenry demonstrated that soil drenching with metam sodium was nearly as effective as methyl bromide. Several granular and liquid nematicides have exhibited yield increases and nematode control (Badra and Caveness, 1983; Bergeson, 1978; Di Sanzo, 1973; Johnson and Chalfant, 1973; Norton and Hinz, 1976; Philis, 1997; San Martín and Magunacelaya, 2005; Zirakparvar, 1979). While these products are quite effective, there are negative environmental aspects that far out-weigh the benefits. These chemicals have been shown to kill beneficial soil microorganisms (Nyczepir and Lewis, 1979). Nematodes are

classified as animals so the chemicals used for their control (such as carbamates and organophosphates) are harmful to humans, too. Contact with these chemicals through mixing, application, cleaning, and storage can be very dangerous to the producers, and thus a shift has appeared in the nematicide market. The government, due to groundwater contamination, has restricted their use (Duncan, 1991). Producers have turned to corn seed treated with nematicidal and nematostatic chemicals. Seed treatments are making nematode control much safer, more economical, and more effective (Truelove, et. al., 1977). Since only a small amount of chemical is applied to each seed, farmers are not coming into contact with, paying for, or applying excessive amounts of harmful chemicals (Windham, 1998). However, nematicides may not always be economically feasible. Chemicals can be expensive and, depending on the nematode species and population density, control may not be warranted (Duncan and Moens, 2006; Windham and Edwards, 1999).

To combat the use of chemicals altogether, producers may rely on biological treatments. Fungal antagonists of nematodes can assist with population reduction (Sikora, 1992; Timper and Brodie, 1993). These predators trap nematodes with adhesive webs or constrictive rings of mycelia. A similar approach is parasitic bacteria; the bacterial spores adhere to nematodes with sticky exudates (Tian, et. al., 2007). Spores germinate and enter the nematode with the use of enzymes to break down the cuticle and utilize nutrients from the nematode as a food source. Predatory nematodes are also an alternative for plant parasitic nematode control (Bilgrami, et. al., 2008). As with any biological organism used for pathogen management, population establishment can be

very difficult. Competition with other soil microorganisms for water, space, and nutrients can be fierce and newly introduced species may be at a disadvantage. Several other methods have been studied for nematode control. The choice of one, or a combination of several, is heavily dependent upon cost, efficacy, and potential for economic return.

Host resistance is a popular choice for many plant pathogens. However, it is not a readily available option for all nematode species. So far, there are few corn lines, all inbred, with known resistance genes for plant parasitic nematodes (Windham and Edwards, 1999). Four of the 129 known corn germplasms have successful resistance genes to nematodes (Young, 1998). Difficulties corn breeders face include the expansive variability of nematode susceptibility to resistance, even within a genus, and the highly aggregated geographical distribution of more economically important nematodes (Duncan and Moens, 2006; Norton, 1983; Windham and Edwards, 1999). Migratory nematodes pose a more difficult challenge in finding resistance. Their feeding relationship with host plants is not as intricate or detailed as sedentary parasites and so, unfortunately, breeding efforts have been mostly unsuccessful or limited (De Waele and Elsen, 2002). Some commercial corn seed companies do not see nematodes as a major economic problem of corn, and thus very little money and time has been devoted to the issue (Windham, 1998). Another potential result of using resistance is the high selection pressure it places on the nematode community (Young, 1998). It is very expensive to breed corn with specific resistance genes, so the overall cost is much too great for a broad

spectrum hybrid to be utilized efficiently (Windham and Edwards, 1999). No resistance has been found in corn against ectoparasites (Windham, 1998).

Integrated pest management (IPM) is an important part of many producer's farm practices. The use of IPM has proven very beneficial to farmers by using multiple techniques for pathogen and pest control. This strategy combines biological, cultural, chemical, and genetic practices to aid in pathogen control all the while reducing the application of chemical products. It can assist with the management of several pathogens with similar control methods while at the same time helping to reduce input costs. Unfortunately, studies conducted on nematode control have indicated that no single treatment has the same effect for all genera and species of plant parasitic nematodes, giving more reasons why an integrated pest management strategy is the best alternative for producers (Norton, et. al., 1978). However, IPM requires greater accuracy and reduced input costs for identification of pathogens to work more successfully (Duncan, 1991; McSorley and Gallaher, 1993).

PRATYLENCHUS SPP. & *HOPLOLAIMUS* SPP. NEMATODES

Regarding corn production in Nebraska, and most of the Midwest, there are two economically important endoparasitic nematodes: *Pratylenchus* spp. and *Hoplolaimus* spp. The two genera are similar in taxonomic relationship (Appendix A). Observing the damage caused, not only through feeding, but also in the root-invading habits of endoparasites, the economic impact of these types of nematodes is of great concern (Norton, 1983; Norton and Hinz, 1976). When sampling for nematodes of corn, a soil

analysis does not always provide the full spectrum of the genera that may be present. MacGuidwin determined that during the growing season, only 20% of total nematodes found in a nematode analysis were from the soil fraction (MacGuidwin, 1989). Other scientists observed similar results with populations of *P. penetrans*, *P. scribneri* and *H. galeatus* (Merrifield and Ingham, 1996; Miller, et. al., 1963; Norton and Edwards, 1988). Results from nematode assays conducted on samples submitted to the UNL Plant & Pest Diagnostic Clinic for analysis suggest that the soil community may not include any endoparasites (or at low population densities). But, endoparasites were sometimes found in staggering numbers from root analyses. Relying on the soil information alone may not be sufficient in giving accurate recommendations for a farmer.

Along with the characteristics mentioned previously for phytoparasites, these genera are both migratory in their feeding habits and can be either endo- or ectoparasitic. They have a vermiform body shape for the entirety of their life cycle. All motile life stages are infectious (Windham and Edwards, 1999). Their head region is composed of cephalic framework that is highly sclerotized, along with their stylet. Reproduction can be either sexual or parthenogenic, depending upon species. Root exudates attract the nematodes for feeding (Krall, 1978; Ogiga and Estey, 1975). These endoparasites produce nematode-made enzymes to aid in root penetration and utilization of root cortical cells (Khan, 2008). These enzymes include β-glucosidase, cellulose, pectinase, and invertase, among others. As these nematodes migrate through root tissue, cells are destroyed (Duncan and Moens, 2006). Visually, this can be seen as dark brown tissue on the outer surface of infected roots. *Pratylenchus* spp. and *Hoplolaimus* spp. limit their

root penetration to the cortical cells (Krall, 1978; Ogiga and Estey, 1975). Feeding by these genera can also cause fewer feeder roots to develop or more lateral roots to emerge (Ogiga and Estey, 1975; Windham and Edwards, 1999).

Pratylenchus spp., the root-lesion nematode, as stated previously is a highly damaging nematode. It is also extremely common, being found in every agricultural region of the world and on every continent (Castillo and Vovlas, 2007). It is one of the most important nematodes of corn because it is more often associated with corn than any other plant parasitic nematode (Windham and Edwards, 1999). Their geographic distribution is often termed "zonal", referring to the species-specific temperature constraints. In a nematode survey conducted in Nebraska in 2007, 93% of the corn fields tested had *Pratylenchus* spp. present (Jackson, unpublished). Of soil samples submitted to the University of Nebraska's Plant and Pest Diagnostic Clinic from 2008-2011, 83% of samples tested positive for *Pratylenchus* spp. A survey across Nebraska alfalfa and fallow fields from 1993 determined that nematodes from the Pratylenchidae family were more frequently discovered than either Hoplolaimidae or Heteroderidae families, 65% compared to 38% and 12% respectively (Neher, et.al., 1998). There are 68 known species of *Pratylenchus* worldwide, 27 in North America, and at least 5 of those cause significant damage to corn: *P. penetrans*, *P. hexincisus*, *P. scribneri*, *P. brachyurus*, and *P. zeae* (Castillo and Vovlas, 2007; Windham and Edwards, 1999). The first three of these five cause the most damage in the Midwest (Castillo and Vovlas, 2007; Duncan and Moens, 2006, Windham and Edwards, 1999). Yield losses can vary by population density and species, but are estimated at 10% (Castillo and Vovlas, 2007) to 26%

(Windham and Edwards, 1999). Population densities of *Pratylenchus* spp. have been negatively related to yield of corn (McSorley and Dickson, 1989; Tarte, 1971). Parasitism by *Pratylenchus* spp. is well adapted, knowing that severe infestations of the nematode rarely kills host plants (Castillo and Vovlas, 2007). The host range of *Pratylenchus* spp. is quite substantial, including soybean, sorghum, rye, potato, as well as a variety of grasses and weed species (Barker and Olthof, 1976; Bélair et. al., 2007; Castillo and Vovlas, 2007; Jordaan and De Waele, 1988). Pathogenicity varies by species and can be a determining factor in effective use of rotation control methods.

Overall, the genus of *Pratylenchus* has a slender and worm-like body tapering towards the posterior end of the animal. The size of an adult *Pratylenchus* nematode is small and varies by species, but is generally from 36-74 µm (Agrios, 2008; Castillo and Vovlas, 2007). Their head region consists of a flattened, heavily sclerotized cephalic framework and a strong stylet with rounded knobs (Duncan and Moens, 2006). The labial region of the head can be offset slightly from the body by a narrowing of the body contour (Castillo and Vovlas, 2007). *Pratylenchus* spp. feed on all root types and in all areas of the root tips, except the root cap, and within cortical root cells (Windham and Edwards, 1999). They may migrate towards the zone of differentiation and areas of ruptured epidermis where lateral roots are emerging (Duncan and Moens, 2006; Ogiga and Estey, 1975). Maize root presence has been shown to influence *Pratylenchus* spp. egg hatch, as well (De Waele, et. al., 1988). Feeding on corn roots causes dark red-brown necrotic lesions on the root tissue, root pruning, sloughing of cortical tissues, as

well as the development of more lateral roots (Castillo and Vovlas, 2007; Windham and Edwards, 1999).

Hoplolaimus spp., also known as the lance nematode, can be moderately damaging as an ecto- or endoparasite of corn. While not as common as *Pratylenchus* spp., it is still a nematode of concern for corn producers (Neher, et. al., 1998). The University of Nebraska Plant and Pest Diagnostic Clinic observed only a 23% occurrence of *Hoplolaimus* spp. in samples submitted between 2008 and 2011. More than 30 species exist today, but the most common in the United States are *H. columbus* and *H. galeatus* (Decraemer and Geraert, 2006; Windham and Edwards, 1999). This nematode can cause an estimated 26% yield loss in corn (Windham and Edwards, 1999). The main hosts of this nematode include, but are not limited to, corn, wheat, cotton and soybean (Fassuliotis, 1974; Krall, 1978; Lewis and Smith, 1976; Noe, 1993). They may also reproduce on a variety of weed species (Fassuliotis, 1974).

Hoplolaimus spp. is a larger nematode, averaging between 1-2 mm as an adult (Decraemer and Geraert, 2006; Krall, 1978). Not only is the body length longer than *Pratylenchus* spp., it also has a more robust body shape, maintaining the same width from head to tail. The tail is short and bluntly rounded (Decraemer and Geraert, 2006). The lip region of a *Hoplolaimus* spp. is heavily sclerotized and offset from the body in a convex, or cap-like, shape (Decraemer and Geraert, 2006; Krall, 1978). Their powerful stylet has tulip-shaped knobs. *Hoplolaimus* spp. generally feed in the maturation zone of both young and old roots (Fassuliotis, 1975; Ferris and Ferris, 1998).

Several factors lead up to a parasitic relationship of nematodes with host plants. Pathogenicity by nematodes is defined as the capacity of a species to establish a successful host-parasite relationship and by the expression of damage caused in the host (Castillo and Vovlas, 2007). Nematodes have evolved to become plant parasites, establishing specific features necessary for parasitism of plants (Gheysen and Jones, 2006). These features include the stylet, digestive enzymes, and sensory organs (Hussey and Williamson, 1998). For these genera, the stylet is a hollow, strong, needle-like structure that is used to pierce tough plant cell walls, secrete digestive enzymes, and for uptake of cell cytoplasm. Several nematode species secrete enzymes to aid in breaking down cell walls and digesting plant cell contents (Castillo and Vovlas, 2007). These enzymes are produced in the pharyngeal glands of the nematode and are usually only present in plant parasitic bacteria and fungi. The secretory glands are considerably larger in plant parasites than other types of nematodes (Hussey and Williamson, 1998). They have never been found in animals before their discovery in nematodes. Phytoparasitic nematodes rely heavily on chemical stimuli for recognition of suitable hosts, migration in soil and roots, orientation at possible feeding sites (Hussey and Williamson, 1998). The body of nematodes contains numerous sensory organs; these organs sense gradients in various chemicals and environmental factors. The nematode processes these data to direct itself towards food, away from predators, or in the direction of more hospitable environments. Nematodes establish specific feeding sites on root surfaces or in root tissue. The feeding sites have commonalities. They have metabolically active tissues, usually including cytoplasm (Gheysen and Jones, 2006). DNA replication within the site

is another characteristic that is commonly found. Enlarged nuclei or multiple nuclei can also be attractive for feeding sites.

DIAGNOSTIC TOOLS

Morphological characteristics are commonly used to identify plant parasitic nematode genera and species. Several of these characteristics include size, body shape, stylet, tail, esophageal organs, reproductive organs, and cuticular patterns (Windham and Edwards, 1999). Under a dissecting microscope, nematodes can be observed within plant tissues or in water after extraction. For ease, some nematologists prefer to stain nematode-infested plant material for quantification (Khan, 2008). The dyes are used to stain the nematodes, not the plant tissue. This technique is only applicable for those plant specimens with a known nematode population. Staining cannot distinguish between genera, so populations with several endoparasitic genera are not feasible with this type of technique. Using microscopy can be a labor intensive method for quantification, but it is generally inexpensive and fast, given the quality of extracted nematode samples.

The use of molecular diagnostic tools is increasing among nematologists around the world and there are several reasons why. DNA characteristics are not altered by environmental changes, unlike morphological characteristics (Subbotin and Moens, 2006). Molecular characteristics are far more abundant than morphological ones. Protein electrophoresis, DNA sequencing, polymerase chain reaction (PCR), restriction fragment length polymorphisms (RFLPs), PCR-RFLPs and multiplex PCR are all helping laboratories to successfully identify and quantify nematodes to species (Castillo and

Vovlas, 2007; Duncan and Moens, 2006; Subbotin and Moens, 2006). The sensitivity of PCR amplification requires extra care to prevent misdiagnosis due to contamination.

EXTRACTION METHODS

Much research has been completed comparing methods for extracting plant parasitic nematodes from soil (Barker et. al., 1969a; Barker et. al., 1969b; Bell and Watson, 2001; Caveness and Jensen, 1955; Oostenbrink, 1960; Persmark, et. al., 1992; Robinson and Heald, 1989; Seinhorst, 1956; Viglierchio and Schmitt, 1983b; Whitehead and Hemming, 1965). Their results show that methods vary in recovery by soil type and nematode genera. It appears that extraction methods for plant material are still widely variable among nematologists, too. Extraction efficiency within the same method can vary by host plant or nematode genus and species (Chapman, 1957; McSorley et. al., 1984; Prot, et. al., 1993). Among nematology laboratories, many extraction methods have been employed, but there are four common procedures used for extraction of endoparasitic nematodes from plant tissues: aerated incubation, modified Seinhorst mist chamber, modified Baermann funnel, and shaken incubation (Bélair et. al., 2007; Forge et. al., 1998; Georgi et. al., 1983; LaMondia, 2002; Lindsey and Cairns, 1971; MacGuidwin, 1989; Niblack, 1992; Norton and Edwards, 1988; Todd and Oakley, 1996). Within published results studying plant tissue extraction technique comparisons, differing conclusions are found. For several experiments, a modified Baermann funnel method was shown to be one of the most effective extraction methods (Prot, et. al., 1993). However, in other studies, Seinhorst's mistifier extracted with greater efficiency (McSorley, et. al., 1984). Still others show that aeration is the most important method to

use for endoparasites (Chapman, 1957; Minderman, 1956). Among these main methods used, many modifications have been tested for use with specific plant material, nematode genera or species, and time constraints (Bird, 1971; Chapman, 1957; Gowen and Edmunds, 1973; Griesbach, et. al., 1999; Kaplan and Davis, 1990; McSorley, et. al., 1984; Robinson and Heald, 1989; Russel, 1987; Sturrock, 1961; Tarjan, 1960; Tarjan, 1967; Tarjan, 1972; Viglierchio and Schmitt, 1983a; Webster, 1962; Young, 1954).

The Baermann funnel (BF) was one of the original nematode extraction methods, and the basis for several new and modified techniques (Baermann, 1917). The BF utilizes incubation of plant material in shallow water to extract nematodes. Nematodes must be alive to move out of plant material into the water; gravity then pulls the nematodes down through the funnel into a closed tube. After extraction, the nematodes are drained from the tube and the sample is then ready for examination. MacGuidwin found extraction efficiencies for *Pratylenchus scribneri* between 9.5 and 36%, varying by developmental stage (MacGuidwin, 1989). There have been several modifications to the original set-up (Appendix B).

Aerated incubation (AI) involves direct aeration of plant material with a constant flow of air (Ladell, 1936; Filipjev & Stekhoven, 1941). Plant material is placed in a container and water is added. A tube connected to an air source is placed inside the container and the air flow is adjusted to maintain a constant, slow aeration. After extraction, the plant-water suspension is separated from the nematodes and condensed for further examination.

Seinhorst's mistifier (MI) requires plant material to be sprayed with a fine mist of water (Seinhorst, 1950). Similar to the BF, active nematodes move out of plant material but, instead of nematodes being directed by gravity to a closed tube, the nematodes are rinsed by the mist through plant tissue into a collection tube. The extraction is set-up to allow overflow water to be released without disrupting the collection of nematodes. After extraction is complete, the nematode sample is condensed for ease in counting. Extraction efficiency between 41-63% can be expected for endoparasites under this technique (Viglierchio and Schmitt, 1983a). Like the BF, the mist chamber technique has been through several changes over the years (Appendix C).

Using shaker incubation (SI) to extract nematodes is very similar to the set up for the aeration incubation (Chapman, 1957; Minderman, 1956). Roots are placed inside a container and water or incubation solution is added (Bird, 1971). The container is placed on a rotary-arm shaker for the desired amount of extraction time. After the extraction is complete, the plant material and solution are poured through sieves to separate nematodes from plant material. The plant material is carefully rinsed to ensure all nematodes have been removed. The SI and AI methods have also been highly modified since their inception (Appendix D).

RATIONALE FOR RESEARCH

Several studies have shown the importance of extracting nematodes from plant tissues in addition to soil fractions when examining nematode populations (MacGuidwin, 1989; Merrifield and Ingham, 1996; Miller, et. al., 1963). MacGuidwin found that during

the growing season, only 20% of total nematodes found in a nematode analysis were from the soil fraction (MacGuidwin, 1989). She also reported that half of the nematode populations found at planting time reside in dead roots from the previous season. Disregarding the endoparasitic nematode population for diagnostic and advisory purposes is both irresponsible and potentially misleading for producers of corn.

After reviewing several hundred articles for endoparasitic nematode extraction techniques, their modifications, and efficiencies, there are still several questions that must be addressed. One issue of concern found within most method comparison studies is the inconsistencies found for each technique's protocol under observation (McSorley, et. al., 1984; Tarjan, 1967). It is inaccurate to compare extraction rates when there are no consistencies between the protocols as far as tissue collection and preparation, incubation temperature, or length of incubation for each method tested. Additionally, the literature shows differences between extraction method results by nematode examined, time of year, and host (McSorley, et. al., 1984). If this is indeed the case, there needs to be extraction method comparisons performed for every host, time of year, and genus combination to have the most accurate analyses achieved.

As previously discussed, there are high amounts of variability among the roots of the corn plant including function, emergence, and physical attributes. In a study involving endoparasitic nematodes on strawberry, researchers observed preferences for certain root types by nematode populations (LaMondia, 2002). The question can therefore be raised that there is a possibility to see the same trend in endoparasites of maize. Nematologists still disagree about which maize root type should be used to

extract endoparasitic nematodes from for analysis. Some suggest that seminal roots contain the majority of nematode populations early in the season (Georgi, et. al., 1983). Still others believe that sampling from the fine feeder roots can achieve a reasonable population assay (MacGuidwin and Stanger, 1991). Some nematology labs use the root types that best fit their individual preferences, such as equipment availability, space limitations, and personal experiences. Most studies of nematodes in corn roots only tested the two main root types, nodal and seminal (Todd and Oakley, 1996; Zirakparvar, 1979). However, there are more than just two root types on corn; lateral roots, fine feeder roots, and root hairs are also feeding sites for plant parasitic nematodes. Within root types, there are age differences as well; roots emerge throughout the growing season. Knowing that nematodes can migrate during the year, finding the roots they prefer for feeding and when they feed on them is invaluable when evaluating populations and chemical efficacies. Sampling for corn root systems is highly labor intensive and therefore expensive. Finding where these endoparasites feed could provide a chance to avoid sampling the entire root system. The less labor involved with both sampling and processing the roots would be ideal for producers, crop consultants, and researchers of nematodes, as well as the labs that process nematode root analyses. Processing time and cost would be greatly reduced resulting in a more cost effective and quicker analysis.

In the search to find an efficient and effective nematode extraction technique for plant roots, processing time is a key factor for most nematology labs. The potential for loss of nematodes increases directly with the number of steps on the protocol (Viglierchio and Schmitt, 1983b). Some protocols suggest maceration, enzymatic tissue breakdown,

or centrifugation for endoparasitic extraction (Caveness and Jensen, 1955; Fallis, 1943; Gowen and Edmunds, 1973; Kaplan and Davis, 1990; Moore, et. al., 1992). For a laboratory processing several hundred samples at any given time, long and laborious extraction protocols are not cost effective and should be re-evaluated for relevance to lab results. Also, incubation length determines the turnaround time for diagnostics of each sample. Research should be conducted to determine the length of time necessary for a sufficient extraction and whether or not endoparasitic nematode genera have different time requirements.

Maize is an important agricultural crop, especially in the Midwest. Protecting yields from pathogens, including endoparasitic nematodes, is imperative to maintain the increasing need for global food production. Proper management of these pathogens involves detailed and accurate diagnosis in a timely manner. Endoparasitic nematode extraction should be re-evaluated to provide both qualitative and quantitative results of the highest level of accuracy achievable.

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CHAPTER II

COMPARISON OF EXTRACTION METHODS AND CORN ROOT TYPES FOR EFFICIENT EXTRACTION OF ENDOPARASITIC NEMATODES

INTRODUCTION

Maize, commonly known as corn, is the most commonly produced field crop grown in the United States today comprising 50% of the world's total maize production. Nebraska ranked third in 2010 for maize production in the U.S. growing 1.4 billion bushels (National Agricultural Statistics Service, 2010). Every year, demands for corn and other crops steadily increase as the world population increases. Producers need to increase yields without an increase of area in production (Farnham, et. al., 2003). Acquiring the greatest yield from each acre is becoming more important as these demands increase.

Corn has a variety of pathogens that diminish yield potentials every season. Of those, nematodes are some of the least understood by producers. More than 60 species of plant parasitic nematodes feed on corn in North America (Norton, 1983; Windham and Edwards, 1999). The three genera having the greatest economic impact are, in order of importance, cyst (*Heterodera* spp.), root-knot (*Meloidogyne* spp.), and root-lesion (*Pratylenchus* spp.) (Sasser and Freckman, 1987). It is no surprise that these highly detrimental pathogens are all endoparasites. Endoparasitic nematodes can enter the root and feed on the inner root tissues (Decraemer and Hunt, 2006). The entry and internal migration of the endoparasitic nematode can cause significant physical damage to the root system, therefore reducing yield potentials very early in the infection process (Hussey and Williamson, 1998). Openings in the root tissue made by nematodes allow secondary pathogens access to the damaged tissue, further decreasing the plant's ability for growth, production, and in some cases, survival (Duncan and Moens, 2006; Krall,

1978; Windham and Edwards, 1999). In 1994, the estimated loss of corn yield in Nebraska due to phytoparasitic nematodes was 0-1% (Koenning, et al., 1999). However, this equated to over $2 billion dollars lost. This illustrates how relatively small levels of nematode damage, even as little as 1%, can greatly impact commodity production on a much larger scale.

Life cycles of most nematodes have six stages, beginning with an embryo followed by four juvenile stages, then an adult (Decraemer and Hunt, 2006). Eggs can be laid in soil or root tissue (Ferris and Ferris, 1998). On average, life cycles range from 2 to 6 weeks depending on species and environmental factors (Agrios, 2008; Castillo and Vovlas, 2007; Khan, 2008; Windham and Edwards, 1999). Reproduction occurs either between mating partners or through parthenogenesis, where females bear only female offspring without need of fertilization (Windham and Edwards, 1999). Optimum temperature for nematode activity is from 16-32 °C (Windham and Edwards, 1999). Of course, ideal temperatures vary by species, environment, and stage of development (Windham and Edwards, 1999).

Once the nematode is ready to feed, it searches for a suitable host. Nematodes can find host tissues through chemotaxis, chemokinesis, or by random movement within soil (Khan, 2008). The nematode is attracted to root exudates of host plants (Krall, 1978). Once a host is found, the nematode then searches for a suitable feeding site by touching various areas on the root surface (Khan, 2008; Zunke, 1990). The nematode begins feeding with the insertion of its stylet into the root tissue. The stylet is used primarily for feeding and is generally a hollow sclerotized tooth-like structure (Agrios,

2008; Ferris and Ferris, 1998). Most plant parasitic nematodes are obligate parasites, needing live plant material to feed upon (Khan, 2008; Windham and Edwards, 1999). However, many species can feed on a variety of plants, so a suitable host is not always a limiting factor for populations.

Nematode spatial distribution is highly aggregated in soils and can be irregularly distributed within fields (Norton and Niblack, 1991). Soil texture, soil type, moisture, temperature, root distribution and host cultivar/variety can influence the presence and reproduction of phytoparasitic nematodes (Castillo and Vovlas, 2007; Kimpinski et. al., 1976; Norton and Niblack, 1991; Nyczepir and Lewis, 1979). Nematodes can be classified as migratory or sedentary. Migratory plant parasites move frequently, feeding on several areas of the root system. Sedentary nematodes find a suitable feeding source and remain in that location for the rest of their lifetime or the host's lifetime. Nematodes require a film of water for movement through pore spaces between soil particles (Decraemer and Hunt, 2006). Movement in a season can range from 0.3 to 2 meters in a year (Agrios, 2008; Castillo and Vovlas, 2007; Khan, 2008; Windham and Edwards, 1999). Since nematodes have little range of dispersal on their own, their long distance transportation is dependent on other means. Nematodes can be carried by water or wind-blown soil particles and plant tissue, but mechanical transfer of infested material is the primary mode of nematode dispersal (Castillo and Vovlas, 2007; Duncan and Moens, 2006; Norton and Niblack, 1991; Windham and Edwards, 1999). The inconsistency of population distribution within a field causes very high statistical variability when conducting research on nematodes. Having a precise assessment of nematode

populations is imperative for proper management strategies to be implemented (Decraemer and Hunt, 2006). By understanding the fragile and delicate balance of profitability within field crop production, severe economic consequences due to over- or under-management can be avoided.

Symptoms of nematode infection are vague and can often be mistaken for numerous other pathogens as well as abiotic factors (Castillo and Vovlas, 2007; Windham and Edwards, 1999), making accurate diagnosis a more involved process. Symptoms on upper plant parts may include stunting, chlorosis, lodging and wilting (Castillo and Vovlas, 2007; Duncan and Moens, 2006; Norton, 1983; Norton and Hinz, 1976). These symptoms can mimic other known corn problems, such as low fertility, poor drainage, or herbicide injury (Windham and Edwards, 1999). Root systems can exhibit heavily branched root tips, stunted root growth, lack of root hairs, and dark red-brown lesions (Agrios, 2008; Windham and Edwards, 1999). Either by the mechanical or chemical injury to root cells, the nematode renders the root less productive in absorbing water and nutrients from the soil. As feeding continues, cortical root tissue begins to breakdown (Windham and Edwards, 1999). The rate of tissue decay is enhanced as the number of nematodes feeding on the root increases. Studies have shown that corn seminal root damage, as early as three week old plants, can reduce yields up to 9% (Kiesselbach, 1999). Estimates of yield loss by nematode feeding could range from 10-26% in maize (Khan, 2008; Windham and Edwards, 1999). Normal plant stresses can add to the degree of damage accrued on nematode parasitized corn. Also, nematodes are usually found in mixed populations, so determining the actual species that caused yield

loss may be difficult to assess (Windham and Edwards, 1999). The only way to correctly identify a nematode population is by analysis of a sample collected from the infested soil and/or root material (Windham and Edwards, 1999).

Maize has an extensive fibrous root system that requires abundant moisture throughout the year (Farnham, et. al., 2003). There are two main root types: seminal and nodal (Abendroth, et. al., 2011; Kiesselbach, 1999). From these roots grow many lateral roots and fine root hairs. The radicle is the first root to grow from the seed, by several branch or lateral roots (Farnham, et. al., 2003; Kiesselbach, 1999). These roots comprise the seminal root system. Their primary function is water uptake for the first 2-3 weeks after germination, but they have been shown to still function for the duration of the corn plant's life (Abendroth, et. al., 2011; Farnham et. al., 2003; Kiesselbach, 1999). Each set of nodal roots develops in accordance to a leaf emerging from the stalk; therefore, the age of the roots depends on its location within the root system (Abendroth, et. al., 2011; Kiesselbach, 1999). These roots, once present, are responsible for water and nutrient absorption from the soil (Farnham, et. al., 2003). The nodal roots comprise the majority of total corn roots by the emergence of the sixth leaf and for the remainder of the plant's life (Kiesselbach, 1999). From each nodal root, several lateral roots emerge along with numerous root hairs. Lateral roots aid in water uptake and stability of the plant. Root hairs are single-cell extensions of the root epidermis (Kiesselbach, 1999). Their main function is to increase the surface area of the root system. Once established, they become the main root tissues used in water and nutrient absorption. The depth of the root system depends on environmental and soil factors, but generally the root system of corn can be

1-2 meters deep (Abendroth, et. al., 2011; Farnham et. al., 2003; Kiesselbach, 1999; Robertson, et. al., 1979). The plant achieves this depth at maturity, 80-90 days after emergence (Farnham et. al., 2003). For the duration of the life cycle of corn, the majority of root tissue is concentrated in the top 12-15 inches of soil (Robertson, et.al., 1979). There is a positive relationship between root density in the upper 15 cm and corn yield (Kuchenbuch and Barber, 1987). Interestingly, MacGuidwin and Stanger found approximately 50% of *Pratylenchus scribneri* populations were also found in the upper 15 cm of corn root systems (MacGuidwin and Stanger, 1991). *Pratylenchus* spp. have been shown to migrate vertically depending on where feeding sites may be located (Pudasaini, et. al., 2006; Windham and Edwards, 1999). In addition, maize roots have been shown to influence nematode egg hatch (De Waele, et. al., 1988). Knowing this, it seems reasonable to sample for nematodes where the most roots are actively growing, assuming to find the most nematode attraction and egg hatch.

There is some degree of debate where nematodes prefer to feed. Zunke studied the feeding habits of *P. penetrans* on various hosts; his results concluded that the majority of nematodes moved directly to the root hair region of each host, regardless of host type or growth stage of nematode (Zunke, 1990). Georgi et. al., found more *Pratylenchus hexincisus* per gram of root in seminal roots, at least for the first 9 weeks of a corn plant's life (Georgi et. al., 1983). Todd and Oakley detected similar results with *P. neglectus* and *P. scribneri*, but also showed negative correlations between test weights of corn and late-season collections of nematodes from adventitious roots (Todd and Oakley, 1996). Kimpinski, et. al., observed more *Pratylenchus minyus* (syn. *P. neglectus*) in the

seminal roots of wheat than any other root type; they concluded this was due to seminal roots of cereals having greater physiological activity than the other root types tested (Kimpinski, et. al., 1976). LaMondia studied strawberry roots and saw that *P. penetrans* inhabited new feeder roots in much greater quantities than older structural roots (LaMondia, 2002). LaMondia's study agrees with earlier work by Zirakparvar on *P. hexincisus* on corn (Zirakparvar, 1979).

A number of cultural, chemical, and biological techniques for nematode management have been utilized over decades of corn production. Many cultural practices have been shown to aid in reducing nematode population densities. Rotation and cover crops using non-host plants have proven very beneficial in reducing plant parasitic nematodes (Jackson et. al., 2005; Koenning, et. al., 1985; Kratochvil et. al., 2004; McSorley and Gallaher, 1993). Delayed planting dates can influence nematode infection (Koenning et. al., 1985). Leaving land fallow for a growing season is also an effective cultural practice for control of nematodes, however, can be very costly to the producer (Koenning et. al., 1985; Kratochvil, et. al., 2004; Windham, 1998). Tillage practices have demonstrated beneficial effects, but those results vary by nematode species, soil type, host plant and location (McSorely and Gallaher, 1993; Windham and Edwards, 1999). Sanitation is always a good practice for reducing the spread of plant pathogens (Duncan and Moens, 2006). For decades, fumigation was a popular choice for nematode control with methyl bromide being a very popular choice, particularly in high value crops (Nyczepir and Lewis, 1979; Olthof, 1989). However, the use of methyl bromide has been severely limited by government regulations so fumigation has proven less economically

efficient (McKenry et. al., 1994). Several granular and liquid nematicides have exhibited yield increases and nematode control (Johnson and Chalfant, 1973; Norton and Hinz, 1976; Zirakparvar, 1979). However, these chemicals have been shown to kill beneficial soil microorganisms (Nyczepir and Lewis, 1979). Producers have turned to planting seed treated with nematicidal and nematostatic chemicals. Seed treatments are making nematode control much safer, more economical, and more effective (Truelove, et. al., 1977). Fungal antagonists of nematodes can also assist with population reduction (Timper and Brodie, 1993). These predators trap nematodes with adhesive webs or constrictive rings of mycelia. Parasitic bacteria use a similar approach; the bacterial spores adhere to nematodes with sticky exudates (Tian, et. al., 2007). Predatory nematodes are another alternative for plant parasitic nematode control (Bilgrami, et. al., 2008). Host resistance is a popular choice for many plant pathogens. However, it is not a readily available option for all nematode species. Four of the 129 known corn germplasms have successful resistance genes to nematodes (Young, 1998). Migratory nematodes pose a more difficult challenge in finding resistance. Their feeding relationship with host plants is not as intricate or detailed as sedentary parasites and so, unfortunately, breeding efforts have been mostly unsuccessful or limited (De Waele and Elsen, 2002). Integrated pest management (IPM) is an important part of many producer's farm practices. The use of IPM has proven very beneficial to farmers by using multiple techniques for pathogen and pest control. This strategy combines biological, cultural, chemical, and genetic practices to aid in pathogen control all the while reducing the application of chemical products. However, IPM requires greater accuracy and reduced

input costs for identification of pathogens to work more successfully (McSorley and Gallaher, 1993).

Regarding corn production in Nebraska, and most of the Midwest, there are two economically important endoparasitic nematodes: *Pratylenchus* spp. and *Hoplolaimus* spp. Observing the damage caused, not only through feeding, but also in the root-invading habits of endoparasites, the economic impact of these types of nematodes is of great concern (Norton, 1983; Norton and Hinz, 1976). *Pratylenchus* spp. and *Hoplolaimus* spp. have shown feeding preferences for host species, as well as root types within hosts. However, their preferences, if any, have not been well documented within the root types of corn. When sampling for nematodes of corn, a soil analysis does not always provide the full spectrum of the genera that may be present. MacGuidwin determined that during the growing season, only 20% of total nematodes found in a nematode analysis were from the soil fraction (MacGuidwin, 1989). Results from nematode assays conducted on samples submitted to the UNL Plant & Pest Diagnostic Clinic for analyses suggest that the soil community may not include any endoparasites (or at low population densities). Relying on the soil information alone may not be sufficient in giving accurate recommendations for a farmer. These genera are both migratory in their feeding habits and can be either endo- or ectoparasitic. They have a vermiform body shape for the entirety of their life cycles. All motile life stages are infectious (Windham and Edwards, 1999). These endoparasites produce nematode-made enzymes to aid in root penetration and utilization of root cortical cells (Khan, 2008). As these nematodes migrate through root tissue, cells are destroyed (Duncan and Moens, 2006).

Pratylenchus spp., the root-lesion nematode, is a highly damaging nematode. It is also extremely common, being found in every agricultural region of the world and on every continent (Castillo and Vovlas, 2007). It is one of the most important nematodes of corn because it is more often associated with corn than any other plant parasitic nematode (Windham and Edwards, 1999). In a nematode survey conducted in Nebraska in 2007, 93% of the corn fields tested had *Pratylenchus* spp. present (Jackson, unpublished). Of soil samples submitted to the University of Nebraska's Plant and Pest Diagnostic Clinic from 2008-2011, 83% of samples tested positive for *Pratylenchus* spp. There are 68 known species of *Pratylenchus* worldwide, 27 in North America, and at least 5 of those cause significant damage to corn: *P. penetrans*, *P. hexincisus*, *P. scribneri*, *P. brachyurus*, and *P. zeae* (Castillo and Vovlas, 2007; Windham and Edwards, 1999). The first three of these five cause the most damage in the Midwest (Castillo and Vovlas, 2007; Duncan and Moens, 2006, Windham and Edwards, 1999). Yield losses can vary by population density and species, but are estimated at 10% (Castillo and Vovlas, 2007) to 26% (Windham and Edwards, 1999). The host range of *Pratylenchus* spp. is quite substantial, including soybean, sorghum, rye, potato, as well as a variety of grasses and weed species (Barker and Olthof, 1976; Bélair et. al., 2007; Castillo and Vovlas, 2007). *Pratylenchus* spp. feed on all root types and in all areas of the root tips, except the root cap, and within cortical root cells (Windham and Edwards, 1999). They may migrate towards the zone of differentiation and areas of ruptured epidermis where lateral roots are emerging (Duncan and Moens, 2006).

Hoplolaimus spp., also known as the lance nematode, can be moderately damaging as an ecto- or endoparasite of corn. While not as common as *Pratylenchus* spp., it is still a nematode of concern for corn producers (Neher, et. al., 1998). The University of Nebraska Plant and Pest Diagnostic Clinic observed only a 23% occurrence of *Hoplolaimus* spp. in samples submitted between 2008 and 2011. More than 30 species exist today, but the most common in the United States are *H. columbus* and *H. galeatus* (Windham and Edwards, 1999). This nematode can cause an estimated 26% yield loss in corn (Windham and Edwards, 1999). The main hosts of this nematode include, but are not limited to, corn, wheat, cotton and soybean (Fassuliotis, 1974; Krall, 1978). They may also reproduce on a variety of weed species (Fassuliotis, 1974). *Hoplolaimus* spp. generally feed in the maturation zone of both young and old roots (Fassuliotis, 1975; Ferris and Ferris, 1998).

Morphological characteristics are commonly used to identify plant parasitic nematode genera and species. Several of these characteristics include size, body shape, stylet, tail, esophageal organs, reproductive organs, and cuticular patterns (Windham and Edwards, 1999). Using microscopy can be a labor intensive method for quantification, but it is generally inexpensive and fast, given the quality of extracted nematode samples. The use of molecular diagnostic tools is increasing among nematologists around the world and there are several reasons why. DNA characteristics are not altered by environmental changes, unlike morphological characteristics (Subbotin and Moens, 2006). Molecular characteristics are far more abundant than morphological ones. Protein electrophoresis, DNA sequencing, polymerase chain reaction (PCR), restriction fragment

length polymorphisms (RFLPs), PCR-RFLPs and multiplex PCR are all helping laboratories to successfully identify and quantify nematodes to species (Castillo and Vovlas, 2007; Duncan and Moens, 2006; Subbotin and Moens, 2006). The sensitivity of PCR amplification requires extra care to prevent misdiagnosis due to contamination.

Extraction methods for plant material are still widely variable among nematologists. Extraction efficiency within the same method can vary by host plant or nematode genus and species (Chapman, 1957; McSorley et. al., 1984; Prot, et. al., 1993). Four common procedures used for extraction of endoparasitic nematodes from plant tissues are aerated incubation, modified Seinhorst mist chamber, modified Baermann funnel, and shaker incubation (Bélair et. al., 2007; Georgi et. al., 1983; Jackson et. al., 2005; LaMondia, 2002; MacGuidwin, 1989; Todd and Oakley, 1996). The Baermann funnel (BF) utilizes incubation of plant material in shallow water to extract nematodes. Aerated incubation (AI) involves direct aeration of plant material with a constant flow of air (Ladell, 1936). Seinhorst's mister (MI) requires plant material to be sprayed with a fine mist of water (Seinhorst, 1950). Using shaker incubation (SI), plant material is incubated on a rotary-arm shaker.

Disregarding the endoparasitic nematode population for diagnostic and advisory purposes is both irresponsible and potentially misleading for producers of corn. Within published results studying plant tissue extraction technique comparisons, differing conclusions are found. One issue of concern found within most method comparison studies is the inconsistencies found for each technique's protocol under observation (McSorley, et. al., 1984; Tarjan, 1967). Additionally, the literature shows differences

between extraction method results by nematode examined, time of year, and host (McSorley, et. al., 1984). As previously discussed, there are high amounts of variability among the roots of the corn plant including function, emergence, and physical attributes. Nematologists still disagree about which maize root type should be used to extract endoparasitic nematodes for analysis. Most studies of nematodes in corn roots only tested the two main root types, nodal and seminal (Todd and Oakley, 1996; Zirakparvar, 1979). Knowing that nematodes can migrate during the year, finding the roots they prefer for feeding and when they feed on them is invaluable when evaluating populations and chemical efficacies. Sampling for corn root systems is highly labor intensive and therefore expensive. Finding where these endoparasites feed could provide a chance to avoid sampling the entire root system. Processing time and cost would be greatly reduced resulting in a more cost effective and quicker analysis. The potential for loss of nematodes increases directly with the number of steps on the extraction protocol (Viglierchio and Schmitt, 1983). For a laboratory processing several hundred samples at any given time, long and laborious extraction protocols are not cost effective and should be re-evaluated for relevance to lab results.

Maize is an important agricultural crop, especially in the Midwest. Protecting yields from pathogens, including endoparasitic nematodes, is imperative to maintain the increasing need for global food production. Proper management of these pathogens involves detailed and accurate diagnosis in a timely manner. Endoparasitic nematode extraction should be re-evaluated to provide both qualitative and quantitative results of the highest level of accuracy achievable. Nematologists have a responsibility to provide

the most accurate information achievable. The lack of accuracy within nematode quantification can hinder recommendations to clientele, experiment conclusions, and the general understanding of the nematode phylum.

In a preliminary study, it was shown that endoparasites are extracted differently by various extraction methods (Appendix T, Appendix U). It was also observed that fine feeder roots, collected from taking soil cores, yielded the greatest population densities of these nematodes. Seeing these results, an experiment was created to include the major extraction techniques being utilized in corn nematology laboratories in the Midwest. The experiment had three main objectives: 1) to determine the most efficient extraction method for endoparasites of corn, 2) to observe differences, if any, between several root types of corn, and 3) to determine if incubation time impacts nematode extraction.

MATERIALS AND METHODS

The study was a factorial treatment design (6 x 4 x 3) arranged in a randomized complete block with six replications. Each of six root types was tested with four extraction methods. Each combination of root type and extraction method was subjected to three incubation lengths: 3, 5, and 7 days. Corn root samples were collected from three sites in Nebraska: North Bend (Nov. 1 and Nov. 22 of 2009), Ewing (Nov. 8 and Nov. 15 of 2009 and Dec. 2010), and St. Libory (Dec. 2010). Each site was selected for having a documented history of high endoparasitic nematode population densities and had been planted to corn for at least 2 consecutive seasons.

ROOT COLLECTION AND PREPARATION

Within each site, six research plots were randomly selected, each approximately 0.0003 hectare in area. These locations were marked with GPS coordinates, mapped, and sampled for corn roots. Within each plot, four corn plants were removed from the soil by shovel to minimize root damage, for a total of 24 plants collected per site. The area dug was approximately 0.6 m in diameter, trying to include as many roots as possible without damaging them and excess soil was removed by gently tapping. The roots were placed in plastic bags, sealed, and put into insulated coolers for transport to the laboratory. A soil sample was also collected from each of the six plots per site with a soil probe 20.3 cm in length and 2.5 cm in diameter. The soil probe was inserted into the soil profile at approximately 45° angle within 10 cm of the corn stalk. Fifty to sixty soil cores were removed from each of the six plots per site. Soil cores were combined to create a composite sample and mixed in a plastic bag, sealed, and placed in the insulated coolers.

The roots from each location were removed from the insulated cooler and washed with water to remove debris and soil within 24 hours after collection. After washing, roots were placed back in the insulated cooler until processed. The root types were identified and separated from the corn plant with scissors or hand-pruners. The four root systems collected within each plot were combined. The roots in each root type were again washed with water ensuring all debris and soil removal. The roots selected for extraction were: seminal, first nodal, second nodal, third nodal, and fourth nodal. As the root types were cut from the root system, they were placed between wet paper towels to

prevent desiccation during processing. Because of the concern over desiccation, only one sample location was processed at a time.

Once cleaned and separated, each root type was cut into 1 cm or smaller pieces. The root pieces were then mixed by hand to homogenize each root type sample. After mixing, the roots were separated into 1 g aliquots. One aliquot of each root type was arbitrarily assigned into each extraction method. Not all root types, especially seminal roots, had sufficient root mass to make 1 g sub-samples; in these cases, the root mass was divided evenly by weight between the extraction methods tested. After each root type was completed, the protocol was repeated for the next root type until all roots for that research plot were processed. This protocol was repeated for all roots collected in this study.

The final root type examined in this experiment was fine feeder roots. This root type was collected in the soil cores as small root fragments. The soil cores collected were mixed within a plastic bag and root fragments extracted during sieving. A total of 100 cm^3 soil was selected by water displacement for root extraction and manually mixed in water to break clods. The soil suspension was allowed to settle for 10 seconds to let debris and heavy soil particles settle to the bottom. The suspension was decanted through a 25-mesh (710 µm) sieve three times. The sieve was rinsed gently with tap water to remove all small debris and soil. Any large debris (>3mm) was removed with forceps and discarded. The rest of the material and roots left on the sieve were then arbitrarily assigned to one of the four extraction techniques. The soil processing protocol was repeated for each extraction method per research plot.

EXTRACTION METHODS

The four extraction methods examined were a modified Baermann funnel (BF), a modified Seinhorst mist chamber (MI), shaker incubation (SI), and aeration incubation (AI). For the MI extraction in 2009, the chamber was located in a greenhouse head house due to space limitations. This room was kept at 27 °C, whereas the other methods were tested in a lab at 22 °C.

The Baermann funnel (BF) technique, modified from G. Baermann, 1917, was setup similarly to Stoller, 1957. The root material was placed on a 2-ply facial tissue (Kleenex® brand), wrapped with the excess facial tissue and placed on the screen inside a funnel (Anderson & Yanagihara, 1955). The funnel was 65 mm in diameter and made of polypropylene. Tissue weights for each BF were weighed and recorded before testing. The roots were then suspended in distilled water. The water level was adjusted to the base of the screen, but did not exceed more than 1 mm above the screen. A disposable Petri dish was placed on top of the funnel to inhibit evaporation (Robinson and Heald, 1989). A 10.2 cm piece of rubber tubing was attached to the base of the funnel stem and clamped with a polypropylene tubing pinch clamp to prevent leakage. Once the extraction was complete, the nematode suspension in the tubing under the funnel was collected in a 15 mL conical centrifuge tube. For the additional five and seven days of incubation, distilled water was added to the funnel. The nematode suspension was stored in a 6 °C refrigerator until counted. After the seven day extraction was complete, the enclosed tissue paper containing roots was removed and dried in an oven at 60 °C for 48 hours. Dry root weights were recorded, original tissue weights subtracted, and nematode

population densities for each BF unit were calculated and adjusted to reflect nematodes per gram dried root.

The shaker incubation (SI) method protocol was similar to the process described by Jackson, et al., in 2005. Roots were placed in labeled 250 mL glass Erlenmeyer flasks. Twenty to thirty mL of 0.5% chlorhexidine diacetate solution was added to each flask to cover roots. Parafilm® was used to seal each flask to prevent evaporation. Flasks were shaken on a wrist-action shaker at 140 rpm. After three, five, or seven days, flasks were removed from the shaker and contents poured over a 200-mesh (75 µm) sieve nested over a 500-mesh (25 µm) sieve. Flasks were triple rinsed with tap water and poured over the sieves to ensure removal of all root pieces and nematodes. Root pieces were caught on the top sieve while nematodes and small debris passed through to the bottom sieve. The 200-mesh sieve was gently rinsed with tap water to remove debris and nematodes. For the three and five day incubated samples, roots were removed from the sieve and placed back into the flask with a fresh aliquot of chlorhexidine diacetate solution (Hibitane) for further incubation on the shaker. The 500-mesh sieve was rinsed and the nematodes and any debris were removed. Using a funnel, the nematode/debris mixture was washed into a 15 mL conical centrifuge tube and stored in a 6 °C refrigerator prior to counting. For the seven day samples, roots were removed from the sieve and dried similarly to the BF samples. Nematode counts from the SI method were adjusted with the root weights.

The aeration incubation (AI) was set up similarly to the SI method. Root pieces were placed in labeled 250 mL Erlenmeyer flasks, but 100 mL of distilled water was

added to each flask before sealing with Parafilm®. The flasks were placed on the countertop, the Parafilm® pulled back slightly from one edge and an air hose was placed inside each flask. The Parafilm® was maneuvered to aid in holding the air hose in place while sealing off the rest of the mouth of the flask. The air supply was turned on and air flow adjusted to allow a constant, slow bubbling within each flask. The air flow was distributed to individual flasks via multiple hoses by an aquarium air control valve. This allowed each flask to receive a similar air flow from the air supply. After extraction, the contents of each flask were separated by sieving as described for the SI technique. Tubes of nematodes recovered by the method were stored as stated previously for both BF and SI methods. Roots were dried in the same manner as for the SI technique. Nematode population densities were re-calculated to reflect nematodes per gram dried root.

For the Seinhorst mist (MI) method, a mist chamber was built in the Biological Systems Engineering shop on the University of Nebraska-Lincoln campus according to Ayoub's specifications with a few modifications to customize to this study (Ayoub, 1980). The mist chamber was built to reflect space limitations of both the lab and the experiment. The chamber accommodated up to 36 samples simultaneously. Brass misting nozzles were used and had an output of approximately 4.5 L/hr. The 2009 mist chamber had one PVC pipe across the top with three mist nozzles attached. Since the mist overlap was not sufficient for even distribution and recovery of nematodes may have been compromised, a new mist chamber was constructed. The 2010 mist chamber was built with two PVC pipes with a total of five mist nozzles overlapping the floor space of the chamber. The PVC pipe was suspended across the top of the chamber to allow at

least 45.7 cm between the nozzles and the funnels. The nozzles require at least this amount of distance to achieve the maximum width and overlap of spray. The mist chamber was a completely enclosed system to minimize evaporation or escape of mist. The root samples were placed in a pre-weighed tissue onto a modified BF set-up. Contradictory to the BF, the tissue was left open for the MI system and the stem of the funnel was not clamped so water and nematodes coming through the tissue paper could drain freely into a 250 mL Erlenmeyer flask (2009) or a 50 mL conical tube (2010). A piece of wire mesh was bent over the mouth of the collection container stabilizing the funnel while allowing overflow water to exit the tube. The funnels were placed directly under a misting nozzle for extraction. After extraction, contents in each collection container were condensed to 10 mL by pouring through a 500-mesh (25 µm) sieve and washed collected material into a centrifuge tube. The nematode sample was stored at 6 °C until counted. Roots were treated as described previously for three and five incubation samples. Seven day incubation tissue and roots were removed from the funnel and dried similar to the other methods. Dry roots were weighed and nematode population densities adjusted as mentioned previously.

The sum of the nematode populations densities were calculated for 5 and 7 day total nematode extraction. The 3-, 5-, and 7-day totals were the data used for analysis. Nematode population densities were transformed to log 10 (x+1) values before statistical analysis to reduce the correlation between means and variances.

STATISTICAL ANALYSIS

Due to the high variability between populations and locations, the data were not combined between years, nematode genera, or locations. Analysis of variance (ANOVA) for nematode population densities and tests of significance were performed with PROC MIXED in SAS (SAS Institute, Inc, 2006). Using this program, individual sample dates were analyzed for a three-way interaction between root type, extraction method, and incubation length. The data from each sample date were then separated by incubation length. Each incubation length for each location was then tested for a two-way interaction between root type and extraction method. All analyses were performed at $\alpha=0.05$ and $\alpha=0.10$. SAS PROC GLM was used for each analysis to acquire coefficient of variance, r-square, and mean square error values. For those interactions or main effects that were found to be statistically significant, the LSMEANS command was utilized in SAS to compare p-values. These comparisons aided in generating means separation letters to show statistical significance within the levels of factors tested.

RESULTS

Due to variability caused by a malfunction in the mist chamber's mist coverage area, results of all mist extraction treatments were removed for all sites in 2009. *Pratylenchus* spp. were identified at all locations. Ewing exhibited a low population density ranging from 1 to 769 nematodes per gram dried root. North Bend and St. Libory had higher population densities of *Pratylenchus* spp. (115-2,289 and 4-1,131 nematodes per gram dried root, respectively). While *Hoplolaimus* spp. were observed at all

locations, population densities at the North Bend and St. Libory locations were too low for accurate assessments and so were not included in the analyses. Population densities ranged from 1-7 nematodes per gram dried root. At the Ewing location, *Hoplolaimus* spp. population density was 1-1,252 nematodes per gram dried root.

Three-way interactions between root type, extraction method, and incubation time were found for *Pratylenchus* spp. at Ewing Nov. 8, 2009 (*P*=0.0022), Ewing Nov. 15, 2009 (*P*=0.0002), North Bend Nov. 22, 2009 (*P*=0.0025), St. Libory (*P*=0.0038) (Table 1). *Hoplolaimus* spp. was found to have a three-way interaction at only the Ewing Nov. 15, 2009 sample date (*P*=0.0314) (Table 2).

Two-way interactions between root type and extraction method were identified for *Pratylenchus* spp. at several sample dates (Table 3). Ewing Nov. 8, 2009 (Figure 1) was significant at the three day incubation time (*P*=0.0043). The anchor 1 root type on BF had the highest extraction; with anchor 1 and 2 on the AI, these three root type by extraction method combinations had significantly higher nematode population densities than other treatment combinations (Table 5). Ewing Nov. 15, 2009 was significant for only the seven day incubation (*P*=0.0201). Anchor 1 on AI had the highest population density but was not statistically different from anchor 2-4 on AI, feeder roots on AI, seminal on BF, and anchor 2 roots on BF (Figure 2). Ewing Dec. 2010 had a significant interaction for the three day incubation (*P*=0.0010). The highest population density following extraction was from feeder roots in the MI treatment (Figure 3). This was significantly greater than all other root type x extraction method combinations except anchor 1-3 on MI, anchor 1-2 on AI, and feeder roots on AI. Extraction from samples

collected from the North Bend location on Nov. 22, 2009 had significant differences at 3, 5 and 7 day incubation periods (*P*=0.0024, <0.0001, <0.0001, respectively). In the three day incubation treatment of seminal roots on BF and AI and anchor 1 on AI had the greatest recovery rate of *Pratylenchus* spp. (Figure 4). They were found to be statistically similar to anchor 3-4 on AI and anchor 1 on BF. For the five (Appendix N) and seven (Appendix O) day incubation treatments, anchor 1-4 and seminal root types in AI, as well as anchor 1 and seminal root types on BF, were statistically different from all other root and method combinations.

Hoplolaimus spp. nematodes showed significant two-way interactions (Table 4) for all incubation time periods from samples collected at the Ewing location on Nov. 8, 2009 (three day *P*=0.0435, five day *P*=0.0004, seven day *P*=0.0017). Extraction of endoparasites from seminal roots on BF were the greatest yielding for all incubation times. Results from the three day incubation exhibited anchor 1 roots on BF to also be statistically greater than other root type and method combinations (Figure 5). Results from the five and seven day incubation periods showed that feeder roots on AI were statistically similar to the previously mentioned root/method treatments for this location (Appendix G, Appendix H). Endoparasitic nematodes extracted from samples collected at the Ewing location on Nov. 15, 2009 also had significant interactions between root type and extraction method at all time periods (three day *P*=0.0052, five day *P*=<0.0001, seven day *P*=0.0006). For the three day incubation (Figure 6), seminal and anchor 3 root types on BF were the greatest yielding, but not significantly different from all other root types from the BF or anchor 4 on AI extraction techniques. At five and seven days,

seminal roots on BF were statistically significant from all root type x extraction method combinations (Appendix J, Appendix K). The Ewing samples collected Dec. 2010 had interactions during the five day (Figure 7) and seven day (Appendix P) incubations (P=0.0278 and 0.0043, respectively). Both five and seven day incubations were consistent: feeder roots on MI were the highest, but not different from anchor 1-4 on MI or feeder roots on AI.

Main effect significance, either for root type or extraction method, was identified at several locations for *Pratylenchus* spp. Extraction methods were significantly different when used on samples collected from Ewing on Nov. 8, 2009 at five (Table 5) and seven (Appendix F) day incubations (P=<0.0001 for both). AI and BF had significantly greater extraction rates than SI for both incubation times. The samples collected from Ewing one week later on Nov. 15, 2009 showed significant difference between root types and extraction methods for both three day (P=0.0087 and 0.0379, respectively) and five day (P=0.0264 and 0.0008, respectively) incubation. Three day incubation treatments resulted in AI and BF being significantly greater than SI treatments, while anchor 1-4 and feeder roots were statistically different from seminal roots (Table 6). Five day incubation treatments showed similar results, with the exception of BF not being statistically greater than SI (Appendix I). Ewing Dec. 2010 had significant root types and methods for both five (P=0.0039 and <0.0001, respectively) and seven day (P=0.0050 and <0.0001, respectively) incubation periods. Five day extraction periods showed that the AI and MI methods were statistically different from BF or SI (Table 7). Feeder anchor 2 roots were significant from other root types. Seven day incubation (Appendix P) had similar results

with the addition of anchor 3 roots as significant from other types. Samples collected from North Bend on Nov. 1, 2009, after seven days incubation, showed significant differences between extraction methods ($P=0.0025$). BF and AI were found to be statistically greater than SI (Table 8). Samples collected from St. Libory exhibited significant differences among root types and extraction methods for all incubation times ($P<0.0001$ for all). At three day incubation (Table 9), the AI and MI extraction methods extracted significantly more *Pratylenchus* spp. than other methods tested. Five and seven day incubation resulted in MI extraction to be the greatest (Appendix R, S). For all incubation times on samples collected from St. Libory, population densities of nematodes extracted from feeder roots were significantly greater than all other root types.

Three day incubation at the Ewing location on Dec. 2010 was the only *Hoplolaimus* spp. location to show significant main effects (Table 10). Of the root types ($P=0.0003$), feeder roots yielded statistically more nematodes than all other root types examined. For the extraction techniques ($P<0.0001$), MI was significantly different from other methods, showing the highest nematode recovery rate.

DISCUSSION

Variability in extraction efficiencies among differing nematode populations can be caused by numerous factors. There are many environmental characteristics and genetic traits that contribute to life processes for nematodes. These environmental and genetic factors can play a role in the efficiency of endoparasitic nematode extraction. Oxygen and temperature are two factors that have been heavily scrutinized when dealing

with endoparasite extraction. Many of the modifications within the extraction techniques are aimed at targeting the needs of nematodes for the highest quality and quantity of extraction (Appendix B-D). However, some aspects of nematode life cannot be easily manipulated or are not well understood, making "efficient" extraction of endoparasites a relative term.

Sampling date for accurate nematode population densities is important, considering the migratory habits of some genera. For this experiment, locations with high population densities were specifically chosen, as well as delaying sample collection until late fall to ensure the highest possible nematode population densities. While this time frame is not ideal for most research on nematode population dynamics, it may be necessary to observe differences between root types and extraction methods. The late sampling date may have played a role in the variation observed in this study. The Ewing site was sampled twice in 2009 and once in 2010, all in the same six research plots. The population densities of *Hoplolaimus* spp. found in 2010 appeared to have decreased compared to those observed in both sample dates in 2009. In 2010, sampling occurred after the ground froze whereas the sampling conducted in 2009 was prior to cold weather. This may have increased the mortality or occurrence of dormancy in the nematode population, resulting in the overall reduction of extraction yields. The freeze could have also had an impact on the amount of root material that was available for collection. The process of digging in frozen soil may have led to damaged roots, possibly losing more heavily infested root material needed for analysis than the previous year and skewing the results observed.

Root weights are critical for calculating endoparasitic population densities. A very small root weight can skew the population density to seem abnormally large. In this experiment, root sub-samples for use in each extraction method were measured in grams of fresh weight to standardize the root tissue, with the exception of the feeder roots from soil cores. The feeder roots are very fine and the average weight of this root type can be quite small, which could potentially inflate the final population densities. In addition, the methods used to extract the roots from soil particles resulted in the collection of other debris and organic material from the soil as well. Much of this residue was too small to be easily and quickly removed manually from the sieve of feeder roots and would have been included in the calculations of root weight, therefore potentially reducing final population densities of nematodes. However time consuming, it did aid in keeping feeder root weights more consistent with the weights of root material examined for other root types.

In 2009, *Pratylenchus* spp. were extracted from seminal or anchor 1 roots at the greatest rates from North Bend samples and anchor 1 at Ewing on either the AI or BF for both sites. Extractions from seminal roots on BF were consistently the greatest root type for *Hoplolaimus* spp. at Ewing in 2009. This contradicts the 2010 data. It is apparent that the MI extraction method and fine feeder roots resulted in the greatest extraction of both genera in 2010. Interestingly, the SI technique had the poorest results in every test of this experiment.

Based on the population densities, the MI extraction method of fine feeder roots was clearly the better choice of the root types and extraction methods examined, once the

set-up was optimized. The AI method with feeder roots was also consistently one of the top root type by method treatment combinations and often found to be statistically similar to MI. For a high volume throughput laboratory, these techniques may have some disadvantages. For example, they both can require substantial counter space for a large amount of samples to be processed, which can limit overall laboratory productivity. Also, if a greater quantity of samples needs to be processed simultaneously, the additional space and time for extraction can impede other experiments and processes occurring in the laboratory during those periods. In addition, cost can be high for assembling the necessary equipment. The final mist chamber constructed for this experiment cost approximately $1,000, but only had a capacity of up to 36 samples simultaneously. By comparison, the BF set-up cost $160 with plastic funnels ($360 with glass funnels) with a capacity of 36 samples and required no counter space. The AI cost was slightly higher than the BF assuming a constant air supply was already present in the laboratory. If not, expensive specialized equipment would be needed, increasing the overall cost and maintenance. Furthermore, the consistency of extraction for MI relies heavily on the even distribution of the mist generated. During these experiments, several samples received little to no mist during their incubation time despite being placed directly under a mist nozzle. Finally, an additional limitation of the MI and AI extraction techniques was the additional time required for sample processing. After mist extraction was complete, nematode suspensions had to be condensed to a more manageable volume for counting under the microscope, requiring further handling steps that averaged approximately an additional minute per sample. In addition, both the SI and AI methods needed the roots to be separated from the nematode suspension prior to counting with the

microscope. These additional steps increased handling time per sample by at least two minutes and increased the amount of debris within the nematode suspension, especially for the feeder root type. The increased debris made quantification and identification of nematodes in the suspension more difficult, therefore also increasing counting time. Furthermore, nematode suspensions from extractions from the feeder roots on either the AI or SI methods were impossible to accurately count without diluting the sample several times. The BF was the only extraction method that did not require additional time after extraction for preparation of counting. For a laboratory processing a large number of samples, the extra processing and handling time can costly.

While all extraction methods had their advantages and disadvantages, the mist chamber and aerated incubation methods had greater extraction efficiencies than the other methods tested, especially when using feeder roots. However, where space and time are limitations, the modified Baermann funnel method would likely be the best choice. This extraction method provides consistent high-yielding results, clean nematode samples that are less cumbersome to count under the microscope and is inexpensive to set up and maintain. To extract both of the genera examined in this study, it may be necessary to collect both the seminal and anchor 1 root types for the greatest nematode representation. For all of these methods and their indicated root types, three day incubation extracted approximately 50% of the population density that was eventually extracted after seven days. Three days of incubation was adequate for identifying differences between these treatment combinations, even in the sites with low overall population densities. For

advisory purposes, a three day incubation period would be sufficient in most cases. For research purposes, a five or seven day incubation period may provide better accuracy.

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Table 1.

P-Values for Root Type x Extraction Method x Incubation Time Interactions

Pratylenchus spp. Population Densities

Location	Date	Mean	C.V. %	Root MSE	R-Square	Pr > F
Ewing, NE	Nov. 8, 2009	35	21.3	0.328	0.955	0.0022
	Nov. 15, 2009	20	21.3	0.279	0.961	0.0002
	Dec. 2010	10	29.8	0.293	0.965	0.5707
North Bend, NE	Nov. 1, 2009	267	10.1	0.246	0.939	0.7903
	Nov. 22, 2009	329	9.5	0.238	0.959	0.0025
St. Libory, NE	Dec. 2010	100	12.7	0.254	0.960	0.0038

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at $\alpha=0.05$ and $\alpha=0.10$.

Pr>F values represent the test of the hypothesis of an interaction between the levels of each factor: root type, extraction method, incubation time.

Table 2.

P-Values for Root Type x Extraction Method x Incubation Time Interactions
Hoplolaimus spp. Population Densities

Location	Date	Mean	C.V. %	Root MSE	R-Square	Pr > F
Ewing, NE	Nov. 8, 2009	40	13.5	0.216	0.976	0.2364
	Nov. 15, 2009	29	18.9	0.276	0.957	0.0314
	Dec. 2010	11	28.6	0.297	0.967	0.2185

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Pr>F values represent the test of the hypothesis of an interaction between the levels of each factor: root type, extraction method, incubation time.

Table 3.

P-Values for Root Type x Extraction Method Interactions
Pratylenchus spp. Population Densities

Location	Time	Mean	C.V. %	Root MSE	R-Square	Pr > F
Ewing, NE Nov. 8, 2009	3 Day	23	47.5	0.647	0.739	0.0043
	5 Day	42	42.5	0.688	0.675	0.1308
	7 Day	44	42.5	0.697	0.685	0.1413
Ewing, NE Nov. 15, 2009	3 Day	14	63.1	0.725	0.583	0.4168
	5 Day	22	52.5	0.701	0.632	0.4471
	7 Day	27	48.8	0.700	0.620	0.0201
Ewing, NE Dec. 2010	3 Day	7	73.3	0.626	0.728	0.0010
	5 Day	10	69.5	0.699	0.702	0.1582
	7 Day	12	63.2	0.690	0.709	0.1986
North Bend, NE Nov. 1, 2009	3 Day	179	30.2	0.680	0.418	0.8623
	5 Day	284	22.9	0.562	0.474	0.9124
	7 Day	381	15.7	0.405	0.625	0.6311
North Bend, NE Nov. 22, 2009	3 Day	239	23.7	0.563	0.714	0.0024
	5 Day	360	12.9	0.329	0.862	<0.0001
	7 Day	415	12.0	0.314	0.874	<0.0001
St. Libory Dec. 2010	3 Day	70	37.3	0.689	0.614	0.8982
	5 Day	106	29.3	0.593	0.666	0.9518
	7 Day	134	24.0	0.511	0.706	0.2711

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Pr>F values represent the test of the hypothesis of an interaction between the levels of each factor: root type and extraction method.

Table 4.

P-Values for Root Type x Extraction Method Interactions

Hoplolaimus spp. Population Densities

Location	Time	Mean	C.V. %	Root MSE	R-Square	Pr > F
Ewing, NE Nov. 8, 2009	3 Day	25	46.3	0.646	0.667	0.0435
	5 Day	46	32.6	0.543	0.741	0.0004
	7 Day	56	34.0	0.594	0.720	0.0017
Ewing, NE Nov. 15, 2009	3 Day	17	52.0	0.636	0.664	0.0052
	5 Day	33	35.7	0.542	0.732	<0.0001
	7 Day	44	36.3	0.597	0.661	0.0006
Ewing, NE Dec. 2010	3 Day	8	77.3	0.683	0.693	0.2482
	5 Day	12	64.2	0.691	0.730	0.0278
	7 Day	14	59.0	0.683	0.744	0.0043

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at $\alpha=0.05$ and $\alpha=0.10$.

Pr>F values represent the test of the hypothesis of an interaction between the levels of each factor: root type and extraction method.

Table 5.

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

Ewing, NE Nov. 8, 2009 5 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean*
Anchor 1	208	674	13	122 a
Anchor 2	263	55	7	46 ab
Anchor 3	138	45	11	42 ab
Anchor 4	90	33	8	29 b
Feeder	64	45	24	41 ab
Seminal	21	137	3	20 b
Extraction Method Mean	99 a	84 a	9 b	

R-Square	C. V. %	Root MSE	Mean
0.675206	42.5	0.688	1.619

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Root type means only significant at α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Table 6.

Root Type x Extraction Method Interaction
Pratylenchus spp. Population Densities
Ewing, NE Nov. 15, 2009 3 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean
Anchor 1	138	11	15	29 a
Anchor 2	78	26	10	28 a
Anchor 3	15	26	14	18 a
Anchor 4	31	10	5	12 a
Feeder	10	10	21	13 a
Seminal	5	4	3	4 b
Extraction Method Mean	25 a	12 ab	9 b	

R-Square	C. V. %	Root MSE	Mean
0.583	63.1	0.725	1.150

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at $\alpha=0.05$ and $\alpha=0.10$.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Table 7.

Root Type x Extraction Method Interaction
Pratylenchus spp. Population Densities
Ewing, NE Dec. 2010 5 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Mist Mean	Shaker Mean	Root Type Mean
Anchor 1	11	1	21	1	4 bc
Anchor 2	37	9	80	2	15 ab
Anchor 3	21	4	29	15	14 bc
Anchor 4	8	14	21	1	7 bc
Feeder	74	31	207	24	58 a
Seminal	7	7	3	1	3 c
Extraction Method Mean	18 a	7 b	29 a	3 c	

R-Square	C. V. %	Root MSE	Mean
0.702	69.5	0.699	1.006

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Table 8.

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

North Bend, NE Nov. 1, 2009 7 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean*
Anchor 1	1133	906	408	748 a
Anchor 2	595	281	295	367 b
Anchor 3	527	243	177	283 b
Anchor 4	467	506	116	301 b
Feeder	275	276	260	270 b
Seminal	754	397	375	483 ab
Extraction Method Mean	570 a	389 ab	249 b	

R-Square	C. V. %	Root MSE	Mean
0.625	15.7	0.405	2.581

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Root type means only significant at α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Table 9.

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

St. Libory, NE Dec. 2010 3 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Mist Mean	Shaker Mean	Root Type Mean
Anchor 1	282	37	204	39	96 bc
Anchor 2	145	45	161	31	76 bc
Anchor 3	120	44	250	24	75 bc
Anchor 4	35	26	199	17	42 bc
Feeder	314	478	730	193	382 a
Seminal	25	18	18	4	14 c
Extraction Method Mean	105 ab	50 bc	166 a	27 c	

R-Square	C. V. %	Root MSE	Mean
0.614	37.3	0.689	1.847

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Table 10.

Root Type x Extraction Method Interaction

Hoplolaimus spp. Population Densities

Ewing, NE Dec. 2010 3 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Mist Mean	Shaker Mean	Root Type Mean
Anchor 1	1	2	26	1	3 b
Anchor 2	2	12	135	2	9 b
Anchor 3	3	2	31	1	3 b
Anchor 4	3	5	76	1	6 b
Feeder	46	22	303	29	54 a
Seminal	9	5	8	5	6 b
Extraction Method Mean	4 bc	5 b	52 a	3 c	

R-Square	C. V. %	Root MSE	Mean
0.693	77.3	0.683	0.884

Notes:

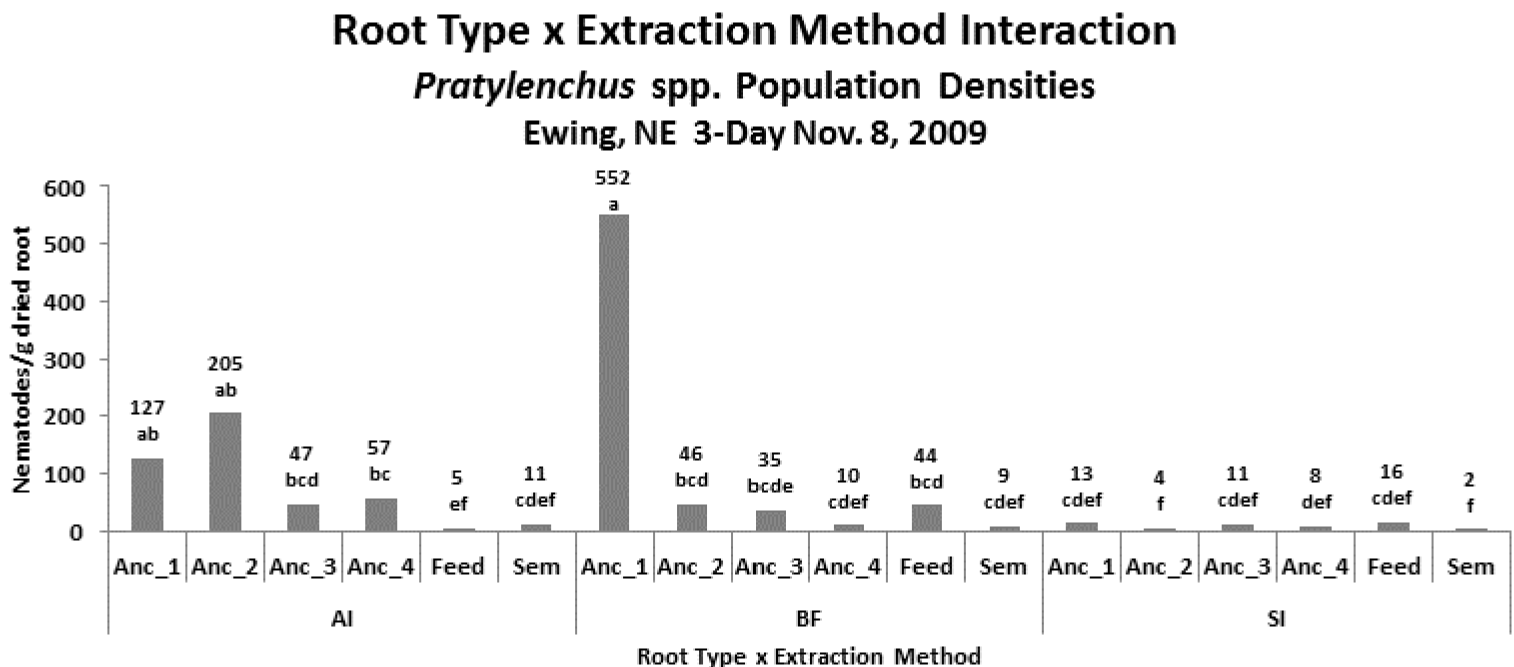
Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

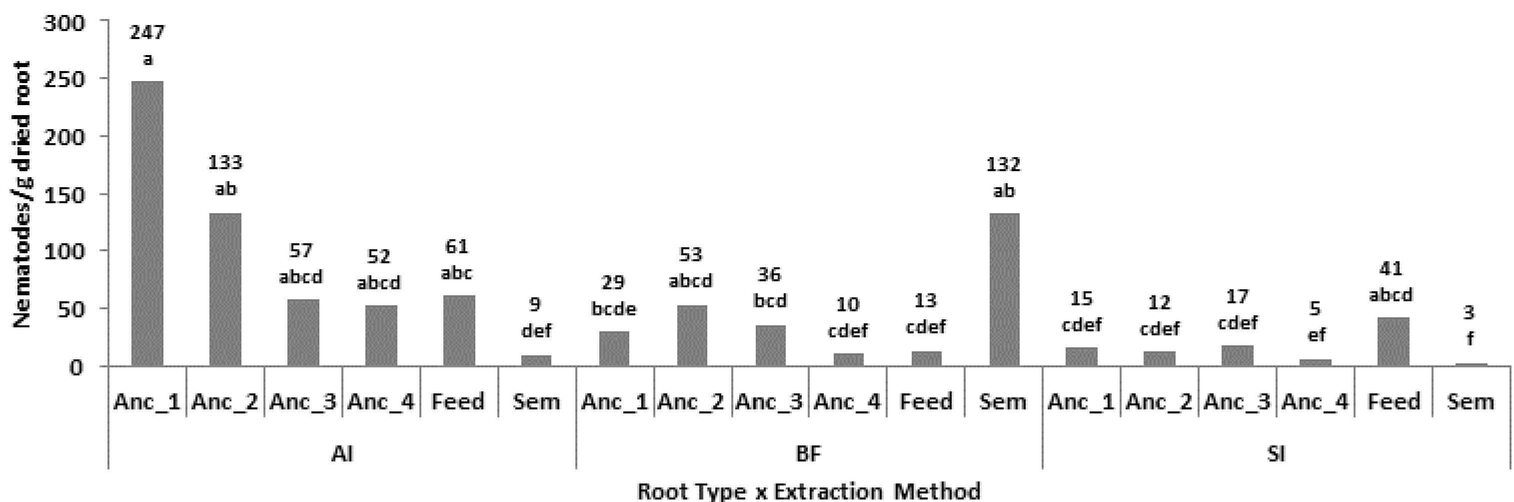
Values followed by the same means separation letter were found to be statistically similar.

Figure 1.



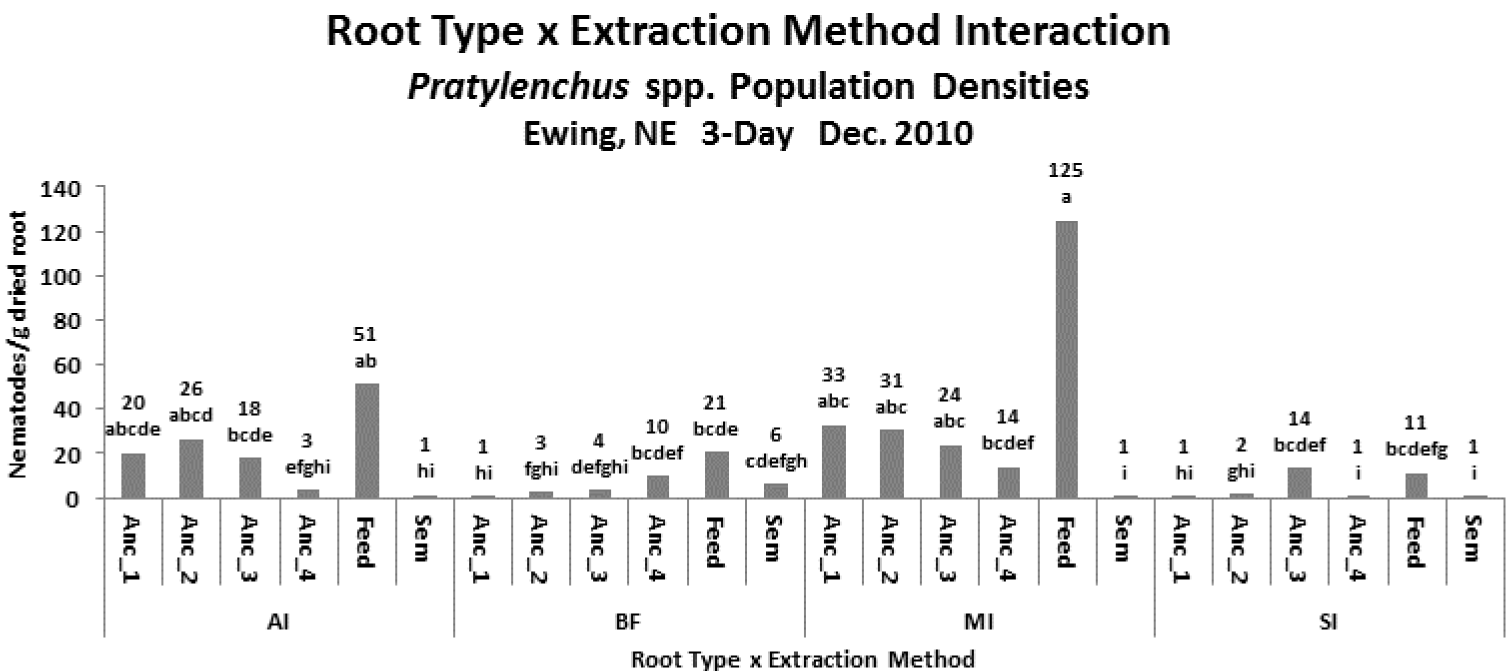
Values with the same means separation letters below were not found to be statistically different according to Fisher's Protected F-Test.

Figure 2.



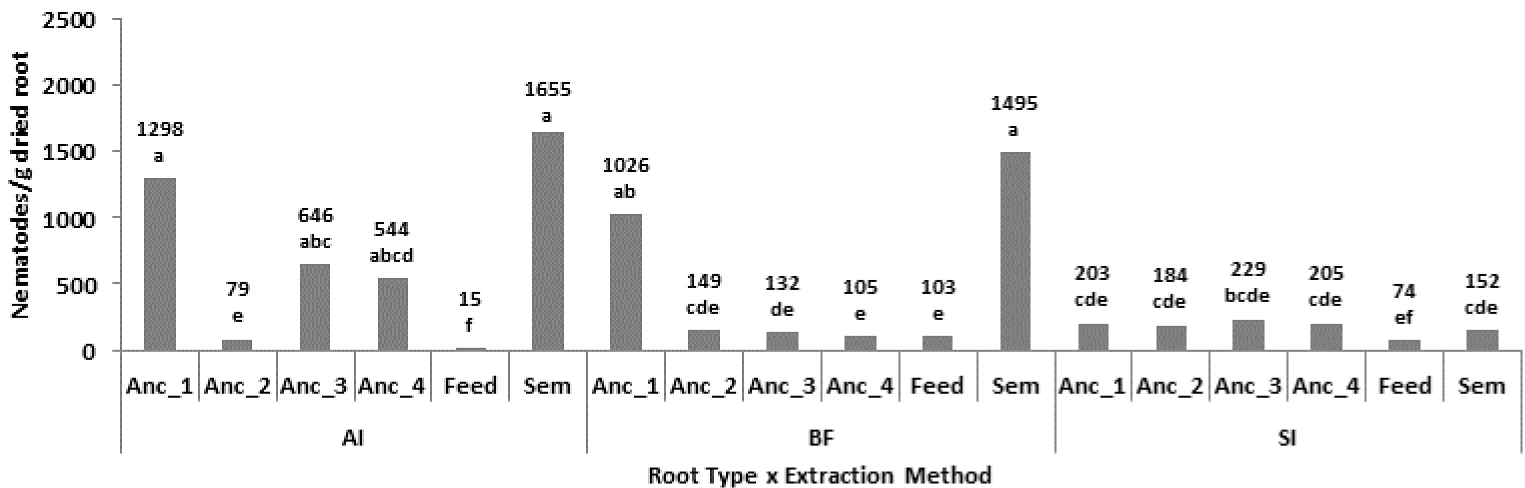
Values with the same means separation letters below were not found to be statistically different according to Fisher's Protected F-Test.

Figure 3.



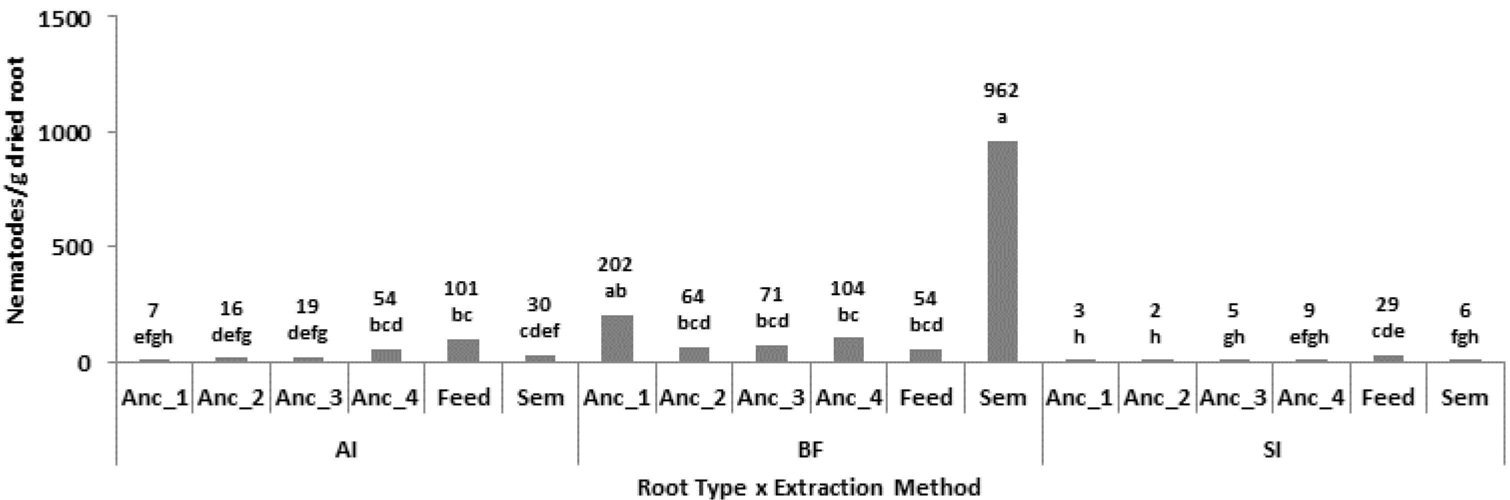
Values with the same means separation letters below were not found to be statistically different according to Fisher's Protected F-Test.

Figure 4.



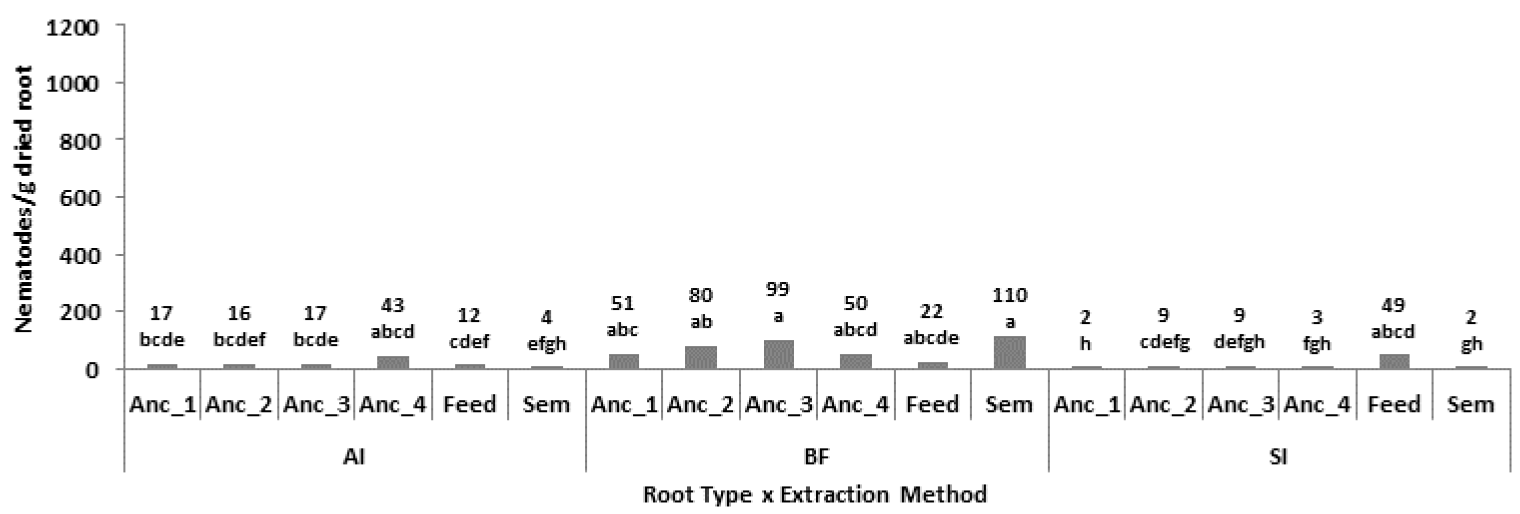
Values with the same means separation letters below were not found to be statistically different according to Fisher's Protected F-Test.

Figure 5.



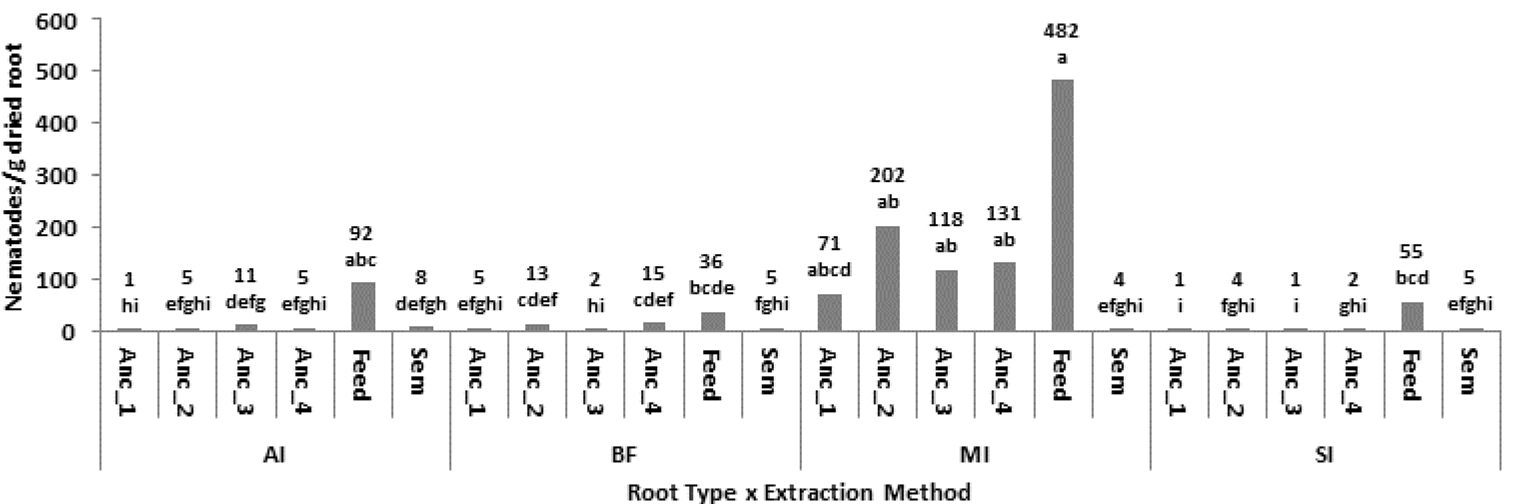
Values with the same means separation letters below were not found to be statistically different according to Fisher's Protected F-Test.

Figure 6.



Values with the same means separation letters below were not found to be statistically different according to Fisher's Protected F-Test.

Figure 7.



Values with the same means separation letters below were not found to be statistically different according to Fisher's Protected F-Test.

Appendix A

Classification of *Hoplolaimus* spp. and *Pratylenchus* spp. (Decraemer and Hunt, 2006)

Taxon	Authority
Phylum Nematoda	Potts, 1932
Class Chromadorea	Inglis, 1983
Subclass Chromadoria	Pearse, 1942
Order Rhabditida	Chitwood, 1933
Suborder Tylenchina	Thorne, 1949
Infraorder Tylenchomorpha	De Ley & Blaxter, 2002
Superfamily Tylenchoidea	Örley, 1880
Family Hoplolaimidae	Filipjev, 1934
Subfamily Hoplolaiminae	Filipjev, 1934
Hoplolaimus	Daday, 1905
Family Pratylenchidae	Thorne, 1949
Subfamily Pratylenchinae	Thorne, 1949
Pratylenchus	Filipjev, 1936

Appendix B

Extraction Method	Modification	Advantage	Disadvantage
Baermann Funnel (Baermann, 1917)	1951-cloth bag used to suspend sample, supported by ring of galvanized wire (Christie & Perry, 1951)	Recovery of active nematodes good (Ayoub, 1980)	Recovery of inactive or sedentary nematodes poor (Ayoub, 1980)
	1954-copper sieve used to support sample; capillary tube at end of funnel used to concentrate nematodes (Staniland, 1954)	Inexpensive materials (Ayoub, 1980)	Recovery from large samples is poor (Ayoub, 1980)
	1955-facial tissue used to contain sample above screen (Anderson & Yanagihara, 1955)	Simple to utilize (Ayoub, 1980)	Funnel too small to be representative (Ayoub, 1980)
	1957-plastic funnel with polythene tube attached to stem for greater oxygen diffusion (Stoller, 1957)	Consistent (Griesbach, et. al., 1999)	Lack of aeration reduces nematode movement (Ayoub, 1980)
	1961-molded wire gauze supports facial tissue inside Petri dish (Schindler, 1961)	Produces clean samples (Griesbach, et. al., 1999)	Tissue in funnel may hinder nematode movement (Ayoub, 1980)
	1989-covering of funnel accelerates nematode movement by maintaining a more constant temperature and reducing evaporation (Robinson and Heald, 1989)		

Appendix C

Extraction Method	Modification	Advantage	Disadvantage
Seinhorst Mistifier (Seinhorst, 1950)	1950?-floor pattern of collection tray changed to concave shape closed with bungs (Peters, 1950?)	Greater recovery due to ideal temperature (Ayoub, 1980)	Expensive and highly specialized equipment (Ayoub, 1980)
	1963-heated water (60 °C) used to improve extraction; intermittent spray of 1.5 min every 10 min (Lownsberry & Serr, 1963)	Downward flow of mist aids in nematode recovery (Ayoub, 1980)	Requires a large amount of space for multiple samples
		No accumulation of toxic materials (Lownsberry and Serr, 1936; Ayoub, 1980)	Recovery of sedentary nematodes poor
			Uneven mist distribution leading to inconsistent results (Sturrock, 1961; Moore, 1992; Griesbach, 1999)

Appendix D

Extraction Method	Modification	Advantage	Disadvantage
Incubation (Ladell, 1936; Filipjev & Stekhoven, 1941)	1954-moist roots left in sealed glass jar; tissue re-wetted periodically with spray bottle (Young, 1954)	Convenient & effective for migratory endo-parasites (Ayoub, 1980)	Recovery of inactive or sedentary nematodes poor (Ayoub, 1980)
	1956-beaker of roots in water intermittently shaken (Minderman, 1956)	Less time required then Baermann funnel or Seinhorst mistifer (Ayoub, 1980)	Tissue must be processed within 24 hours of collection for greatest recover (Ayoub, 1980)
	1957-roots kept in H_2O for 1 day (West, 1957)		Less recovery when compared to Seinhorst mistifier (Ayoub, 1980)
	1960-roots submerged in distilled water with anti-microbial agents; stored at 18 °C; aerated individually and continuously (McKeen & Mountain, 1960)		Shaking can cause samples to become dirty due to excessive plant material breakdown (Chapman, 1957)
	1966-chopped roots in water inside flasks on wrist action shaker for 3 days (Edmunds & Mai, 1966)		
	1967-H_2O_2 used in plastic bag incubation to increase aeration (Tarjan, 1967)		
	1990-maceration enzymes work well with shaken incubation (Kaplan & Davis, 1990)		

Appendix E

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Appendix F

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

Ewing, NE Nov. 8, 2009 7 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean
Anchor 1	227	770	13	131 a
Anchor 2	307	61	7	50 ab
Anchor 3	156	51	12	46 ab
Anchor 4	95	33	9	30 b
Feeder	91	49	26	49 ab
Seminal	23	140	3	20 b
Extraction Method Mean	114 a	90 a	9 b	

R-Square	C. V. %	Root MSE	Mean
0.685247	42.5	0.697	1.642

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Appendix G

Root Type x Extraction Method Interaction

Hoplolaimus spp. Population Densities

Ewing, NE Nov. 8, 2009 5 Day Incubation

Root Type	Aerated Mean		Baermann Mean		Shaker Mean		Root Type Mean	
Anchor 1	23	efg	421	ab	3	h	31	bc
Anchor 2	58	cdef	89	cde	4	h	27	c
Anchor 3	73	cde	111	bcd	9	gh	42	bc
Anchor 4	92	cde	133	bcd	15	fg	57	ab
Feeder	196	abc	67	cde	98	cd	109	a
Seminal	43	def	894	a	8	gh	68	ab
Extraction Method Mean	65	b	179	a	10	c		

R-Square	C. V. %	Root MSE	Mean
0.741	32.6	0.543	1.666

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

A significant two-way interaction was found at this location for this incubation time between root types and extraction methods.

Values followed by the same means separation letter were found to be statistically similar.

Appendix H

Root Type x Extraction Method Interaction

Hoplolaimus spp. Population Densities

Ewing, NE Nov. 8, 2009 7 Day Incubation

Root Type	Aerated Mean		Baermann Mean		Shaker Mean		Root Type Mean	
Anchor 1	34	efg	462	ab	4	i	38	bc
Anchor 2	66	cdef	93	cde	5	hi	32	c
Anchor 3	107	bcde	137	bcde	10	ghi	53	bc
Anchor 4	118	bcde	156	bcd	17	fgh	67	abc
Feeder	269	abc	84	cde	127	bcde	142	a
Seminal	43	defg	1252	a	9	ghi	80	ab
Extraction Method Mean	83	b	214	a	12	c		

R-Square	C. V. %	Root MSE	Mean
0.720	34.0	0.594	1.749

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

A significant two-way interaction was found at this location for this incubation time between root types and extraction methods.

Values followed by the same means separation letter were found to be statistically similar.

Appendix I

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

Ewing, NE Nov. 15, 2009 5 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean
Anchor 1	215	29	15	45 a
Anchor 2	114	46	12	40 a
Anchor 3	54	36	17	32 a
Anchor 4	48	10	5	14 ab
Feeder	36	12	29	23 a
Seminal	5	12	3	5 b
Extraction Method Mean	48 a	20 b	10 b	

R-Square	C. V. %	Root MSE	Mean
0.631518	52.5	0.701	1.334

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at $\alpha=0.05$ and $\alpha=0.10$.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Appendix J

Root Type x Extraction Method Interaction

Hoplolaimus spp. Population Densities

Ewing, NE Nov. 15, 2009 5 Day Incubation

Root Type	Aerated Mean		Baermann Mean		Shaker Mean		Root Type Mean	
Anchor 1	26	bcdef	63	bcd	3	g	16	c
Anchor 2	16	def	114	b	18	cdef	32	abc
Anchor 3	53	bcd	113	b	11	efg	40	ab
Anchor 4	73	bc	51	bcd	8	fg	31	bc
Feeder	97	b	35	bcde	87	b	67	a
Seminal	18	cdef	740	a	3	g	35	abc
Extraction Method Mean	38	b	101	a	10	c		

R-Square	C. V. %	Root MSE	Mean
0.732	35.7	0.542	1.518

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

A significant two-way interaction was found at this location for this incubation time between root types and extraction methods.

Values followed by the same means separation letter were found to be statistically similar.

Appendix K

Root Type x Extraction Method Interaction

Hoplolaimus spp. Population Densities

Ewing, NE Nov. 15, 2009 7 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean
Anchor 1	38 bcd	76 bc	5 e	24
Anchor 2	39 bcd	114 b	19 cde	44
Anchor 3	68 bc	116 b	13 de	47
Anchor 4	97 b	65 bc	13 de	44
Feeder	150 b	43 bcd	107 b	88
Seminal	19 cde	1015 a	5 e	47
Extraction Method Mean	55 b	119 a	14 c	

R-Square	C. V. %	Root MSE	Mean
0.661	36.3	0.597	1.644

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at $\alpha=0.05$ and $\alpha=0.10$.

A significant two-way interaction was found at this location for this incubation time between root types and extraction methods.

Values followed by the same means separation letter were found to be statistically similar.

Appendix L

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

North Bend, NE Nov. 1, 2009 3 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean
Anchor 1	88	431	202	197
Anchor 2	202	206	176	194
Anchor 3	115	111	119	115
Anchor 4	225	284	81	173
Feeder	80	242	155	144
Seminal	377	260	264	296
Extraction Method Mean	155	237	155	

R-Square	C. V. %	Root MSE	Mean
0.418	30.2	0.680	2.252

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Data were not found to be statistically significant at this location for this incubation time.

Appendix M

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

North Bend, NE Nov. 1, 2009 5 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Shaker Mean	Root Type Mean
Anchor 1	324	744	383	452
Anchor 2	363	248	276	292
Anchor 3	203	209	165	191
Anchor 4	290	458	106	241
Feeder	190	263	212	220
Seminal	530	344	365	405
Extraction Method Mean	298	342	229	

R-Square	C. V. %	Root MSE	Mean
0.474	22.9	0.562	2.453

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Data were not found to be statistically significant at this location for this incubation time.

Appendix N

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

North Bend, NE Nov. 22, 2009 5 Day Incubation

Root Type	Aerated Mean		Baermann Mean		Shaker Mean		Root Type Mean	
Anchor 1	1763	a	1557	a	259	b	892	a
Anchor 2	921	a	166	bc	216	b	321	b
Anchor 3	822	a	161	bc	271	b	330	b
Anchor 4	834	a	122	bc	229	b	285	b
Feeder	76	c	113	bc	107	bc	97	c
Seminal	2027	a	1733	a	159	bc	824	a
Extraction Method Mean	745	a	316	b	197	c		

R-Square	C. V. %	Root MSE	Mean
0.862	12.9	0.329	2.557

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

A significant two-way interaction was found at this location for this incubation time between root types and extraction methods.

Values followed by the same means separation letter were found to be statistically similar.

Appendix O

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

North Bend, NE Nov. 22, 2009 7 Day Incubation

Root Type	Aerated Mean		Baermann Mean		Shaker Mean		Root Type Mean	
Anchor 1	1929	a	1869	a	285	b	1009	a
Anchor 2	1233	a	194	b	233	b	383	b
Anchor 3	953	a	194	b	302	b	382	b
Anchor 4	906	a	133	b	245	b	309	b
Feeder	126	b	116	b	127	b	123	c
Seminal	2289	a	1794	a	173	b	892	a
Extraction Method Mean	916	a	353	b	218	c		

R-Square	C. V. %	Root MSE	Mean
0.874	12.0	0.314	2.618

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

A significant two-way interaction was found at this location for this incubation time between root types and extraction methods.

Values followed by the same means separation letter were found to be statistically similar.

Appendix P

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

Ewing, NE Dec. 2010 7 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Mist Mean	Shaker Mean	Root Type Mean
Anchor 1	34	2	28	1	7 bc
Anchor 2	38	9	94	2	16 ab
Anchor 3	36	7	47	16	21 ab
Anchor 4	8	15	35	1	8 bc
Feeder	88	34	244	24	65 a
Seminal	7	7	4	1	4 c
Extraction Method Mean	25 a	9 b	40 a	3 c	

R-Square	C. V. %	Root MSE	Mean
0.709	63.2	0.690	1.091

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Appendix Q

Root Type x Extraction Method Interaction

Hoplolaimus spp. Population Densities

Ewing, NE Dec. 2010 7 Day Incubation

Root Type	Aerated Mean		Baermann Mean		Mist Mean		Shaker Mean		Root Type Mean	
Anchor 1	1	gh	5	efgh	75	abcd	1	h	4	c
Anchor 2	10	defg	13	cdef	259	ab	4	efgh	19	b
Anchor 3	11	cdef	3	efgh	140	ab	1	h	8	bc
Anchor 4	5	efgh	16	cde	141	ab	2	fgh	12	bc
Feeder	121	ab	41	bcd	506	a	78	bc	118	a
Seminal	49	bcd	5	efgh	4	efgh	5	efgh	9	bc
Extraction Method Mean	12	b	9	b	97	a	4	c		

R-Square	C. V. %	Root MSE	Mean
0.744	59.0	0.683	1.159

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

A significant two-way interaction was found at this location for this incubation time between root types and extraction methods.

Values followed by the same means separation letter were found to be statistically similar.

Appendix R

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

St. Libory, NE Dec. 2010 5 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Mist Mean	Shaker Mean	Root Type Mean
Anchor 1	330	178	355	44	174 b
Anchor 2	172	66	497	34	117 bc
Anchor 3	181	49	310	25	92 bc
Anchor 4	69	35	246	31	66 c
Feeder	544	688	999	226	539 a
Seminal	35	19	66	4	21 d
Extraction Method Mean	155 b	80 c	310 a	32 d	

R-Square	C. V. %	Root MSE	Mean
0.666	29.3	0.593	2.025

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at α=0.05 and α=0.10.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Appendix S

Root Type x Extraction Method Interaction

Pratylenchus spp. Population Densities

St. Libory, NE Dec. 2010 7 Day Incubation

Root Type	Aerated Mean	Baermann Mean	Mist Mean	Shaker Mean	Root Type Mean
Anchor 1	346	263	406	46	203 b
Anchor 2	177	72	577	53	140 b
Anchor 3	209	55	331	42	112 bc
Anchor 4	77	38	273	31	71 cd
Feeder	647	801	1131	274	633 a
Seminal	164	42	185	4	49 d
Extraction Method Mean	217 b	105 c	405 a	40 d	

R-Square	C. V. %	Root MSE	Mean
0.706	24.0	0.511	2.127

Notes:

Population Densities expressed as nematodes/gram dried root.

Interactions were tested at $\alpha=0.05$ and $\alpha=0.10$.

Only the main effects were found to be statistically significant at this location and incubation time.

Values followed by the same means separation letter were found to be statistically similar.

Appendix T

Preliminary Extraction Method Comparison

North Bend, NE Sept. 2007

	Pratylenchus spp.			*Hoplolaimus* spp.		
	BF	MI	SI	BF	MI	SI
Anchor 1	206	12	178	16	5	75
Anchor 2	531	4	417	12	0	81
Anchor 3	1577	0	665	18	0	108
Anchor 4	668	3	602	5	0	94
Feeder	3101	.	.	63	.	.
Seminal	678	0	73	29	0	27

* Data were not analyzed statistically due to incomplete factorial treatment design—
Feeder root type not tested on MI or SI extraction methods.

** Shaker extraction method had a two day incubation period, not three day as BF and MI had.

*** Mist chamber used for MI extraction was not reliable and did not provide consistent water output evenly distributed across chamber.

Appendix U

Preliminary Extraction Method Comparison

North Bend, NE Oct. 2007

	Pratylenchus spp.			*Hoplolaimus* spp.		
	BF	MI	SI	BF	MI	SI
Anchor 1	683	9	99	25	1	52
Anchor 2	903	7	150	16	2	36
Anchor 3	1018	2	303	14	0	50
Anchor 4	3211	1	448	69	0	95
Feeder	2918	.	.	77	.	.
Seminal	1016	0	22	104	0	20

* Data were not analyzed statistically due to incomplete factorial treatment design—Feeder root type not tested on MI or SI extraction methods.

** Shaker extraction method had a two day incubation period, not three day as BF and MI had.

*** Mist chamber used for MI extraction was not reliable and did not provide consistent water output evenly distributed across chamber.