Identification of a new nematode species in Ohio and soil factor effects on plant nutrition of soybean

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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2016

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Abstract

Plant nutrition is of great importance to soybean [*Glycine max* (L.) Merr.] growth and grain yield. Nutrient analysis is often difficult to interpret due to the compounding interactions in the soybean rhizosphere. A state-wide survey of Ohio soybean production was done with two objectives: 1) to assess the status of soil fertility and plant nutrition; and 2) to determine the impact of soil factors on the relationship of nutrient uptake to the plant from the soil. Sampling was conducted from 2013 through 2015 in Ohio resulting in 588 total samples. Soil-test and tissue concentrations of phosphorus (P) and potassium (K) were taken as well as soil-test levels of pH, cation exchange capacity (CEC), soil texture, and nematode population densities. Low correlations were observed between the soil and tissue tests with R^2 values of 0.1539 and 0.36781, for P and K respectively. We found that 32.9% of the P soil samples tested below the critical soil test range, but only 2.7% of the samples were below tissue-test critical levels for P, while 23.4% of the K soil test samples were found to be below the critical levels and only 5.9% of the K tissue tests fell below the critical level. All of the soil factors tested in a step-wise regression model were found to influence P uptake, while only soil K, CEC, and Soybean cyst nematode (SCN) were found to influence K uptake. During the nematode analysis, a new species for Ohio was identified as *Paratylenchus neoamblycephalus* with the aid of morphometric, morphological, and molecular examination. We recommend the aid of

tissue-testing as well as a secure knowledge of the soil factors that influence the uptake of nutrients from the soil into the soybean plant.

Acknowledgments

I first thank Dr. Laura Lindsey for advising me during my time as a graduate student at The Ohio State University. She was not only instrumental in helping me succeed as a graduate student but was always willing to provide guidance and advice for my future. Without her support and assistance, I would likely not have decided to continue my education and pursue my PhD. I would also like to thank my committee members Dr. Terry Niblack, and Dr. Kent Harrison for all of their assistance throughout my master's degree program. Their willingness to meet and share their wisdom was immeasurable to my project. Also, thank you to the Ohio Soybean Council for funding this research and to the Dr. Terry Niblack and Dr. Libby Dayton lab groups for sample analysis.

I am also very thankful for all those who assisted with sampling and data collection for my project including Ohio State Extension agents, JD Bethel, Chris Kroon van Diest, Matt Hankinson, and Sin Joe Ng. I would especially like to thank my lab partner Aaron Brooker who also worked on this project. He kept me guided me through the confusing world that is agronomy and accepted my city ways. His words of wisdom and kind nature helped me through the difficult writing process and made even the most medial task entertaining and fun. He was a fantastic partner in every sense of the word.

Finally, I would like to thank my family, friends, and especially Steve for supporting me in not only this process, but in all aspects of my life. I would not be in this position if not for their encouragement and love.

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Chapter 1: Literature Review

1.1 Ohio Soybean Production

Soybean, *Glycine max* (L.) Merr., is an annual herbaceous legume that accounts for one half of the world's oilseed and protein meal (Oerke, 2006). Though this crop originated in eastern Asia in the 11th century, it has become a staple crop for farms in temperate to sub-tropical latitudes. After the introduction of soybeans to the U.S. in the 1900s, it was quickly a crop of interest being tested and promoted as a forage crop. Soybean production soon grew into the U.S. Corn Belt, and the U.S. is the world's top soybean producer with production in 2015 totaling 108.14 billion kg harvested which is a 2% increase from 2014 (NASS, 2015).

According to the USDA National Agricultural Statistics Service (NASS, 2015), there has been a steady increase in Ohio soybean yield through the years from 1,906.8 kg/ha in 1965 to 3,575.25 kg/ha in 2014. With both genetic advancements and changes in agronomic practices, soybean yield has been able to improve, but not enough to keep up with the demand (Specht et al., 1999). In the 2014 growing season, the U.S. exported a total of over 43.53 billion kg of whole soybeans, 13.18 billion kg of soybean meal, and 4.39 billion kg of soybean oil. Overall, these export amounts account for 62% of the total U.S. soybean production from 2014 (USDA, 2015). A further increase of yield is the only way to meet the demand of our projected population increase to 9 billion people world-wide (Specht et al., 1999).

1.2 Soybean Uses

After soybeans are processed, the oil and meal components have a variety of uses. The oil can serve as a food product, has industrial uses, and can even serve as biodiesel. The meal serves as a nutritious livestock feed. With so many known uses of soybean already discovered, it is understandable that there exists an ever increasing amount of research for further applications of soybean production. Soybean is the most widely planted crop in Ohio, accounting for over 1.944 million ha of planted cropland (NASS, 2014). Annually, soybean production in Ohio is a $5.3-billion-dollar business with 26,000 soybean farmers currently active in the state. Ohio ranks 8th overall in the U.S. for soybean acreage, and 6th in the nation for overall soybean production (NASS, 2014).

1.3 Soybean Yield Limitations

There are many yield-limiting factors in a soybean production system, and disease and injury from plant-parasitic nematodes are of great importance. Not only do nematodes rob the plant of nutrients by withdrawing contents through their spear-like stylet, but the wound left as a result of the nematode penetration allows secondary microbial invasion which can often lead to more injury to the host plant. Annually, Barker et al. (1994) estimated that nematodes cause a loss in value of both food and fiber crops worth $78 billion worldwide and about $8 billion in the U.S. Others estimate that plant-parasitic nematodes cause more than $100 billion in losses per year world-wide (Opperman and Bird, 1998). The presence of plant-parasitic nematodes causes many physiological changes in the soybean plant. Water and nutrient relations in both the root and the shoot become altered, leading to poor soybean growth and lowered plant

productivity (Melakeberhan, 1997). The soybean is susceptible to a collection of soil-borne nematode pathogens that feed both endo- and ectoparasitically on the plant's roots. There are 197 genera and 4,300 species of nematode phytoparasites (Lambert and Bekal, 2002). These obligate feeders all have a protrusible, hollow stylet in which to puncture and feed on plant cells (Dordas, 2009).

Roundworms that belong to the phylum Nematoda are the most abundant animals on earth. While most of these creatures are free-living, there is a portion that parasitizes animals or plants. Soil nematodes are extremely small (0.3-5.0 mm long as adults), but are found in a wide range of soil types and in high abundance, commonly in the millions (Yeates, 1979). These worm-like animals are invertebrates and, like many insect species, they undergo molts during their life cycles. Typically, there are four molts from the egg to adult stage, and the second molt produces the infective second-stage juvenile (J2), Nematodes, as omnipresent as they are, have limitations in their ability to thrive in soil systems. They are completely dependent on continuous soil water films in order to move, feed, and reproduce. Nematodes that are obligate parasites of plant tissue are largely controlled by the soil biological and physical conditions (Yeates and Bongers, 1999). Plant-parasitic nematodes have stylet that allows them to penetrate and feed upon plant tissue. The stylet is cuticularized and found in the anterior end of the nematode that functions like a syringe to secrete enzymes and withdraw plant contents through muscular pumping. Plant-parasitic nematodes have a large impact on agricultural systems as different species can attack any part of a growing plant; from the roots to the leaves and bulbs (Barker et al., 1998). Plant-parasitic nematodes cause huge losses in crop yield by suppressing plant growth with their feeding.

1.4 Soybean Cyst Nematode

Of all the nematodes that feed on soybean, the biggest threat is from *Heterodera glycines* Inchinohe, the soybean cyst nematode (SCN). Soybean cyst nematode has been known to cause stunted top growth and root pruning from plant invasion and feeding, but mineral element deficiency is also associated with SCN infection (Blevins et al., 1995). Soybean cyst nematode originated in Asia and was first described in 1952 when the disease it causes was given the name of "soybean yellow dwarf" due to the yellowed areas of poor soybean growth associated with infection. Today, SCN infestations are found world-wide anywhere soybean is grown (Wrather et al. 2001). In the United States, SCN was first discovered in North Carolina in 1954, and has spread to 26 eastern states from Minnesota to Florida. A conservative estimate of the losses caused by SCN based on the assumption of a mean of 3% direct loss from the nematode is over $1.1 billion annually worldwide (Schmitt et al., 2004).

1.4.1 SCN Feeding and Life Cycle

Soybean cyst nematode is a sedentary endoparasite, meaning it feeds from within the root and is not motile once the feeding site is established. To begin the life cycle, the nematode senses environmental and host plant cues to hatch after initially molting once within the egg to its second-stage juvenile (J2) form. These J2s are the infective form of SCN, using their stylet to pierce plant epidermal cells, penetrate the host roots, and migrate to the vascular tissue to establish a feeding site, known as a syncytium (Jones, 1981). Once this feeding site is established, the nematode continues its development into the adult form, which involves sexual differentiation. The male form will retain its somatic muscles in order to leave the root after infection and search for a mate while the

female will lose her somatic musculature and her head will not leave the feeding site. The female nematode swells until the posterior portion of her body protrudes from the root, and deposits 50 to 400 eggs (Triantaphyllou and Hirschmann, 1962) into a gelatinous matrix. Up to several hundred eggs are retained within the exposed female body, which hardens and forms a cyst allowing for the survival of the eggs over winter and lack of a host for many decades, greatly complicating pest management (Young, 1982). This entire cycle can take about 25-40 days.

1.5 Pin Nematode

The pin nematode, *Paratylenchus* spp., the smallest of all the plant-parasitic nematodes, is a migratory ectoparasite. Like most nematodes, the pin nematode has four juvenile stages, but has a unique fourth juvenile stage (J4) in which no feeding occurs and the stylet is greatly reduced in appearance (Rhodes and Linford, 1961). Pin nematodes do not remain in the non-feeding J4 stage for extended periods and soon molt to stylet-bearing feeding adults if the environment allows for it (Eck, 1970). The pin nematode feeds on the lateral root hairs, rather than the main root. Likely, because of their small size, it is easier to feed on proportionally sized roots (Eck, 1970). Feeding is often done on newly emerged lateral roots of the host, but pin nematodes can be found along any part of the outside of the root.

1.5.1 Pin Nematode Feeding

As soon as the J2 stylet has impaled the plant cell, a series of secretions from the nematode will cause cellular changes in the host that allow the nematode to absorb nutrients via pumping through its median bulb muscles. The pin nematode is a solitary

feeder, meaning only one individual nematode will feed from one location. This feeding can occur for up to six days in a single feeding site and will not stop until the nematode development continues with a further molt. The nematode will then migrate along the plant root to a new, suitable feeding site and the process begins again (Eck, 1970). Although known as an ectoparasitic feeder, Rhoades and Linford (1970) observed pin nematodes taking advantage of already-present entry points in a host plant and entering the root entirely to feed endoparasitically. This observation may explain why pin nematodes are occasionally reported from root samples as well as in soil samples.

1.5.2 Pin Nematode Symptoms

In host plants, symptoms range from no noticeable pathology to shallow and localized lesions near the feeding site (Raski and Radewald, 1958). Because these nematodes feed for days at a time, often the rate at which the root will grow is reduced and subsequent root development can even be completely halted (Faulkner, 1964). Plant responses result in a reduced epidermal surface area which can lead to nutrient absorption issues by the host. These symptoms can become more obvious with pin population density increases over several growing seasons (Eck, 1970).

1.6 Importance of Soil Fertility

Proper timing and amount of applied plant nutrients are critical components to successful crop production (Fageria, 2011). In order to maximize the economic advantage from these nutrients, a comprehensive soil testing program must be implemented, followed by strict preservation of nutrient critical levels which are determined at the point in which 95 to 97% of the crop's yield potential will be reached without additional input

of the nutrient (Stanton, 2014). In order to reach the highest yields possible, nutrient management has become a very important and often-studied facet of modern agriculture. In order to combat limited land use and the increasing consumer population, current research must focus on increasing yields in our most important crops. The use of nutrient fertilization will remain an ever-increasing part of the crop production process.

1.7 Nematode Infections Inhibiting Nutrient Uptake

The nematode is able to rob the host plant of nutrients through the act of parasitic feeding. Phytonematodes are able to pierce through the plant cell wall, entering the cellular membrane of the host cell. This forced penetration is possible because the nematode stylet is attached to the pharynx, a specialized muscle that contracts and expands to allow the nematode mouth part to pump the withdrawn plant food to its intestine for digestion and absorption. The pharynx also contains salivary glands that produce secretions that assist in host invasions and parasitism through a series of degradation enzymes and proteins that mimic plant hormones (Lambert and Bekal, 2002).

Plant-parasitic nematodes are able to obtain nutrients directly from the plant cells based on physiological changes due to feeding. Previous research on host plant transcription patterns have shown that nematode infections will initiate very complex changes in the plant gene expression (Gheysen, 2002). These changes include differences to the cell wall architecture caused by degradation enzymes secreted by the nematode and host endoglucanase and polygalacturonase genes being upregulated to provide feeding-site formation (Mahalingam, 1999). The nematode infection also alters the host plants genes that provide metabolic functions such as those involved in cell-cycle progression

and water transportation (Gheyson, 2002). The nematode secretions result in an increased expression of these genes in and around their feeding sites. Once an endoparasitic nematode has successfully infiltrated the plant vascular system and established a modified cell, the nematode essentially draws in solutes and nutrients from the plant (Jones and Dropkin, 1975). Patrick (1976) determined that nematode-modified cells when viewed at the cellular level act in the way of a strong metabolic sink. The plant response to a nematode infection can also lead to a decrease in nutrients being available when a hypersensitive response follows (Jones and Dropkin, 1975).

1.8 Phosphorus

Phosphorus (P) is an extremely important nutrient for plants. It is involved in a number of beneficial processes: enhancing the photosynthesis rate, increasing enzymatic activities, forming roots, development, energy and nutrient transfer, water use efficacy, reproductive growth, and has a synergistic effect with potassium (K) in plant defense (Snyder, 2000). A plant with a suitable amount of P is able to increase root growth and nitrogen (N) fixation capacity (Havlin et al., 2005). Phosphorus primarily stores and transfers energy throughout the plant to be used for growth and reproduction. Adequate levels are necessary to increase shoot and root growth and promote early maturity (Havlin et al., 2005). If P levels drop too low in the soybean plant, growth, production, and stress tolerance suffers. The demand for P is greatest during pod and seed development, and more than half of the P will end up the pods and seeds (Snyder, 2000).

1.8.1 Phosphorus Recommendations for Ohio

If soil P test levels are less than the critical level, fertilizer recommendations will be higher than what is removed at harvest in order to build up the P levels in the soil (Vitosh et al., 1995). The reverse is true if soil test levels are higher than the maintenance range. In such a case, fertilizer recommendations are lower than the crop removal level (Figure 1). For soybean, the critical level for P (Bray extraction) is 15 mg/kg and the maintenance range is 15-30 mg/kg.

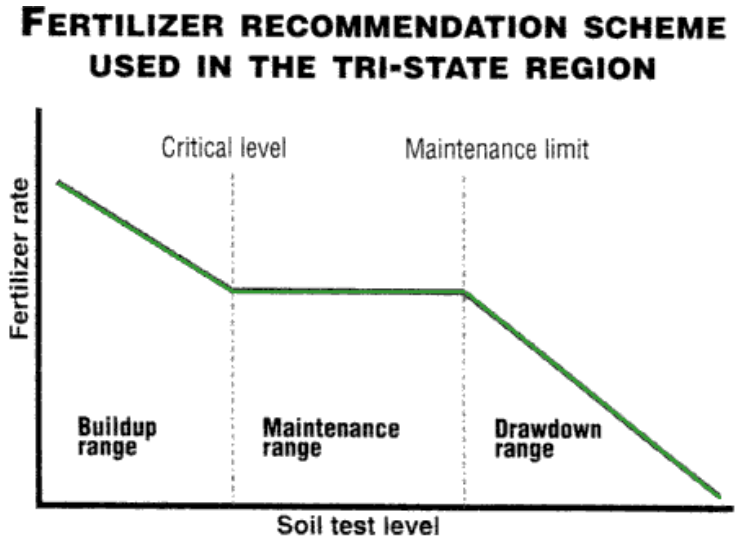

Figure 1. Tri-State Fertilizer Recommendations for Corn, Soybean, Wheat, and Alfalfa based on soil-test levels showing a decrease in fertilizer rate being recommended as the soil test levels show results in the 'buildup range' to the 'drawdown range' (Vitosh et al., 1995).

1.8.2 Phosphorus and SCN Populations

Definitive effects of fertilizer and soil pathogens are not known (Blevins et al., 1995). Specifically, there is little information available on the relationship between P and soybean pathogens and those studies that have been performed have conflicting results. Although Seinhorst and Den Ouden (1971) found a correlation between the formation of

a nematode's feeding site and a decrease in plant nutrient uptake, Oteifa et al. (1958) showed opposing findings when their infested tomatoes showed higher P uptake than the control. Blevins et al. (1995) showed that SCN could influence both calcium and P uptake as well as the mobilization of these macronutrients throughout the host plant in order to supply the nematodes' high demand for P.

1.9 Potassium

Potassium is responsible for many plant functions. It aids water and nutrient transport across the cell walls and regulates water and carbon dioxide exchanges in the plant system and nutrient uptake (Snyder, 2000). In Ohio soils, K is not readily available for plant uptake because almost all of soil K is used for structural components of soil minerals. This leads to a variation in the amount of K fertilizer required across different soil types. When a lack of K is present the plant goes through an increased drought stress response through difficulty absorbing water and N from the soil. Soybean plants are able to conserve their water use and reduce moisture stress by regulating stomate openings, a mechanism regulated by K (Wang et al., 2013). The most important time for the soybean to absorb K is during flowering through early pod development. If inadequate levels are obtained, then yield loss can result without any obvious foliar symptoms.

In order to calculate fertilizer application rates, the soil cation exchange capacity (CEC) must be known. For Ohio soils the CEC is multiplied by 2.5 and 75 is added to give the critical level (Vitosh et al., 1995). Soybeans remove more K than P from the soil, about 0.636 kg K_2O/kg of grain, so the maintenance application rate for a 4,086 kg/ha

crop is 96 kg of K_2O per hectare. A spring application is recommended over the fall especially when it comes to coarse-textured soils to avoid leaching (Staton, 2014).

1.9.1 Potassium and SCN Populations

The effect of K in conjunction with SCN-infested soybean plants has been studied, but lacks a decisive recommendation. Blevins et al (1995) showed soybean roots infected with SCN had significantly less root volume and decreased K root concentration than control roots. Soybean cyst nematode populations have been shown to increase in soils with K fertilizer applied in moderate levels was used, but this outcome was not repeated if no or high fertilizer levels were applied. (Luedders et al., 1979; Hanson et al., 1988; Blevins et al., 1995; Melakeberhan, 1999). There are a number of studies that show plants deficient in K that are treated with fertilizer have an increased immune response to any fungal, bacterial, or parasitic pathogen response, but more specific work to how SCN is affected by K is needed (Huber, 1980; Huber and Graham, 1999; Dordas, 2009). Hanson and Charles (1989) showed a relationship between K fertilizer applied to K-deficient soybeans inhibiting SCN populations. Dordas (2009) explained that K fertilization has a diminishing return for plant defense and once this threshold is reached there is no increase in resistance to SCN or other pathogens. Differing results were found by Smith et al. (2001) where it was suggested that K was not a limiting factor for a soybean plant that is infected with SCN.

1.10 Soil pH

Nutrient availability is strongly influenced by soil pH. A soil pH below 5.2 is detrimental to soybean plant performance as some nutrients including P and K are less

available for uptake in such acidic conditions (Peters et al., 2005). Thus, managing soil pH in the optimal range (6.0-6.8) is essential to produce both high yielding and profitable soybeans (Vitosh et al., 1995). At an optimal pH, the soybean plant is able to absorb a greater amount of nutrients and N fixation. This optimal pH also allows the soybean to fend off pathogens, minimizing soybean cyst nematode (SCN) population growth (Barker and Koenning, 1998).

1.10.1 Relationship of pH and SCN populations

According to a four-year field study conducted in Wisconsin, the greatest populations of SCN were found in soils with a pH of 7.0 or higher (Grau et al., 2003). In soils with pH of 7.0 to 8.0, higher densities of SCN were found compared with soils with pH of 5.9 to 6.5, and throughout the study pH greater than 7.0 was consistently associated with higher initial SCN egg population densities (Grau et al., 2003). This supports the theory that soil pH affects the distribution of SCN along with the point of introduction, as well as playing a role in SCN reproduction in a given field (Anand et al., 1995). The use of SCN-resistant varieties was found to have the highest yield advantage in soils with higher pH and subsequently the lowest advantage in fields with low soil pH. This suggests that any yield advantage of SCN-resistant varieties is gained in SCN infested fields when soil pH is above 6.4. This is of great importance for growers interested in managing SCN infestations. Soil pH could be used along with SCN population density and distribution data to determine management tactics. Overall, SCN populations are found in the greatest numbers with fields with the highest soil pH.

Long-term management efforts to increase soybean yield may focus on decreasing SCN reproduction rather than only on the use of SCN resistance. Even with the use of SCN-resistant varieties, populations have been seen to exceed 2,000 eggs/100 cm^3 soil only in plots with soil pH above 7.0 (Grau et al., 2003). This highlights the notion that SCN resistant varieties cannot reduce already-present SCN populations and soil pH could be used to predict future SCN population changes, regardless of use of susceptible or resistant varieties.

1.11 Soil Texture and SCN Populations

Soil texture has been shown to affect the establishment of SCN, its population density, reproduction, spatial distribution, and the associated negative yield effect of the nematode feeding (Workneh et al., 1999). There is strong evidence that suggests variation in soil texture could be essential to explain the variability of SCN population density within infested fields. Sand has a stronger association with SCN Pi (initial population) and Pf (final population), and it is widely accepted that higher SCN population densities occur in sandy soils (Workneh, et al. 1999). Although there is variation within species of nematode, generally coarse soils favor nematode growth by providing more space for worm movement (Jones et al., 1969). Koenning et al. (1988) found that an increasing sand content was negatively correlated to soybean yield, but had inconsistent results associated with the increase or decrease in SCN populations. This gives rise to a need to address site-specific management of SCN to account for this population density variation within fields (Avendano et al., 2004). Francl (1993) suggested that soil texture or some combination of individual soil separates (sand, silt, and clay) either directly or indirectly

influences SCN population densities and could more accurately describe SCN management zones.

If the soybean roots are damaged early in development, there is a decreased number in potential feeding sides for the J2s which would lead to a decrease in nematode population density. If such damaged roots are grown in clay-rich soils, SCN population densities cannot recover as quickly and cannot build up to damaging levels, maybe even for years. The first report to successfully document a consistent relationship between soil texture and SCN across fields over time, Avendano et al. (2004), found the lowest nematode densities were found in soils with more than 25% clay, given further credence to the accepted relationship between fine soils and low nematode presence. The percentage of soil particulates did have a significant role in affecting SCN levels, but it was found that a very high level of clay (greater than 60% clay) and a very low level of sand (less than 50% sand) were necessary to show an interference with SCN reproductive potential.

The soybean rhizosphere is full of compounding interactions that make nutrient analysis studies difficult to interpret. This research aims to focus on specific soil factors and give plausible explanations for the discrepancies between nutrient levels in the soil and those in soybean leaf tissues. The objectives of this study were to: (i) determine if a correlation exists between the soil-test and plant tissue nutrient levels; and (ii) determine what factors affect nutrient uptake in soybean (soil-test P and K, SCN, pin nematode, CEC, pH, and soil texture).

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Chapter 2: **First Report of *Paratylenchus* Species in Ohio**

Pin nematodes (family Tylenchulidae) are an agronomically and economically important group of ectoparasites that feed on roots and are capable of causing substantial crop loss (1, 2). In June of 2013, 2014, and 2015, 588 soil samples were collected from a fields planted to soybean (*Glycine max*) in thirty-six counties throughout Ohio and submitted to the Nematology Laboratory at The Ohio State University for nematode identification and population analysis. The soil had variable amounts of sand, silt, and clay, and the fields were planted to corn (*Zea mays*) or soybean during the previous year. Nematodes were extracted from 100 cm^3 soil by decanting and sieving followed by sucrose centrifugal flotation. Phytoparasitic nematodes were identified and counted based on morphological traits to genus at 40× to 100× magnification. Nematode genera parasitic to soybean recovered from these samples included *Heterodera.* Pin nematodes (*Paratylenchus* sp.) were detected in 69% of fields in 2013, 52% of fields in 2014, and 36% of fields in 2015 with a maximum count of 3346/100 cm^3 soil. Individual pin nematodes were hand-picked and identified from a single field to species under a compound light microscope as *Paratylenchus neoamblycephalus* (Garaert, 1965) Morphological and morphometric characteristics (1) from females (n = 20) were examined. The females had conical truncate heads, strong spears, and basal knobs that were rounded. The nematode body was distinctly annulated with lateral fields containing 4 incisures. A single, outstretched ovary was noted as well as a posteriorly located vulva.

Spermathecae were present. The anus was often obscure, with tail conical and tapering in shape. Body length ranged from 349.6 to 435.0 μm (mean = 397.0 μm), and stomatostylet length ranged from 29.3 to 33.9 μm (mean = 31.4 μm). DNA was extracted from five adult females (n = 5) and the 18S rRNA region was amplified with 18S (CGCGAATRGCTCATTACAACAGC) and 28S r RNA region was amplified with the D2/D3 (5'ACAAGTACCGTGAGGGAAAGTTG) primers (3, 4). PCR products were purified, sequenced and compared with previously deposited sequences by means of BLASTN search. The BLASTN comparison lead to a 97% identity match with *P. neomablycephalus* (AY284634.1). Molecular results and morphological observations confirmed the presence of *P. neomablycephalus* in the sample. *P. neomablycephalus* is a migratory ectoparasite. The distribution of *P. neomablycephalus* is worldwide previously known to occur in Europe, Africa, North and South America, and Australia. It has been described as a significant pathogen in California prune production (1). *Rosa* spp. (rose), *Prunus persica* (peach), and *Malus domestica* (apple) are known susceptible hosts where stunting, lesions, and crop loss may result from direct damage to roots (1, 2). Nematode management recommendations for soybean will depend on the distribution of this nematode and pathogenicity. To our knowledge, this is the first report of *P. neomablycephalus* in Ohio.

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Chapter 3. Methods Used to Identify *Paratylenchus neoamblycephalus*

3.1 Introduction to Pin Nematode

All nematodes can be defined as nonsegmented worms that are triploblastic noncoelomates with bilateral symmetry. It is important to note the commonalities and the differences among different nematode genera and species in order to correctly identify them. Although the majority of nematodes remain worm-like throughout their life stages, morphological diversity is apparent among different nematode genera and species as well as between adult and juvenile stages. Even plant-parasitic nematodes, who all belong to the plant-feeding functional group and thus have similar structures, can vary greatly in head shape, stylet length form, and tail type. While most nematodes move in a wave-like fashion during active feeding, their position when in a relaxed state can help with genus and species identification.

The genus *Paratylenchus,* known as the pin nematode, is the smallest of the plant parasitic nematodes (0.2-0.5 mm in length). The majority of pin nematodes have well defined stylets that are very long in comparison to their short body length. Once the nematode reaches their second molt or infective juvenile stage (J2), they are able to infect a plant's root system. These migratory ectoparasites freely move from root to root feeding on the outside of newly emerged lateral roots. Like all plant-parasitic nematodes, pin nematodes feed upon individual plant cells by inserting their long stylet and removing nutrients with the aid of their esophageal secretions. Some species of *Paratylenchus* have

been known behave as semi-endoparasites and create feeding sites, withdrawing nutrients for days at a time, often around five or six days total (Eck, 1970). Pin nematode feeding results in both localized lesions and necrotic root development damage, along with disruption to the host's ability to absorb water and nutrients from the rhizosphere. Above ground, plants infected with pin nematode may be stunted. *Paratylenchus* is a cosmopolitan pest with the ability to infect more than 120 plant species, but the damage this nematode causes is often masked by other nematode feeding or other microbial interactions (Siddiqi, 2000).

In order to contain and alleviate the effects of nematode infection, the species must be known in order to determine the proper management strategies. The objective of this experiment was to combine morphological and morphometric measurements with molecular techniques in order to identify a *Paratylenchus* species found frequently in Ohio soils.

3.2 Methods

3.2.1. Morphometric and Morphological Observations of Paratylenchus

For the identification and quantification of the genera of nematodes in Ohio soybean fields, nematodes were extracted from soil samples and observed microscopically. With the aid of both dissecting and compound microscopes, nematodes were enumerated and identified based on overall morphology and morphometric measurements. The microscopy work was done using a scored line petri dish in order to prevent repeated counting of individual nematodes. When a previously undocumented genus of nematode was discovered from Ohio soil samples, detailed morphometric measurements were taken to determine the species of the new nematode.

Twenty female specimens of a genus previously identified as *Paratylenchus* were further examined under a compound microscope to confirm the generic diagnosis. A series of morpho-anatomical attributes were observed and confirmed as *Paratylenchus.* Morphometric measurements of key nematode components included: 1) stylet length measured from the base of the knobs to the lips; 2) body length measured from the lip region to the tail tip; and 3) the de Man ratios which focused on "a" the body length divided by the greatest width of the female nematode, "b" the body length divided by the oesophagus length, and "c" the body length divided by the tail length (Raski, 1976). Morphological characteristics of the cephalic region included determining a lack of head and lip structures as well as conicoid head shape. Morphological characteristics of the cuticle were also noted, along with the position of the singular ovary which is a key diagnostic point for pin nematodes. Once the morphometric and morphological data were collected, species confirmation was provided by use of diagnostic keys from Raski (1965) and Geraert (1976).

3.2.2 Polymerase chain reaction for molecular confirmation of new species

Based on previous experiments done by Subbotin *et al*. (2013) to identify species of nematodes, the D2/D3 primer was used to amplify the 28s ribosomal DNA gene and the 18s primer was used to amplify the 18s ribosomal DNA gene. The DNA digestion methods used to identify the species of *Paratylenchus* were adapted from Hübschen et al. (2004). Individual *Paratylenchus* female nematodes were hand-picked from 600 mL soil suspensions from 100 cm^3 soil samples obtained from Ohio soybean fields and 500 mL of H_2O. In order to quantify and purify DNA for molecular confirmation, the nematode

DNA was extracted by means of NaOH digestion. Three different nematode quantities were initially used in order to assess DNA presence. A single nematode, five nematodes, or ten nematodes were each combined with 20 µl of 1M NaOH in individual PCR tubes and centrifuged briefly before being incubated at 25°C for at least five hours. The PCR tubes and their digested DNA were removed from the incubation chamber and heated for three minutes at 99 °C before 10 µl HCl, 5 µl 0.5 M Tris-HCl (pH 8.0), and 5µl 2% Triton-X were added (Thermo Fisher Scientific, Waltham, MA). The tubes and their contents were centrifuged briefly (~five seconds) and again heated for three minutes at 99°C before being stored at -80°C.

After the nematode DNA was hand-picked from the soil solution and digested for 24 hours it was with absorbance readings of the nucleic acid obtained from a Thermo Scientific NanoDrop ND-2000c (Thermo Fisher Scientific, Waltham, MA). 1.5 µl of the digested nematode solution was used for accurate quantification results. After the machine was cleaned with a 1.5 µl drop of purified water, a "blank" deposit was initially run as a check. This "blank" matched the contents of the nematode DNA solution completely, but lacked DNA and thus was confirmed with a zero absorption reading. Once the accuracy of the NanoDrop was shown, 1.5 µl of the nematode solution was placed on the sensor and the absorption was obtained. Because multiple nematode DNA solution readings needed to be obtained, the NanoDrop sensor was wiped between each separate 1.5 µl drop. Based on the absorption readings, the correct amount of DNA was calculated for the PCR reaction. A quantification of 49.8 ng/µl was obtained and in order to perform a 50 µl PCR reaction, 5 µl of the nematode solution (measuring 49.8 ng/µl) was used.

Table 1. Primers used for molecular identification of pin nematode

Name of Primer	*Fragment (bp)*	*Primer Sequences (5'-3')*	*References*
D2/D3	700	ACAAGTACCGTGAGGGAAAGTTG	*Courtright et al., 2000*
		TCGGAAGGAACCAGCTACTA	
18s	900	CGCGAATRGCTCATTACAACAGC	*Floyd et al., 2005*
		GGGCGGTATCTGATCGCC	

The reagents were thawed completely, centrifuged, and kept on ice for the duration of the reaction. Four reactions were set up with 15µl of H_2O, 25µl of Thermo Scientific™ Phusion™ Flash High-Fidelity PCR Master Mix, 2.5µl of individual primer (D2, D3, 18sF, 18sR), and 5µl of nematode DNA. Next, 2 µl of dye was added to each PCR tube and its contents (1:5 ration of volume of dye per product of PCR) and placed in the thermocycler (Table 2).

Table 2. PCR amplification conditions of primers used for molecular identification of pin nematode

Cycle Step	*2-Step Protocol*		*Cycles*
	Temp.	*Time*	
Initial Denaturation	*98°C*	*10 s*	*1*
Denaturation	*98°C*	*0 or 1 s*	*30*
Annealing	-	-	
Extension	*72°C*	*15s/kb*	
Final Extension	*72°C/ 4°C*	*1 min*	*1*

Once the DNA was amplified it was then run on a 1% agarose gel and subjected to electrophoresis, with the voltage set at 115 and amperes set at 2 for a total of 45 minutes. The gel electrophoresis separated the DNA by base pair size and this was visualized under ultra violet light.

The DNA purification protocol followed the QIAquick® PCR purification kit (Qiagen, Valencia, Ca). The nematode DNA was first inserted into the QIAquick column and centrifuged for 30 seconds in order to assure the DNA would bind to the QIAquick column and any non-DNA molecules would passed through the vacuum. Any remaining liquid was discarded. Next, the bound DNA was washed with 750 µl buffer and was centrifuged for 30 seconds. Any liquid that did not pass through the vacuum was discarded. The clean, bound DNA was placed in a clean 1.5 µl microcentrifuge tube ready for sequencing.

The DNA sequencing was done by the Ohio State plant-microbe genomics lab. An Applied Biosystems 3730 DNA analyzer and BigDye™ cycle sequencing terminator chemistry was used to carry out the DNA sequencing (Applied Biosystems, Foster City, CA). In order to sequence the DNA, the dideoxynucleotides of the submitted nematode DNA were dye-labeled so they could be read. The DNA was then combined with a heat-stable DNA polymerase and subjected to the thermal cycler and generated extension products that were then separated by capillary electrophoresis on the 3730 DNA analyzer. The generated extension products were read by exciting the dyes with a laser. This level of fluorescent emission was then measured with a CCD (charge-coupled device) camera. Finally, the emission signal was interpreted by the Applied Biosystems Sequencing Analysis program and a full sequence of the nematode DNA was determined (Applied Biosystems, Foster City, CA).

3.3 Results and Discussion

3.3.1 Morphometric and Morphological characteristics of Paratylenchus neoamblycephalus

Determining a nematode's morphometric measurements and distinct morphology are the first steps in identifying the genus and species of an individual worm. Twenty *Paratylenchus* females were observed under a compound microscope to view morphological structures (Figure 2) and obtain morphometric measurements (Table 3). Sixteen measurements were taken for each nematode observed, in order to follow the dichotomous keys of Raski (1965) and Geraert (1976). The position of the single vulva as well as the position of the excretory pore were significant to confirming the correct genus, and aided in separating species. The key diagnostic features for *Paratylenchus* were the shape of the head, length of the stylet, and form of the tail. The females all possessed a conical-truncated head with poorly developed submedian lobes. These twenty females also had an average stylet length of 31.4 µm, which is a stylet length only a few *Paratylenchus* species possess. Finally, the tail shape of the females, slightly tapering to a point, was the morphological key that separated this species from the others. The female specimens found in Ohio were identified as *Paratylenchus neoamblycephalus.*

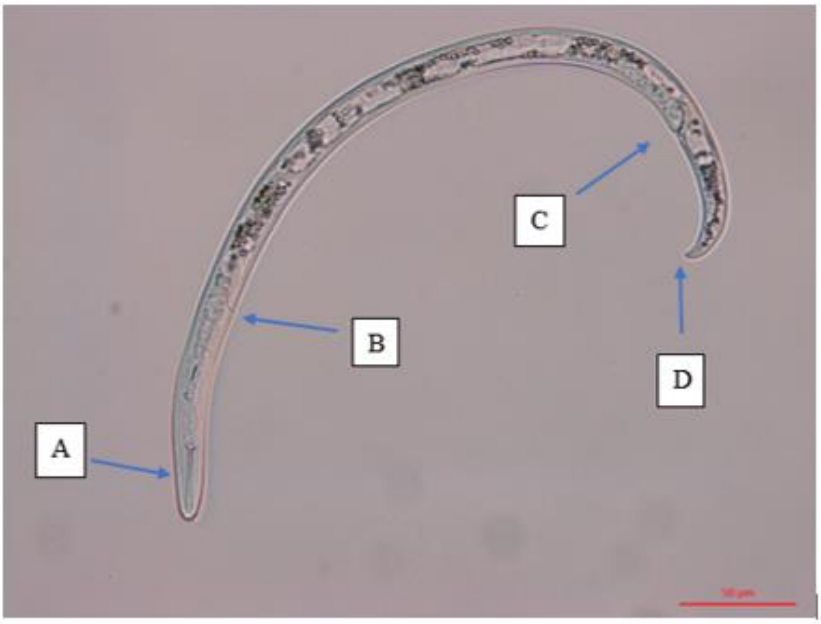

Figure 2. An adult *Paratylenchus* female with key morphological structures labeled A-D. The text box "A" represents the nematode mouthpiece (stylet). This nematode has a unique stylet length and shape that is particular to Paratylenchus species. "B" is representative of the excretory pore which is the opening from the transverse canal to the outside environment, the positioning of which aligns with the Paratylenchus genus is a taxonomic feature that can be species specific. "C" shows the vulva, an opening in the ventral, transverse section of the nematode, positioned in the lower third of the nematode body. "D" points out the slightly rounded tail both of these features of indicative of the pin nematode.

Table 3. Morphometric measurements of 20 *Paratylenchus* females from Ohio soybean fields.

M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	AVE
L	385.0	404.3	363.3	392.2	402.6	356.4	407.3	349.6	435.0	382.1	427.7	408.5	392.5	406.2	366.9	394.6	412.9	417.3	372.6	425.5	397.0
GW	17.4	17.6	16.7	17.0	17.2	15.3	15.6	14.0	17.4	16.5	16.1	16.0	16.2	15.9	17.3	16.8	15.5	15.7	13.2	18.8	16.0
OL	81.1	74.9	66.6	70.9	84.3	65.8	73.5	70.8	76.9	70.4	86.8	79.1	73.4	75.8	63.9	75.1	79.1	79.1	78.5	69.4	74.5
OL TO L	111.9	108.0	98.3	98.7	113.1	95.2	104.0	102.1	109.3	103.0	118.7	11;	.04.1	109.4	96.6	104.7	111.4	111.2	107.8	103.8	106.2
TL	8.7	8.4	6.3	8.6	8.6	5.8	8.6	9.0	11.0	7.5	7.6	6.0	10.0	6.9	6.2	9.9	7.0	9.4	6.1	7.8	7.9
BW AT A	5.1	6.5	4.5	6.1	6.6	5.0	6.3	6.1	5.7	6.2	6.1	4.2	5.5	4.7	6.1	6.6	5.2	6.2	4.8	4.7	5.6
V TO L	333.7	331.2	315.7	325.8	329.0	295.2	329.8	290.8	357.4	317.9	358.1	335.3	325.8	338.4	305.6	337.0	337.3	340.6	334.0	356.3	328.4
A	22.1	23.0	21.8	23.1	23.4	23.3	26.1	25.0	25.0	23.2	26.6	25.5	24.2	25.5	21.2	23.5	26.6	26.6	28.2	22.6	24.8
B	4.7	5.4	5.5	5.5	4.8	5.4	5.5	4.9	5.7	5.4	4.9	5.2	5.3	5.4	5.7	5.3	5.2	5.3	4.7	6.1	5.3
B’	3.4	3.7	3.7	4.0	3.6	3.7	3.9	3.4	4.0	3.7	3.6	3.7	3.8	3.7	3.8	3.8	3.7	3.8	3.5	4.1	3.7
C	44.3	48.1	57.7	45.6	46.8	61.4	47.4	38.8	39.5	50.9	56.3	68.1	39.3	58.9	59.2	39.9	59.0	44.4	61.1	54.6	51.0
C”	22.1	23.0	21.8	23.1	23.4	23.3	26.1	25.0	25.0	23.2	26.6	25.5	24.2	25.5	21.2	23.5	26.6	26.6	28.2	22.6	24.8
V	86.7	81.9	86.9	83.1	81.7	82.8	81.0	83.2	82.2	83.2	83.7	82.1	83.0	83.3	83.3	85.4	81.7	81.6	89.6	83.7	82.8
STYLET	31.2	32.2	30.6	29.8	29.3	29.4	30.5	30.6	32.1	31.3	31.9	31.9	30.7	33.6	31.7	29.7	31.4	32.6	29.6	33.9	31.4
DGO	7.3	7.4	7.0	7.3	6.5	6.7	6.1	6.1	6.1	5.5	6.5	6.1	7.1	7.4	7.1	7.8	6.1	7.5	6.4	6.3	6.6
EP	91.4	86.1	82.3	87.2	82.2	80.8	89.8	80.0	92.6	92.5	93.3	87.0	85.7	85.5	82.0	83.3	86.2	87.0	82.7	88.7	86.5

* M = Measurement in µm, L = Total body length, GW= Greatest width, OL= Oesophagus length, OL to L= Oesophagus length from the lips, TL= Tail length, BW at A= Body width at the anus, V to L = Distance of the vulva from the lips, A = Body length/ Greatest width, B= Body length/ Oesophagus length, B’= Body length/ Oesophagus length from the lips, C= Body length/ Tail length, V= Distance of vulva from the lips x100/ Body length, DGO= Dorsal esophageal gland orifice, EP= Excretory pore.

3.3.2 Molecular identification of Paratylenchus neoamblycephalus

Paratylenchus species are morphologically very similar to each other and identification to the species level is difficult. It is therefore necessary to use molecular techniques in order to quickly and accurately confirm *Paratylenchus* species. In this study, gene-specific primers were used for identification of *Paratylenchus neoamblycephalus*. The primer pairs used for diagnosis of *Paratylenchus neoamblycephalus* were D2/D3 and 18S and were used to amplify regions on both the 28s and 18s ribosomal subunits, respectively. These primers resulted in consisted amplifications by DNA obtained from females with band widths reading at ~700 for the D2/D3 primer and ~900 for the 18s primer (figures 3 and 4). Courtright et al. (2000) previously reported on the 28s primer and Floyd (2005) previously reported on the 18s primer and showed consistent DNA amplification at their respective genes.

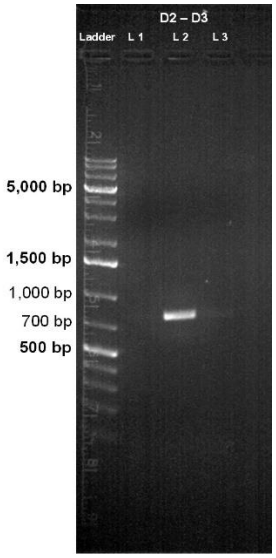

Figure 3. Amplification products with the D2/D3 primers, DNA ladder and DNA samples in L1, L2, and L3. Only L2 showed DNA amplification with a band with ~750 bp.

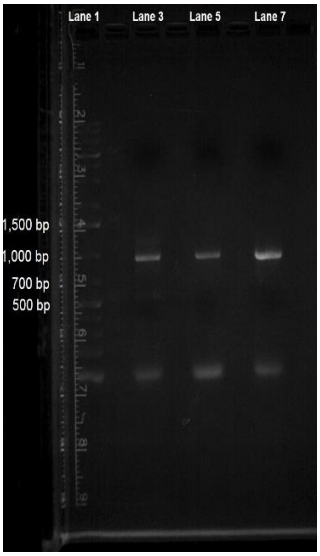

Figure 4. Amplification products with the 18s primers, DNA ladder (lane 1), and DNA samples in lanes 3, 5, and 7 showing band widths at ~900 bp.

DNA sequences obtained from Ohio specimens were used for BLASTN comparisons against previously deposited GenBank accessions for *Paratylenchus* nematodes (Altschul et al., 1997). The sequence our lab submitted was found to have a 97% identity match (Figure 5) with a *Paratylenchus neoamblycephalus* sequence; accession number AY284634.1 as determined by use of Clustal X (Thompson et al., 1997).

In conclusion, this study reported on the morphological distinctions, morphometric measurements, and the optimization of PCR for accurate identification of new nematode species for Ohio, *Paratylenchus neoamblycephalus*.

Figure 5. DNA full sequence aligned with Clustal X. It shows a BLASTN match of a previously deposited 18s *Paratylenchus neoamblycephalus* DNA had a 97% identity match with our deposited DNA.

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Chapter 4: Influence of Soil Characteristics on Soybean Plant Nutrition

4.1 Abstract

Plant mineral nutrition is one of the major factors that influences soybean [*Glycine max* (L.) Merr.] grain yield. It is important to understand the availability of nutrients in the soil and to know their uptake into the plant. A survey was completed in Ohio with the objectives 1.) to assess soil fertility and plant mineral nutrition of soybeans grown in Ohio, and 2.) to determine what soil factors are influencing the lack of correlation between soil-test and tissue test nutrient levels. In Ohio, soil sampling was conducted from 2013 through 2015 resulting in 588 total samples. Soil phosphorus (P), potassium (K), pH, cation exchange capacity (CEC), soil texture, and nematode quantities were measured. Nutrient concentrations of P and K were measured in the soybean leaf tissue of the upper most developed trifoliates for each farm site. There was a low correlation between the soil and tissue tests with R^2 values of 0.1539 and 0.36781 for P and K, respectively. We found 32.9% of the soil samples tested below the critical soil test range, but only 2.7% of the samples were below tissue-test critical levels for P, while 23.4% of the K soil test samples were found to below the critical levels and only 5.9% of the tissue tests fell below the critical level. All of the soil factors tested in a step-wise regression model were found to influence P uptake, while only soil K, CEC, and SCN were found to influence K uptake. To prevent yield loss, producers should soil and tissue

sample to monitor fertility levels and maintain levels within the state guidelines as well as examine the soil factors that influence nutrient uptake.

4.2 Introduction

Plant nutrition is directly related to the growth and development of a crop (Fageria, 2011). Phosphorus (P) and potassium (K) are two macronutrients that have a large impact on the growth and development of soybean. Phosphorus uptake is most important to soybean plants during pod development and grain fill, although it is an essential nutrient for the plant at every growth stage (Barker et al., 2005). Phosphorus is especially vital to soybean plants during the nodulation process, as nitrogen fixation demands a large requirement of P in the form of ATP (Shanmugam et al., 1978).

Potassium is an essential nutrient for the soybean which requires its constant uptake throughout the growing season (Pettigrew, 2008). Borges and Mallarino (2000) showed K had a large impact on soybean throughout each growth stage because of the involvement K has with photosynthesis and water relations. Stomatal openings are controlled primarily by K, and when K deficiency is found, stomatal conductance has been reported to be a principal limiting factor in photosynthesis (Talbott and Zeiger, 1996; Bednarz et al., 1998). Apart from its critical role in stomata control, K is a predominant osmoticum in the phloem providing the necessary turgor pressure for proper tissue growth (Mengel and Dou, 1998). When an insufficient level of K is seen in soybean, it is associated with reduced leaf area that precedes an overall reduction in leaf size (Huber, 1985).

In order to assess a plant's nutritional status, both soil and tissue sampling have been employed. Although there are recommendations on the proper soil test P and K levels, there is an inconsistency between the nutrient levels reported by the soil tests and those nutrient levels seen from plant tissue testing (Wolf, 1982; Tisdale and Nelson, 1975). One reason for this inconsistency is that soil analysis can influence the composition of the soluble nutrient constituents and increases the need for pairing such soil testing with sampling plant tissues (Schwab, 2000). The low correlation of soil and tissue nutrient levels is a complex problem that is likely influenced by a number of factors including cation exchange capacity (CEC), soil pH, soil texture, and nematode presence.

Soil texture has a well-documented effect on nutrient movement as does the soil CEC (Olsen and Watanabe, 1970). Soil with a high clay content and a high CEC leads to the positively charged nutrients binding tightly to the soil (Stanton, 2014). Soils with high sand content and a smaller CEC are associated with a lower nutrient holding capacity. Furthermore, sandier soils have larger pores than those of clay soils which may cause nutrients to leach (Workneh, 1999). Soil pH can affect nutrient availability by changing the form of the nutrient which leads to different absorption abilities (Stanton, 2014). In general, many nutrients are more available to the plant in slightly acidic soil. For example, P and K are most available in soils with a pH between 6.0 and 7.0 (Peters et al., 2005).

Increasing knowledge of soil microbes, especially phyto-nematodes, have allowed further research into how their presence and abundance results in physiological changes

of the plant. Of all the nematodes that are known to feed on soybean, the biggest threat is from *Heterodera glycines,* the soybean cyst nematode (SCN). Soybean cyst nematode has been known to cause stunted top growth and root pruning from plant invasion and feeding, but mineral element deficiency is also associated with SCN infection such as a decrease in both root and tissue K and magnesium (Mg) (Blevins et al., 1995). During an SCN infection, the lateral plant roots are damaged which results in a decrease in both water and nutrient absorption by the host plant. Specifically, chlorosis and necrosis that can been seen in soybean plants supporting high SCN populations show a deficiency in K (Horsfall, 2012).

Recently discovered in Ohio soils by Simon (2015), *Paratylenchus,* or pin nematode, is a known ectoparasitic feeder that feeds on lateral roots and causes lowered epidermal root surface area leading to nutrient absorption issues in the host plant (Eck, 1970). The wounds made by the emergence of the pin nematode's stylet into the lateral roots allows them entry into the plant's cortex where a "sucking" type feeding will withdraw any available nutrients directly from the roots. This nutrient withdrawal can be seen as a symptom of pin nematode feeding in an extensive host range, including rose (*Rosa* spp.) and pineapple (*Ananas comosus*) (Siddiqi, 2000). Soybean cyst nematode and pin nematode rob host plants of a multitude of macro- and micro-nutrients because the nematodes require both P and K for biosynthesis and metabolism (Blevins et al., 1995).

This research aims to provide explanations to the inconsistencies between nutrient levels in the soil and in soybean leaf tissues. The objectives of this study were to (i) determine if a correlation exists between the soil-test and plant tissue nutrient levels, and

(ii) determine what factors affect nutrient uptake in soybean (soil-test P and K, SCN, pin nematode, CEC, pH, and soil texture).

4.3 Methods

4.3.1 Soil and Tissue Sampling

An on-farm survey of Ohio soybean fields was conducted from 2013 through 2015 to assess the status of nematode populations throughout the state and to determine the impact of nematode abundance on soybean plant nutrition. Ohio soybean farmers across the state volunteered to participate in this three-year project. There were 65, 83, and 60 soybean fields sampled in 2013, 2014, and 2015, respectively. Farmers were allowed to participate in the survey as many years as desired, but the same fields were not sampled in subsequent years. In each field, there were three sampling areas. The size of these sampling areas were variable, but within each sampling area GPS coordinates were recorded from the north, south, east, and west extremes of each soil sampling area in order to ensure subsequent leaf tissue samples were collected from the same area. In total, there were 588 samples collected, with the majority of these samples collected by Ohio State University Extension educators using an established protocol (Lindsey et al., 2014).

Soil samples were collected in either May or June of each year. A soil sample consisted of 10 to 15 homogenized soil cores collected with a 2.5 cm diameter soil probe to a depth of 20 cm. Soil samples were collected in a zig-zag pattern within each sampling area. Soil samples were analyzed for P, K, pH, CEC, and texture as well as SCN and pin nematode abundance. Soil used for nematode abundance was stored in a plastic bag in a cooler before it was taken to the nematode laboratory and stored at 4°C

until being processed. Soil P and K were extracted using the Mehlich 3 extractant followed by the inductively coupled plasma (ICP) method. A 1:1 soil to water ratio was used to determine the soil pH. Soil texture was determined by use of the Boyoucos hydrometer method (Day, 1965). The CEC of the soil was calculated by the following equations:

$CEC = cmol_+ Ca + cmol_+ Mg + cmol_+ K + cmol_+ Na + cmol_+ H$, where, $cmol_+ Ca$ = mg/kg Ca divided by 200;

$cmol_+ Mg$ = mg/kg Mg divided by 100;

$cmol_+ K$ = mg/kg K divided by 390;

$cmol_+ Na$ = mg/kg Na divided by 230;

$cmol_+ H$ = 0 if buffer pH is > 7.0 or = 12 x (7 – buffer pH) if buffer pH < 7.0.

Soybean leaf tissue samples were collected between the R1 (initial flowering) and R2 (full flowering) growth stages. Ten of the uppermost, fully developed soybean trifoliates were collected in a zig-zag pattern within each sampling area. Leaf tissue samples were analyzed by use of HNO_3 digestion to extract P and K and then the amounts were measured by means of ICP (Nathan and Gelderman, 2012).

4.3.2 Nematode Extraction

Nematodes were extracted from soil by means of the Cobb sieving and flotation-centrifugation method (Jenkins, 1964). A 100 cm^3 subsample of the soil was mixed with 500 mL distilled water and a series of sieves were used to separate the nematodes from their aqueous environment. Centrifugal sugar flotation techniques were applied to the nematode-rich aqueous solution in order to process several samples and to obtain both active and inactive nematodes at a variety of life-stages. A modified Baermann funnel

method was used on denser plant and root matter (Goodey, 1951). A funnel method was done in order to extract migratory and sedentary endoparasites by separating the nematodes from debris and mineral particles by pouring the aqueous suspension onto a tissue paper laden wire mesh and submerging the tissue with water. This allows any actively moving nematodes to swim through the tissue paper into clean water where they can be decanted and centrifuged.

4.3.3 Statistical Procedures

The correlation between soil test P and leaf P was determined using the Proc Corr procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The Proc Corr procedure was also used to determine the correlation between soil test K and leaf K. Multivariate analyses through stepwise regressions were done in order to determine the impact of multiple factors including soil P and K, pin nematode, SCN, CEC, and soil texture on plant nutrition. This was performed in order to assess which factors were influencing the uptake of P and K by the soybean plant. Significance was determined at $\alpha=0.05$. The nematode quantities were subjected to a natural log +1 transformation in order to fit the assumption of normality.

4.4 Results and Discussion

4.4.1 Correlation Between Soil-Test Phosphorus and Leaf Tissue Phosphorus

There was a low correlation between soil-test P and leaf tissue P concentration (R^2 value of 0.1539 and p-value of 0.0005) (Figure 6). The critical levels for soil test and tissue test P are 21.4 mg/kg and <0.3%, respectively (Vitosh et al., 1995). In this study,

32.9% of the soil samples tested were below the critical level for P, while 2.7% of the leaf samples were found to be below the tissue-test critical level.

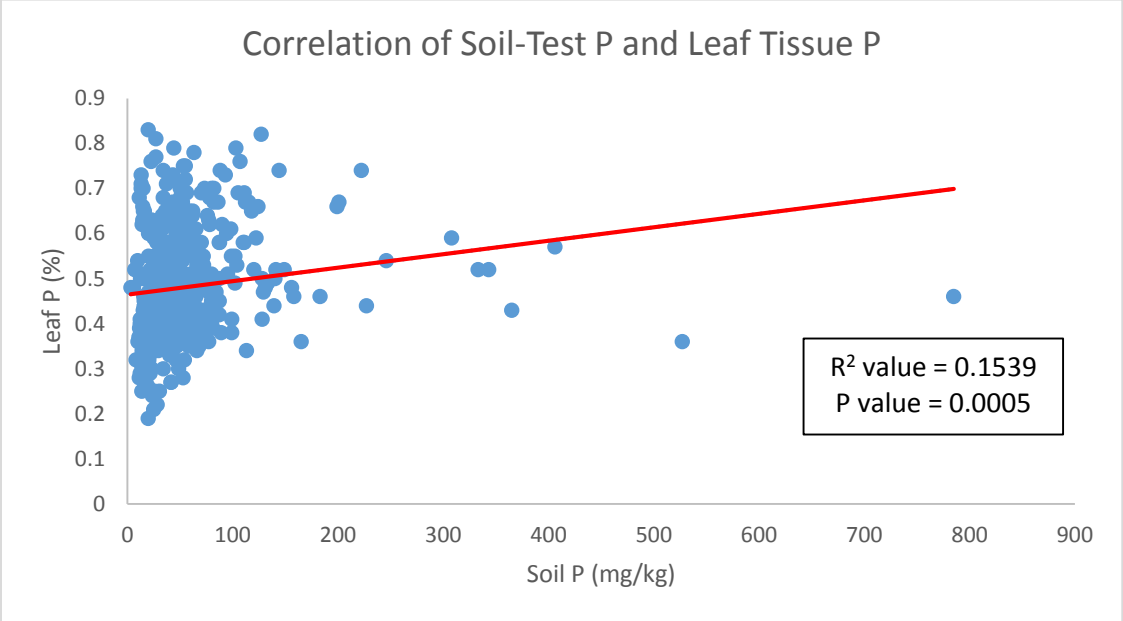

Fig. 6. Correlation between soil test P and leaf P of soybean leaves collected at the R1-R2 growth stage.

4.4.2 Factors Influencing Soil P Uptake by the Soybean Plant

Due to the low, but significant linear relationship of leaf P and soil P (Figure 6), further analysis was done to explore factors that could influence the relationship of P uptake from the soil into the plant. A stepwise model was run on the independent variables of soil P, soil texture (percent sand), CEC, pH, pin nematode abundance, SCN abundance, and year to determine which factors had the most influence on P uptake by the soybean plant. The stepwise model had a p value of <0.0001 and an F value of 10.55

(Table 4). The stepwise regression included various combinations of the independent variables tested together, removing any factors that did not exhibit a significant effect on the dependent factor (leaf P).

Table 4. Stepwise analysis for leaf P as influenced by year, soil P, CEC, pH, soil texture (% sand), SCN abundance, and pin nematode abundance. All of the factors tested were deemed significant with at $\alpha = 0.05$. The R^2 value of the model increased with the addition of each independent variable.

Stepwise Selection Summary

	F value	P value	R-Square value	Parameter estimate
Model	10.55	<.0001		
Intercept	7.06	0.0082		38.856
SoilP	13.38	0.0003	0.0237	0.0003
Sand	23.08	<.0001	0.0518	-0.002
CEC	26.39	<.0001	0.0895	-0.005
pH	7.33	0.007	0.1025	0.026
Pin	5.81	0.0163	0.1106	-0.006
Year	6.88	0.009	0.1179	-0.019
SCN	6.31	0.0123	0.1289	-0.005

The seven factors; soil P, soil texture, CEC, pH, pin nematode, SCN, and year, that were run through the multiple regression analysis were significant in the model so no factors were removed. The order of appearance does indicate the highest influence; meaning soil P affected leaf P uptake the greatest. This relationship is expected and the low parameter estimate may be explained by human error. Multiple Extension educators and students collected leaf samples and a mistake in determining the proper growth stage of the leaf samples could have occurred. Such an error would result in testing a tissue that may be more mature; such as the R3 (beginning pod fill) growth stage. At this maturity

the soybean would be allocating more nutrients to the pod rather than the leaf and a misrepresentation of nutrient levels would result (Grabau et al, 1986).

There were several coefficients that were found to have a negative mean change effect in leaf P uptake including year, CEC, soil texture, SCN, and pin nematode. This information is extremely valuable in explaining the low correlation between soil test P and leaf tissue P. Phosphorus primarily enters the plant root through diffusion from the surrounding soil solution. Plant parasitic nematodes occupy this same habitat competing for entry into the roots. The presence of these SCN and pin nematode alters the plant's physiological processes of water and nutrient uptake first in the root and then reduces plant productivity when as they modify chlorophyll synthesis, photosynthesis, and respiration of the soybean shoot (Atkinson, 1985). This alteration of the plant's physiology and metabolism is a result of the nematode's destructive feeding habits that impede nutrient uptake by their host (Melakeberhan, 1997). The specific results of which nutrients and the degree of loss varies amongst the scientist that study this phenomenon, but it is clear that the feeding site is able to modify vascular cells to sequester nutrients away from the plant and allow for direct ingestion by the nematode (Gunning, 1977; Jones, 1981). While both SCN and pin nematode did have a negative effect on P uptake by the plant, it was a relatively small effect. For example, an increase of 10% in the pin nematode population would result in a -0.006% change in leaf P.

Cation exchange capacity has a huge influence on nutrient availability and is greatly affected by soil texture and organic matter (Havlin, 2005). The negative response of P uptake to increases in sand for soil texture could be explained by a lack of P being

diffused into the root system due to not being bound to soils with high CEC and being lost through the coarser soils. As the CEC value increased, the amount of P taken up by the soybean plant decreased. This negative relationship between the CEC leaf P could be explained by the fact that P is negatively charged in its available form, PO_4^{3-} and a larger CEC value means positively charged ions are bound tighter and therefore P is lost to water logged soils or soil erosion.

4.4.3 Correlation Between Soil-Test Potassium and Leaf Tissue Potassium

The correlation between soil test K and leaf tissue K was low with an R^2 value of 0.36781 (Figure 7). The critical levels of soil test and tissue test K are 148.8 ppm (for a CEC of 20 cmol/100 g) and <2.01%, respectively (Vitosh et al., 1995). The samples tested resulted in 23.4% of the samples below the soil test critical levels and 5.9% of the samples below the critical tissue level tests.

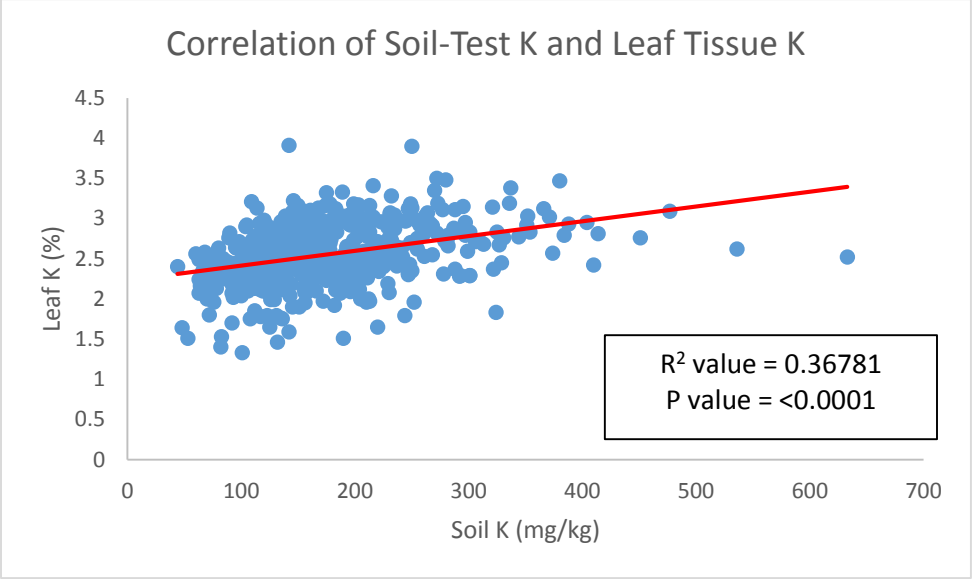

Fig. 7. Correlation between soil test K and leaf K in soybean leaves at the R1-R2 growth stage.

4.4.4 Factors Influencing Soil K Uptake by the Soybean Plant

A stepwise model was run on the factors to determine which variables had the most influence on K uptake by the soybean plant. Any factors that were input that did not result in a significant p value were removed, table 5 shows only soil K, CEC, and SCN had a strong influence on leaf K.

Table 5. Stepwise analysis for leaf K as influenced by year, soil P, CEC, pH, soil texture (% sand), SCN abundance, and pin nematode abundance. The variables that did not contribute to the significance of the model were removed during the final regression step, leaving only soil K, CEC, and SCN as significant independent factors influencing leaf K. The R^2 value of the model increased with the addition of each independent variable.

Stepwise Selection Summary

	F value	*P value*	*R-Square value*	*Parameter estimate*
Model	*78.85*	*<.0001*		
Intercept	*2132.3*	*<.0001*		*2.4399*
Soil K	*104.18*	*<.0001*	*0.1353*	*0.0021*
CEC	*26.63*	*<.0001*	*0.1842*	*-0.0150*
SCN	*4.99*	*0.0259*	*0.1922*	*-0.0127*

Again the stepwise results show soil nutrition had the greatest influence on leaf nutrient uptake. The soil K had the greatest influence, the largest parameter estimate, and the only positive correlation with increased nutrient uptake. This result is not unexpected. The mass flow rate is much greater in K than P (20% versus 4%) which results in a greater dependency on the rate of water flow at the soil surface as well as the plant's transpirational water uptake. Because SCN is a sedentary endoparasite that establishes its

feeding site in the vascular cells of soybean, it could be concluded that this serves as a blockage of plant transpiration and would inhibit K uptake. Significant effect and a negative parameter estimate could be explained by the life style of the nematode. (Havlin, 2005).

It is possible that the parasitic feeding of SCN would account for the low correlation by directly removing K from the plant (Dordas, 2007). Soybean cyst nematode had a negative impact on the total uptake of leaf K by soybean plants showing an estimated -0.012 which can be interpreted as every 10% increase in SCN would decrease the amount of leaf P by 0.012%. Potassium uptake is also reduced if the roots are damaged which could occur either by the initial nematode feeding or secondary invaders that using feeding sites as opportunist entry points (Silberbush and Barber, 1983). Potassium flows into a plant from the surrounding soil so root destruction by mobile, infective juveniles could block the initial entry of K into the plant. A further impedance of this nutrient could occur by the vascular tissue being blocked by the enlarged sedentary female nematodes. Once a nematode has successfully infiltrated the plant vascular system and established a modified cell, the nematode draws in solutes and nutrients from the plant which would have a greater effect on the nutrients being carried by mass flow (Jones and Dropkin, 1975). This would equate a syncytium to a normal transfer cell in the plant vascular system, but the nematode receives the nutrients rather than the soybean. Patrick (1976) determined that nematode-modified cells act in the way of a strong sink. Potassium is mostly moved from the soil to the roots by mass flow and is therefore greatly impacted by vascular blockage as it is dependent on transpiration for

uptake. The immune response of the soybean can also lead to a decrease in K as an apoptosis response often follows nematode infections.

4.5 Conclusion

For any study of a complicated agroecosystem, pinpointing the exact cause of a poor correlation between the soil and plant tissue is unlikely, but the stepwise regression model chosen to analyze the soil factors selected was significant and had a strong predictive ability so the results shown can be inferred with confidence. Of the soil factors chosen to for this study; soil nutrient level, pH, soil texture, CEC, and nematode abundance, the most ignored factor is the nematode presence. In attempting to rid a plant system of nematode infections many methods are applied, quarantines, eradication, and exclusion strategies are chief among these attempts, but are not often successful due to the resilience of nematodes and their great ability to adapt to new, harsh environments. Therefore, a better way to help infected plants is to focus more on suppressing the current nematode populations. This has been attempted with breeding efforts and crop rotation, but more focus should be placed on managing the soil nutrient profile, as a plant with sufficient nutrition is much less affected and able to fend off invaders such as nematodes (Dordas, 2009). Thus farmers with known nematode infections need to have an understanding of their soil test nutrient results and soil tests should be complimented with tissue testing.

The other factors that were tested: pH (if it is too high), CEC, and soil texture are more difficult to change to benefit nutrient availability and absorption. Knowing if soils are acidic and P or K uptake is poor by comparison of soil and tissue testing can direct a

producer to the proper lime application if the environment allows for it. The CEC levels and soil texture are not fixed with fertilizer application, but knowing these levels can lead to more precise management strategies with nutrient applications based on whether a high or low CEC is found or if the soil texture is sandy and requires split applications of nutrients. Testing both the soil and the tissue is the only way in which these factors can be known and an accurate assessment of nutrient uptake can be inferred.

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Appendix A: Results Figures

Sample ID	*# of Nematodes*	*ng/µl*	*A260*	*A280*	*260/280*	*260/230*
A	*1*	*25.64*	*0.513*	*0.558*	*0.92*	*0.18*
B	*5*	*36.58*	*0.732*	*0.962*	*0.76*	*0.17*
C	*1*	*38.64*	*0.773*	*1.029*	*0.75*	*0.16*
D	*10*	*35.52*	*0.71*	*1.093*	*0.65*	*0.15*
E	*5*	*49.81*	*0.996*	*1.387*	*0.72*	*0.17*

PCR Phusion Flash Master Mix, 50 µl rxn:

Component	*50µl rxn*
H20	*15µl*
2x Phusion Flash PCR Master Mix	*25µl*
D2/D3 Primer	*2.5µl*
ITS Primer	*2.5µl*
Nematode DNA	*5µl*

Appendix B: SAS Code

SAS Code for Correlation

```
ods rtf;
title 'Correlations of Leaf P and Soil P';
proc corr CSSCP data=P outp=CorrOutp;
  var LeafP SoilP;
run;
ods rtf close;
```

*Note: This code was used determine the correlation of soil P and leaf P

```
ods rtf;
title 'Correlations of Leaf K and Soil K';
proc corr CSSCP data=K outp=CorrOutp;
```

SAS Code for Linear Regression

Stepwise Regression:

```
ods graphics on;
proc reg data=P;
model LeafP =Year SoilP CEC pH sand SCN PIN:/Selection=stepwise;
run;
ods graphics off;

proc print data=P;
run;
```

*Note: This code was used to order effects of soil P, CEC, pH, sand, SCN, and Pin on Leaf P

```
ods graphics on;
proc reg data=K;
model LeafK =Year SoilK CEC pH _sand SCN PIN:/Selection=stepwise;
run;
ods graphics off;
proc print data=K;
run;
```

*Note: This code was used to order effects of soil K, CEC, pH, sand, SCN, and Pin on Leaf K