Nematode Community Structure and Effects on Peanut Production Systems

by

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Abstract

Understanding the total nematode community in agronomic systems and its impact on crop health may provide insight into more sustainable management strategies. In this study the focus was on management of the peanut root-knot nematode, *Meloidogyne arenaria* race 1 and the aflatoxigenic fungi *Aspergillus flavus* and *A. parasiticus*, a toxic food contaminate that poses a threat to humans and animals, to increase peanut yields while lowering toxins. The overall approach of management is to suppress plant-parasitic nematodes that facilitate invasion of the toxin producing fungi through manipulation of free-living nematode populations that act to increase plant health. The objectives of this research were 1) evaluate nematode consensus primers and Denaturing Gradient Gel Electrophoresis (DGGE) techniques for effectiveness in identification of nematode populations and monitoring community shifts; 2) develop nematode genetic profiles of selected soil samples, using DGGE fingerprinting, from different rotation sequences: continuous peanut, continuous bahiagrass, peanut/cotton, and peanut/corn, to determine if any factors exist that result in nematode population shifts; and 3) identify individual populations in the nematode community and determine their relationship with peanut yields and aflatoxin contamination. Nematode populations were established through various methods including *in vitro* culturing methods, after which total genomic DNA was extracted from each species to evaluate the specificity of nematode consensus primers. The primers amplified a wide trophic range of nematode DNA and fungal DNA, showing that the primers may be universal to all eukaryotes. DGGE techniques were then evaluated by amplifying a portion of the 18S rDNA

per species collected and subsequently separating the species through denaturing gradient gel electrophoresis. The DGGE technique successfully separated nematodes at the generic level. Nematode genetic profiles were created from peanut soils under different cropping sequences which revealed individual banding patterns, indicating population shifts between rotation sequences and shifts between sampling periods. Free-living nematodes accounted for the majority of sequences recovered from profiles, although plant-parasitic, animal-parasitic, and entomopathogenic nematodes, as well as nematophagus fungi were identified in recovered sequences. Bahiagrass rotations supported higher population levels of microbivore nematodes and significantly lower levels of aflatoxins when planted in rotation with peanuts. Negative correlations occurred between microbivore populations and total aflatoxin levels, suggesting that free-living nematodes may play a role in the suppression of aflatoxin contamination in peanuts.

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Chapter I. Introduction and Literature Review

There is a complex biotic structure within the soil that affects plant health. Some specific communities of soil organisms can lead to suppression of detrimental soil-borne bacterial, fungal and nematode populations, with subsequent alleviation of plant disease (Cook and Baker, 1983; Dickson et al., 1994). These organisms include bacteria, fungi and non-plant parasitic nematodes called free-living nematodes (Neher, 2001).

Free-living nematodes consist of bacterial-feeders, fungal-feeders and predatory nematodes. These free-living nematodes have direct and indirect effects on soil nutrition that can affect other organisms (Neher, 2001). They can affect the growth of plants and the metabolic activities of other soil microbes by regulating rates of decomposition and nutrient mineralization (Ingham et al., 1985). Free-living nematodes are commonly attributed to increased plant growth, increased nitrogen (N) uptake by plants, decreased or increased bacterial populations, increased CO_2 evolution, increased N and phosphorous (P) mineralization, and increased substrate utilization (Ingham et al., 1985).

Understanding soil suppression of plant diseases requires understanding the soil microbial community composition, including interactions between the populations. Traditional techniques employed to describe the composition and diversity of the nematode community relies on phenotypic characteristics. Traditional morphological identification by light microscopy is time-consuming and requires extensive training.Molecular analytical tools can overcome these limitations by directly exploring the composition present in a soil sample. One method is the utilization of a set of molecular analytical tools to generate population specific fingerprints by

displaying the ribosomal polymorphisms naturally present in microbial communities. Among these DNA fingerprinting methods, Denatured Gradient Gel Electrophoresis (DGGE) is a staple in environmental microbiology for studying microbial population structure and dynamics.

Previous studies have used DGGE to determine nematode species biodiversity by comparing molecular fingerprints (Foucher and Wilson, 2002; Foucher et al., 2004). Other studies have designed and evaluated nematode primers for DGGE analysis of soil community DNA (Waite et al., 2003). These previous studies have assessed nematode biodiversity without making an analysis of the populations present.

Peanuts are an important crop in the southeastern United States. They can be detrimentally affected by a number of soil-borne organisms, including plant parasitic nematodes and the ubiquitous *Aspergillus flavus* fungal group. Aflatoxins, produced by the *A. flavus* group, are highly carcinogenic, are strictly regulated to ensure a safe food supply, and can decrease the economic return from a peanut crop (Dorner et al., 2003). There is no highly effective control for aflatoxigenic fungi and aflatoxins, but minimization of this problem may be possible through a greater understanding of the microbial community that influences *A. flavus* production of aflatoxins including the nematode community.

The objective of this study was to determine the potential of DGGE to monitor populations within the nematode community and then apply this analysis to a peanut rotation system to determine if the nematode community affects plant health and yield quality. Evaluating the use of DGGE in identifying nematode populations was accomplished by choosing primers and testing their specificity to determine how robust the analysis is on a trophic level. This was followed by an evaluation of DGGE efficiency to determine denaturant characteristics of DNA for common species. The technique was then used to create genetic fingerprints of nematode

communities from peanut fields under various crop rotations in order to determine if free-living nematodes contribute to the health of the peanut crop by decreasing aflatoxin contamination or increasing yield.

Suppression of Soil-borne Diseases

Agricultural pests, such as microbial pathogens, insects and weeds, infest crops, causing significant losses in plant yield or quality. Disease suppression is usually achieved through cultural management practices, including crop rotations, resistant varieties, soil amendments and solarization. Beyond cultural management practices, growers usually depend on chemicals including herbicides, insecticides, fungicides and nematicides (Rosas, 2007).

The overuse of chemical pesticides to prevent or decrease pest populations has caused soil pollution and environmental contamination. Biological control offers answers to the many serious problems of modern agriculture and it is an essential component in the development of sustainable agriculture (Rosa, 2007). Baker and Cook (1974) defined biological control as "the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists." Biological control answers many agricultural problems such as the need to increase crop production within existing resources, avoiding development of pathogen resistance to chemicals, maintaining pollution- and risk-free control, and adopting practices compatible to sustainable agriculture (Cook and Baker, 1983).

Disease suppressive soils are one type of biological control, and are defined as soils where the pathogen does not establish or persist, establishes but causes low levels of damage or

no damage, or establishes and causes disease for a certain period of time until disease levels begin to lower and become insignificant (Baker and Cook, 1974). Suppressive soils are known for many plant pathogens and diseases including: *Gaeumannomyces graminis* var. *tritici* (Raaijmakers and Weller, 1998), Peach Tree Short Life (Kluepfel et al., 2002), root-knot nematodes (Dickson *et al*., 1994), and cyst nematodes (Kerry et al., 1982; Meyer et al., 1990; Carris et al., 1989; Yin et al., 2003).

Disease suppressive soils may be characterized as providing either general or specific suppression. General suppression is directly related to the total amount of microbial activity at a time critical to the pathogen (e.g. propagule germination and penetration). The type of microorganism present during this period is less important than the total active microbial biomass, which will compete with the pathogen for resources. Specific suppression is an effect of an individual or select group of microorganisms antagonistic to the pathogen during a stage in its life cycle (Cook and Baker, 1983).

Take-all decline (TAD) is a classic example of a specific suppressiveness. TAD is the natural biological control of take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici*. TAD is defined as the spontaneous reduction in disease and increase in yield with extended monoculture of wheat or barley (Slope and Cox, 1964). This phenomenon was first described in the 1930's (Glynne, 1935), and within 50 years it was recognized worldwide (Hornby, 1983). In 1976, Cook and Rovira suggested that TAD was based on microbiological interactions between the take-all pathogen and specific root-associated microorganisms. In 1998, Raaijmaker and Weller demonstrated that root-associated fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-diacetylphoroglucinol (Phl) are the key components of the natural biological control that operates in TAD soils. This was demonstrated by showing that

suppression of take-all was lost when Phl-producing fluorescent *Pseudomonas* spp. were eliminated, and conducive soils gained suppressiveness to take-all when Phl-producing *Pseudomonas* spp. were introduced to the soil.

Fluorescent *Pseudomonas* species have also been associated with the suppression of other plant diseases including Peach Tree Short Life (PTSL) (Kluepfel et al., 2002). PTSL is a syndrome that results in premature mortality of peach trees in the southeast United States. One major factor in PTSL is the migratory ectoparasitic nematode *Mesocriconema xenoplax* (the ring nematode). Kluepfel et al. (2002) isolated *Pseudomonas* sp. BG33R and demonstrated its ability to inhibit *M. xenoplax* multiplication *in vivo* and egg hatch *in vitro*. They also cloned and sequenced five genes from BG33R that were involved in production of the egg-kill factor. It was suggested that salicylic acid and a fluorescent siderophore plays a role in egg-kill. This study showed that shifting the soil microbial community toward *Pseudomonas* sp. BG33R, through soil solarization and microbial inoculation, inhibited ring nematode reproduction and suppressed PTSL.

Plant parasitic nematodes can also be suppressed by soil-borne organisms. Of these nematode antagonists, *Pasteuria* spp. have the greatest potential for biological control of plant parasitic nematodes (Dickson et al., 1994). *Pasteuria* spp. are Gram-positive, endospore-forming bacteria that are obligate parasites of several plant parasitic nematodes. Three nematode parasitic species have been characterized: *P. thornei*, a parasite of the root lesion nematode *Pratylenchus* spp., *P. nishizawae*, a parasite of cyst nematodes *Heterodera* spp. and *Globodera* spp., and *P. penetrans*, a parasite of the root-knot nematodes *Meloidogyne* spp. (Sayre and Starr, 1989).

Pasteuria spp. produce nonmotile endospores that are resistant to desiccation. The endospores readily attach to the cuticle of host nematodes on contact in soil or water. In root-

knot nematodes this usually occurs during the second-stage juvenile (J2). After attachment the endospore germinates, producing a germ tube that penetrates the nematode's cuticle. Inside the nematode's body, the germ tube develops into a vegetative colony. Sporangia develop; giving rise to more endospores that will eventually fill the nematode's body. Parasitized nematodes usually reach the adult stage, but fecundity is reduced or blocked. Disintegration of the parasitized nematode's body occurs, during which the endospores are released back into the soil (Dickson et al., 1994).

The potential of *Pasteuria* species as a biological control agent has mainly focused on *P. penetrans* (Tzortzakakis et al., 1997). In 1997, Chen et al. showed that peanut fields heavily infested with *Meloidogyne arenaria*, race 1, had yield increases and reductions of population densities of nematodes with continuous planting to peanut when inoculated with *P. penetrans*.

One of the classical studies on suppressive soils with fungal antagonists of nematodes was an investigation of cereal monoculture sites at the Rothamstead Experimental Station in Great Britain. This study showed a continuous decrease in the population levels of the cereal cyst nematode, *Heterodera avenae*, after a short population peak. Kerry et al. (1982) reported the fungi *Nematophthora gynophila* and *Verticillium chlamydosporium* as parasites of the cereal cyst nematode eggs and cysts responsible for the specific soil suppression.

Carris et al. (1989) compared fungal isolates from two soybean fields: one with high levels of the soybean cyst nematode, *Heterodera glycines*, and the second with suppressed *H. glycines* populations despite years of continuous cropping with susceptible soybean cultivars. They showed that *Fusarium oxysporum* and *Paraphoma radicina* were predominant in the field with the suppressed nematode population.

Meyer et al. (1990) found that a complex of soil-borne fungi could suppress egg hatch and juvenile mobility of the soybean cyst nematode, *H. glycines*, under laboratory conditions. This bioassay was conducted on eggs from nematodes that had been grown monoxenically on excised root tips. They showed that a combination of *Phoma chrysanthemicola*, one strain of *Verticillium chlamydosporium*, and one strain of *V. lecanii* decreased the number of viable eggs.

Yin et al. (2003) attempted to identify fungi associated with *Heterodera schachtii*, the sugar beet cyst nematode, obtained from soils possessing various levels of suppressiveness in California. The fungi were identified through an rDNA analysis termed oligonucleotide fingerprinting of ribosomal RNA genes (OFRG). Cysts obtained from the suppressive soil predominantly contained fungal rDNA with high sequence identity to *Dactylella oviparasitica*.

Identification of the biological properties contributing to the function of suppressive soils is necessary to manage such systems for use in the control of soilborne diseases. The development and application of molecular methods for monitoring soil microbial properties will enable a more rapid and detailed assessment of the biological nature of suppressive soils (Mazzola, 2004).

Dynamics of the Nematode Community

Free-living nematodes have direct and indirect effects on soil nutrition that can affect other soil organisms (Neher, 2001). Free-living nematodes are commonly attributed to increased plant growth, increased N uptake by plants, decreased or increased bacterial populations, increased CO_2 evolution, increased N and phosphorous (P) mineralization, and increased substrate utilization (Ingham et al., 1985).

Free-living nematodes indirectly affect the growth of plants and the metabolic activities of other soil microbes by regulating rates of decomposition and nutrient mineralization. Bacteria can act as a nutrient sink in soils, immobilizing nutrients from organic compounds (Ingham et al., 1985). Several studies have shown that microbial grazers, such as bacterial-feeding nematodes, can mineralize some of these immobilized nutrients, including N and to some extent P (Cole et al., 1978; Gould et al., 1981; Woods et al., 1982).

Nematodes contribute to nitrogen mineralization specifically by grazing on decomposer microbes and excreting ammonium (Ingham et al., 1985), which is the main excretory product of nematodes (Wright and Newall, 1976). De Ruiter et al. (1993) showed that bacterial-feeding and predatory nematodes contribute 13% and 9% of nitrogen mineralization, respectively, in conventional management practices.

Ingham et al. (1985) developed a conceptual model in which microfloral grazers were considered separate variables and evaluated the effects of bacterial-feeding nematodes on microbial growth, nutrient cycling, plant growth, and nutrient uptake. They showed that plants grow faster in the presence of microbial grazing nematodes than in their absence, and that the growth response was caused by the increase in nitrogen mineralization from the nematodes.

Understanding the impact of the nematode community on plant health requires identifation of the populations present within the community, and identifying interactions between the populations. Traditional techniques employed to describe the composition and diversity of nematode populations in the soil relies on phenotypic characteristics, which are evolutionarily highly conserved. Such a technique provides an incomplete assessment of diversity, is time-consuming and requires extensive training. In addition, identification of nematodes at the species level can be problematic in many cases. Most species can only be

identified from adult male- or female-specific structures. Van Der Knaap et al. (1993) noted that *Caenorhabditis elegans* and *C. briggsae* can only be differentiated by males (based on the arrangement of bursal rays at the tail) which can form less than 0.1% of the population.

Researchers usually classify nematodes into trophic groups instead of identifying each species for community analyses. According to Bernard (1992) soil inhabiting nematodes can be separated into five trophic groups: microbivores (bacterial-feeders), fungivores (fungal-feeders), plant-parasites, predators, and omnivores. The problem with using trophic groups when analyzing functionality in nematode communities is that these categories are not mutually exclusive. Species placed in one category may have developmental stages that fit another category (Bernard, 1992). For example, juvenile stages of some species of the predacious orders Mononchida and Diplogasterida may feed on bacteria in their initial juvenile stages (Yeates, 1987). Yeates also reported maintaining cultures of the predacious nematode *Mononchus propapillatus* on bacteria for over eight months.

The total number of nematode species described from a single site can also complicate identification. Hodda and Wanless (1994) identified 154 nematode species from an English Chalk Grassland, 44 of which could not be assigned positively to previously described species. Beier and Traunspurger (2003) identified 113 species from a coarse-grained sub-mountain carbonate stream in southwest Germany. Baird and Bernard (1984) reported 100 species in two wheat-soybean fields in Tennessee. Orr and Dickerson (1966) found 228 nematode species, representing 80 genera, in 61 soil samples taken from a prairie pasture in Kansas. This is the maximum number of nematode species described from a single soil site (Boag and Yeates, 1998). Furthermore, terrestrial nematodes can easily exceed one million individuals per square meter of soil (Floyd et al., 2002).

It has been recognized that there is a severe shortage of taxonomically oriented nematologists, especially for free-living nematodes (Bernard, 1992; Coomans, 2002). Nematodes are mainly studied with the compound light microscope and the observations are usually made based on numerous fixed specimens, which can take a considerable amount of time to prepare. The limitations to nematode community analysis may be overcome by using molecular analytical tools to directly explore the composition in a soil sample based on the nucleic acids present.

Molecular Analytical Techniques

Soil microbial communities affect crop health and in turn affect yields. Monitoring these communities has become important in sustainable agriculture. The soil microbial community may be altered to increase beneficial organisms by manipulating cropping conditions. A reliable, reproducible and sensitive method to profile these populations is needed. Molecular analytical tools have recently been applied to characterize the biology resident to soil ecosystems and have provided new insight into the diversity of microbial species found in soil habitats (Mazzola, 2004). These molecular analytical methods can directly explore the microbial composition of a sample based on the nucleic acids present within that sample.

Various molecular techniques have been used in nematology for diagnostics, estimation of genetic diversity of populations and inference of phylogenetic relationships between taxa (Subbotin and Moens, 2006). These techniques include protein electrophoresis (Esbenshade and Triantaphhllou, 1985), polymerase chain reaction (PCR) (McCuiston et al., 2007), restriction fragment length polymorphism (RFLP) (Curran et al., 1986), multiplex PCR (Skantar et al., 2007), random amplified polymorphic DNA (RAPD) (Caswell-Chen et al., 1992), amplified fragment length polymorphism (AFLP) (Folkertsma et al., 1996), sequencing of DNA (Bae et al.,

2008), DNA bar-coding (Floyd et al., 2002), and real-time PCR (Madani et al., 2005). Molecular nematology, although, has only recently been applied in an ecological context.

In 1993, Van Der Knaap et al. used an arbitrarily primed polymerase chain reaction (ap-PCR) technique to differentiate closely related bacterial-feeding nematode genera (*Caenorhabditis*, *Acrobeloides*, *Cephalobus*, and *Zeldia*), which are difficult to separate into species. The technique was used to assess biodiversity and required PCR amplification of individual nematodes with at least three different primer sets. However, the technique could not identify the nematodes without considerable calibration.

Vrain et al. (1992) separated populations of the *Xiphinema americanum* group, a plant parasitic nematode vector of nepoviruses, based on their capability to vector viruses using restriction fragment length polymorphism (RFLP). This was accomplished using the restriction fragment length difference in the 5.8S gene and the internal transcribed spacer (ITS) of ribosomal DNA.

In 2002, Floyd et al. developed a molecular operational taxonomic unit (MOTU) method using a molecular barcode derived from single-specimen polymerase chain reaction (PCR) and sequencing of the 5' segment of the small subunit ribosomal RNA (SSU) gene for soil nematodes. The results indicated that this technique allowed a rapid assessment of nematode diversity in soils. This method requires sequencing PCR amplified products from individual nematodes.

Eyualem and Blaxter (2003) used Floyd's molecular barcode system to identify free-living nematode species. They attempted to differentiate five cultured isolates of the taxonomically difficult genus, *Panagrolaimus*. Their results showed that the five populations belonged to two different species.

Qiu et al. (2006) developed a simple PCR assay protocol for detection of the root-knot nematode species *Meloidogyne arenaria*, *M. incognita* and *M. javanica* extracted from soil. The PCR assay was carried out with primers specific for this group of nematodes they developed and with universal primers spanning the ITS region of rRNA genes (Vrain et al., 1992). This analysis can detect the presence of second stage juveniles from large numbers of other plant-parasitic and free-living nematodes.

Griffiths et al. (2005) combined morphology and molecular sequencing to establish the potential for analyzing nematode communities by molecular biological characterization. From their study they concluded that DNA from certain groups of nematodes was under-represented by this analysis. This was attributed to either a mismatch in sequence at the primer site or PCR inhibition from the secondary structure of the template DNA or co-extracted compounds.

Among the molecular analytical techniques available, molecular fingerprinting methods can help monitor changes in microbial communities over time with a simplified representation of the community. These methods generate population specific fingerprints that display the ribosomal polymorphism naturally present in the community at a given time. Among these fingerprinting methods, denatured gradient gel electrophoresis (DGGE) has been successfully applied to study microbial communities from different sources including agricultural soils.

DGGE is used in microbial ecology to investigate population diversity and community dynamics in response to environmental variations. Recent applications study microbial communities within soil, rivers, seas, lake water, gastrointestinal tracts of animals, wastewater treatment bioreactors, insects, clinical samples, and food (Ercolini, 2004).

DGGE separates PCR products based on sequence differences that result in differential denaturing characteristics of the DNA. PCR products encounter increasingly higher

concentrations of chemical denaturants (formamide and urea) as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature at which time migration slows dramatically. Differing sequences of DNA (from different organisms) will denature at different denaturant concentrations, depending on the % GC composition of the sequence, resulting in a pattern of bands. Each band theoretically represents a different sequence present in the community. Fingerprints can be uploaded into an analytical software database in which similarity can be assessed to determine microbial structural differences between environments or treatments (Muyzer et al., 1993).

In a previous study by Foucher and Wilson (2002), DGGE was used to distinguish nematode species from a mixed laboratory culture. This study suggested that DGGE could be used to measure nematode diversity within the soil. In 2003, Waite et al. used DGGE to analyze nematode communities from total genomic DNA extracted from the soil. They showed that the nematode community fingerprint differed between different sites. In 2004, Foucher et al. also used DGGE to assess nematode biodiversity by comparing nematode community fingerprints.

Previous studies using DGGE to assess nematode biodiversity have not made any analysis of the taxonomic populations present or their abundance from the molecular data. One unique characteristic of DGGE is that DNA from each organism can be retrieved, once molecular fingerprints have been analyzed, by excising individual bands from the gel. After the DNA has been retrieved it can be reamplified and sequenced. The sequences can be uploaded into a database and compared to know sequences to identify the populations present in the community.

In this study, DGGE analysis was applied to a peanut rotation cropping system. Nematode populations from continuous peanut, peanut/cotton, peanut/corn, and continuous bahiagrass rotations were assessed in order to determine the affect of the total nematode community on the health of the plant based on aflatoxin contamination and yield.

Peanuts and Aflatoxin Contamination

Peanuts are an important crop in the southeastern United States. They can be detrimentally affected by a number of soil-borne organisms, including plant parasitic nematodes and the *Aspergillus flavus* fungal group. Aflatoxins, produced by the *A. flavus* fungal group, are highly carcinogenic, are strictly regulated to ensure a safe food supply, and can decrease the economic return from a peanut crop (Dorner et al., 2003). Contamination of aflatoxins in peanut seeds results in a loss of $2.6 million per year to peanut growers (Lamb and Sternitzke, 2001). There is no highly effective control for aflatoxigenic fungi and aflatoxins, but minimization of this problem may be possible through a greater understanding of the microbial community that influences *A. flavus* production of aflatoxins.

Aflatoxins are polycyclic, unsaturated highly substituted coumarins. Approximately 20 aflatoxins have been identified but only four of them occur naturally: B_1, B_2, G_1, and G_2. Aflatoxin B_1 is the most potent. There is no threshold dose below which no tumor formation will occur when consumed by animals, and only a zero level of exposure will result in no risk. Besides their liver carcinogenic effect, aflatoxins are also mutagenic, teratogenic, and hepatogenic. When consumed at low doses they can also be responsible for weight loss, loss of reproductive capacity, and imparity of immune systems. Unprocessed foods of plant origin are the most important source of aflatoxins in the diet (Weidenborner, 2001).

Aflatoxins are produced in peanut pod tissues by *A. flavus* and *A. parasiticus*, which are commonly referred to as the *A. flavus* fungal group. The toxins are produced when environmental conditions are hot and dry, three to six weeks prior to peanut maturity. Damaged and immature pods are more susceptible to infection by aflatoxin producing fungi than healthy pods (Hill et al., 1983). Damage to pods is in part due to plant parasitic nematodes. Wounds caused by nematode feeding are generally superficial, although damage may create conditions favorable for invasion of *A. flavus*. The root-knot nematode, *Meloidogyne arenaria*, race 1, and the ring nematode, *Mesocriconema xenoplax*, have been shown to increase aflatoxin contamination of peanut seeds (Timper et al., 2004; Bowen et al., 2003).

There are no reliable methods for control of aflatoxin contamination in peanuts. Irrigation has been shown to reduce *A. flavus* colonization of peanuts, especially in the last 40 to 75 days of growth, but this is not feasible for most growers (Wilson and Stansell, 1983). Biological control of aflatoxin contamination was demonstrated using atoxigenic strains of *A. flavus* and *A. parasiticus*. These strains competitively exclude toxigenic strains in the soil and reduce aflatoxin concentrations. However, these strains can be human allergens (Dorner et al., 1992). Another biological control strategy includes introducing or enhancing bacteria to reduce or eliminate colonization of the fungus through competition, although this strategy has not been optimized yet (Mickler et al., 1995).

Mechanisms by which plant parasitic nematodes increase aflatoxins are unknown. Galls on peanut pods produced by root-knot nematodes may increase kernel colonization by *A. flavus* fungi by serving as entry points for the fungus or by preventing kernel development. Nematodes could also contribute to aflatoxin production because their damage impairs root function, predisposing the plant to drought stress. Plant parasitic nematode infection of roots also causes

physiological changes in the plant that increase the susceptibility of kernels to infection by the fungus (Timper et al., 2004).

Nematode populations are usually controlled through crop rotations with a non-host crop. Bowen et al. (1996) showed that root-knot nematode densities in peanut production fields were lower following two years of cropping corn, cotton or other non-leguminous crops than when peanut was planted in alternating years. These observations suggest that root-knot nematodes limit yields in continuously cropped peanuts. Bahiagrass in rotation with peanuts has also been shown to reduce number of root-knot nematode juveniles and increase yields 36% higher than monocultured peanuts, if planted following two years of bahiagrass (Rodriguez-Kabana et al., 1991).

Dynamics within the nematode community, including the role various free-living nematodes play, may contribute to general or specific suppression of certain soil-borne diseases. Understanding the resident nematode community composition as well as the functional interactions between members present in the population may be the key to understanding how free-living nematodes affect soil-borne disease complexes.

Monitoring these free-living and plant parasitic nematode profiles using molecular fingerprinting methods such as DGGE under different crop rotations and then correlating these results to aflatoxin contamination and yield may shed light on potential interaction that act to suppress nematode/fungal damage to peanuts. An understanding of these interactions under field conditions could indicate possible management schemes to change soil microbial profiles by shifting nematode communities toward beneficial populations and thus ultimately reduce nematode/aflatoxin contamination and increase yields.

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Chapter II. Evaluation of DGGE to Monitor Nematode Populations in Agricultural Soils

Abstract

Denaturing Gradient Gel Electrophoresis (DGGE) can be used to monitor communities of microorganisms by generating population specific fingerprints that display the ribosomal polymorphism naturally present in the community. The objective of this study was to evaluate the potential use of DGGE to monitor nematode populations in agricultural soils. This was accomplished by testing the specificity of nematode consensus primers, determining the efficiency of DGGE to separate a wide range of nematode DNA and then applying the technique to analyze soil samples from a peanut rotation system. The nematode consensus primers amplified a wide trophic range of nematode DNA and fungal DNA, showing that the primers may be universal to all eukaryotes. DGGE separated most nematode species at separate denaturant concentrations except *Meloidogyne arenaria* and *M. incognita*. *Rhabditis* sp. and *Tylenchorhynchus* sp. samples each yielded two bands which were matched to separate genera and separate species, respectively. The DGGE profile indicated similarities among community profiles of replicated plot samples from continuous bahiagrass rotations and continuous peanut rotation with 73% and 55% similarity, respectively. A total of 37 band classes were observed between all plots, 15 of which were excised, re-amplified, sequenced, and matched to closely related sequences held in GenBank's database. These results show that DGGE can be successfully applied to analyze populations within the nematode community and to monitor shifts in the populations due to cropping rotations.

Introduction

The nematode community represents a complex structure within the soil that affects plant health. This community consists of plant-parasitic nematodes and free-living nematodes. Free-living nematodes include microbivores (bacterial-feeders), fungivores (fungal-feeders) and predatory nematodes. These free-living nematodes have direct and indirect effects on soil nutrition that can affect other soil organisms (Neher, 2001). Free-living nematodes are commonly attributed to increased plant growth, increased nitrogen (N) uptake by plants, decreased or increased bacterial populations, increased CO_2 evolution, increased N and phosphorous (P) mineralization, and increased substrate utilization (Ingham et al., 1985).

Free-living nematodes indirectly affect the growth of plants and the metabolic activities of other soil microbes by regulating rates of decomposition and nutrient mineralization. Bacteria can act as a nutrient sink in soils, immobilizing nutrients from organic compounds (Ingham et al., 1985). Several studies have shown that microbial grazers, such as bacterial-feeding nematodes, can mineralize some of these immobilized nutrients, including N and to some extent P (Cole et al., 1978; Gould et al., 1981; Woods et al., 1982).

Nematodes contribute to nitrogen mineralization specifically by grazing on decomposer microbes and excreting ammonium (Ingham et al., 1985), which is the main excretory product of nematodes (Wright and Newall, 1976). De Ruiter et al. (1993) showed that bacterial-feeding and predatory nematodes contribute 13% and 9% of nitrogen mineralization, respectively, in conventional management practices.

Understanding the impact the nematode community has on plant health requires identifying populations present within the community and interactions between those populations. Traditional techniques employed to describe the composition and diversity of

nematode populations in the soil relies on phenotypic characteristics, which are time-consuming and require extensive training. In addition, identification of nematodes to the species level can be problematic in many cases. Most species can only be identified from adult male- or female-specific structures. Limitations to nematode community analysis may be overcome by using molecular analytical tools to directly explore the composition in a soil sample based on the nucleic acids present.

Among the molecular analytical techniques available, molecular fingerprinting methods can help monitor changes in microbial communities over time with a simplified representation of the community. These methods generate population specific fingerprints that display the ribosomal polymorphism naturally present in the community at a given time. Among these fingerprinting methods, denaturing gradient gel electrophoresis (DGGE) has been successfully applied to study microbial communities from different sources including agricultural soils (Ampe et al., 2001; Avrahami et al., 2003).

DGGE separates amplified products based on sequence differences that result in differential denaturing characteristics of the DNA. Amplified products, typically generated through Polymerase Chain Reaction (PCR), encounter increasingly higher concentrations of chemical denaturants (formamide and urea) as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the double-stranded PCR products with lower melting temperatures will begin to denature at which time migration stops. Differing sequences of DNA (from different organisms) will denature at different denaturant concentrations, depending on the % GC composition of the sequence, resulting in a pattern of bands (Muyzer et al., 1993). Each band theoretically representing a different organism present in the community.

Fingerprints can be uploaded into a database and similarity can be assessed to determine microbial structural differences between environments or treatments.

Foucher and Wilson (2002) developed a PCR-DGGE technique to distinguish nematode species from a mixed laboratory culture. They were able to separate PCR fragments from all species tested except those with similar melting behaviors. Waite et al. (2003) designed and evaluated nematode consensus primers for PCR amplification of soil community DNA. Foucher et al. (2004) used PCR-DGGE to estimate nematode species richness from grassland soil samples. Their analysis revealed a relationship between species richness and DGGE estimates for species that represented more than 1% of the population although they did not make any analysis of the populations present.

The objective of this study was to evaluate the potential use of DGGE to monitor nematode populations in agricultural soils by analyzing the populations present within the nematode community. This was accomplished by testing the specificity of nematode consensus primers on a wide trophic range of nematode DNA. The primers were then used to determine the efficiency of DGGE techniques to separate individual nematode species. Finally, DGGE was used to analyze soil samples from a peanut rotation system to determine if the technique can be used to identify nematode populations present and monitor shifts in the populations based on rotation sequence.

Materials and Methods

Primers: Nematode consensus primers designed to amplify a ~630 bp fragment of the 18S rDNA were used in this experiment: nem1 - forward (5'-GCAAGTCTGGTGCCAGCAGC-3') and nem2 - reverse (5'-CCGTGTTGAGTCAAATTAAG-3') (Foucher and Wilson, 2002). The

18S rDNA is a highly conserved gene with somewhat variable regions making it a suitable target for consensus primers. The forward primer contained a 39 bp GC clamp at the 5' end to prevent complete denaturation of the amplified product during electrophoresis (Myers et al., 1985).

DNA collection: Soil naturally infested with *Meloidogyne arenaria* (Neil, 1889) Chitwood, 1949 was collected from peanut fields at the Wiregrass Research and Extension Center (WREC) in Headland, Alabama. Tomato seeds (*Solanum lycopersicum* cv Rutgers) were planted in the nematode infested soil in polystyrene cups to allow nematode colonization of the roots in the Plant Sciences Research Center (PSRC) in Auburn, Alabama. The roots were removed and nematode eggs were extracted using the sodium hypochlorite method (Hussey and Barker, 1973) after 45 days. Eggs were quantified and standardized using a Nikon-T 100 inverted microscope. Approximately 5,000 eggs were used to inoculate three week old tomato plants in a 3:1 ratio of autoclaved field soil and autoclaved sand. This was repeated every 3 generations to maintain populations.

Meloidogyne incognita (Kofoid and White, 1919) Chitwood, 1949 infested soil was collected from tomato plots at the E.V. Smith Research Center (EVSRC) in Tallassee, Alabama. The populations were purified and maintained in the same manner as for *M. arenaria*.

Populations of *M. arenaria* and *M. incognita* were identified to race level using the North Carolina Differential Host Test (Hartman and Sasser, 1985) and protein electrophoresis analysis using esterase and malate dehydrogenase enzymes (Esbenshade and Triantaphyllou, 1990). The *M. arenaria* population was identified as race 1 (peanut root-knot nematode) and the *M. incognita* population was identified as race 3 (southern root-knot nematode).

Rotylenchulus reniformis (Linford and Oliveira, 1940) populations were collected from infested cotton soils at EVSRC in Tallassee, Alabama. These populations were maintained on cotton (*Gossypium hirsutum* cv Stanville 5599) as described for *M. arenaria*.

Meloidogyne arenaria, *M. incognita* and *R. reniformis* were maintained in monoxenic cultures on tomato root-explants according to Huettel (1990) with slight modification. Tomato seeds (*Solanum lycopersicum* cv Rutgers) were disinfected in 95% EtOH for 3 min. The EtOH was poured off and replaced with 10% Clorox solution for 10 min. The seeds were transferred directly to 1% water agar plates and incubated at 27°C in the dark for 3-4 days. Healthy, straight root tips about 2-3 cm in length were excised by cutting with a sterile dissecting blade, under a laminar flow hood. The root tips were transferred to Gamborg's B-5 media (Research Products International Corp., Mt. Prospect, IL) (3 root tips/plate) and incubated at 27˚C for 3 days or until root tips began to grow. *Meloidogyne arenaria* and *M. incognita* egg masses were collected from tomato roots maintained in the PSRC. Egg masses were hand-picked and placed in sterile microcentrifuge tubes. The eggs masses were washed with sterile water and pelletized by centrifugation at 2,000 rpm for 2 min. The supernatant was decanted and 1% streptomycin sulfate was added. The egg masses sat for 10 min at room temperature then centrifugation was repeated. The streptomycin sulfate was removed and the egg masses were washed in sterile water. After the water was decanted, 0.001% mercuric chloride was added and the samples were immediately centrifuged and decanted. The egg masses were washed two more times with sterile water. Gamborg's B-5 plates containing tomato root-explants were inoculated with 3 egg masses each. Populations were maintained by transferring egg masses to new tomato root-explants every 3 generations.

Rotylenchulus reniformis eggs were collected from cotton plants maintained in the PSRC. Eggs were collected using the sodium hypochlorite method and disinfected as earlier described. Tomato root-explants, established on Gamborg's B-5 media, were inoculated with 50 eggs/plate. Roots were transferred every 3 generations to maintain populations.

Soil samples collected from peanut plots at the WREC in Headland, Alabama were subjected to a sieving process followed by sugar flotation to extract nematodes (Jenkins, 1964). Aliquots of 1.0 µl water solution containing nematodes were placed on 1.5% water agar. Individual nematodes were transferred to clean 1.5% water agar plates after 3-4 weeks. Plates with single nematodes were incubated at 27°C for 1-2 months to allow nematode reproduction. All other nematode specimens were hand-picked on a Nikon SMZ800 dissecting microscope after extraction from soil by sieving and sugar flotation.

Permanent mounts (20-30) were prepared for all nematode species, except *M. arenaria*, *M. incognita* and *R. reniformis*. Permanent mounts were prepared according to Seinhorst (1962) with modifications. Individual nematodes were placed in room temperature water in microcentrifuge tubes. The tubes were centrifuged at 2000 rpm for one min and the supernatant was decanted. Boiling 10% formalin was added to the nematode specimens and incubated at room temperature. After one week the formalin was replaced with 2.5% glycerin EtOH. The EtOH was allowed to evaporate in a desecrator for one week. The specimens were mounted in dehydrated glycerin.

Nematode specimens were keyed out to generic level on a Nikon Eclipse 80*i* using one of three keys: 1) Interactive Diagnostic Key to Plant Parasitic, Free-living and Predaceous Nematodes from the UNL Nematology Lab, 2) Identification of Freeliving Nematodes (Secernentea) from UCR Extension, or 3) Rhabditina Generic Identification from the University

of Florida. The nematodes raised in pure culture on 1% water agar were identified as *Panagrolaimus* sp. and *Prismatolaimus* sp. Hand-picked nematode specimens were identified as *Tylenchorhynchus* sp., *Helicotylenchus* sp., *Mesocriconema* sp. (plant-parasitic nematodes), *Mesorhabditis* sp., *Acrobeles* sp. (microbivorous nematodes), *Neoactinolaimus* sp. (fungivorous nematode), and *Monochus* sp. (predacious nematode).

Two common fungi were isolated from the same peanut soils from which nematodes were extracted. *Sclerotium rolfsii* (Sacc. 1911) was cultured on Acidic Potato Dextrose Agar (APDA). *Aspergillus flavus* (Link 1809) was cultured on *Aspergillus flavus* and *parasiticus* agar (AFPA) (Pitt et al., 1983).

Total genomic DNA was extracted from all 14 species (Table 1) using the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories Inc. Carlsbad, CA) following the manufacturer's instructions. DNA quality and quantity was assessed using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). DNA from each sample was diluted to 20 ng/µL and stored at -80°C until required for downstream applications.

Primer specificity: Specificity of the nem1/nem2 primer pair was evaluated to determine the range of nematode trophic group DNA that can be amplified. DNA from the 12 nematode species and two fungal species, in the previously described DNA collection, was PCR amplified in 50 µl volumes consisting of 5 µl 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 5 µl 25mM $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl each 10 µM forward and reverse primers, 0.3 µl 5 U/µl Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA), and 5 µl template DNA (100ng). A negative control sample without template DNA was included. The amplification process was performed in a Techne TC-312 thermocycler. The PCR program consisted of 94ºC

for 5 minutes; 35 cycles of 94ºC for 45 seconds, 48ºC for 45 seconds and 72ºC for 1 minute; followed by a final extension of 72ºC for 10 minutes. PCR products were separated on a 1% agarose gel at 100 V for 1 hour and visualized with UV illumination after staining with ethidium bromide (EtBr) to determine if amplicons of the correct size were detected.

DGGE efficiency and sequence confirmation: DGGE analysis was carried out using the Dcode® Universal Mutation Detection System (Bio-Rad, Hercules, CA). A total of 15 samples were run: 12 nematode species, 2 fungal species and a negative control. PCR amplified products, as described above, were separated on a 20-50% denaturant solution, 1mm, 6% polyacrylamide gel. The polyacrylamide gel was prepared by mixing 15 ml of each denaturant solution with 81 µl 10% ammonium persulfate (APS) and 4.5 µl N,N,N',N'-tetramethylethylenediamine (TEMED). The high and low denaturant solutions were mixed using a manual gradient delivery system (Bio-Rad, Hercules, CA). Electrophoresis was run at 100 V for 16 hours in 1X TAE buffer at 60°C. After electrophoresis, the gel was stained with GelStar® Nucleic Acid Gel Stain (Lonza, Rockland, ME) for 30 minutes and rinsed with deionized water. The gel was subjected to UV illumination using an AlphaImager® HP (Alpha Innothec Corp., San Leandro, CA) to determine banding position.

Bands from samples intended as standards, *M. arenaria*, *M. incognita* and *R. reniformis*, as well as bands from samples containing multiple bands were excised from the gel using a wide borer pipette tip and placed in 30 µl sterile water. The DNA was allowed to diffuse into the water at 4˚C overnight (Ampe et al., 2001). This DNA was used as template and re-amplified using PCR conditions as described above, except the forward primer did not contain a GC clamp. Electrophoresis on a 1% agarose gel was used to confirm the presence of the PCR amplified

product. The remaining amplified product was cleaned using Wizard PCR Prep kits (Promega, Madison, WI) following the manufacturers recommendations. The samples were sequenced by Lucigen Inc. and the results were compared to known sequences in Genbank's nucleotide collection using the basic local alignment search tool (BLASTN) (Altschul et al., 1990).

DGGE analysis of nematode communities from peanut rotation soils: Soil samples were obtained from the Wiregrass Research and Extension Center in Headland, Alabama from a long term rotation study. The rotation sequences used in this study included: continuous peanuts, peanut/cotton, peanut/corn, and continuous bahiagrass. Rotation sequences were arranged in a randomized complete block design with four replications. Soil sampling was conducted at planting (May) 2008. Five soil cores were taken randomly across each plot from the root zone in each replication. Samples were placed in a plastic bag, mixed thoroughly and stored at 10°C until needed.

Nematodes were extracted from 100 cm^3 subsamples from each plot using a sieving process followed by sugar flotation (Jenkins, 1964). The sugar flotation process was repeated to ensure that specimens were clean of any debris (Miller, 1957). DNA was extracted from all samples, a ~630 bp fragment of the 18S rDNA was amplified using the nem1/nem2 primer pair, and the amplified products were separated using DGGE as previously described.

The DGGE gel image was analyzed with BioNumerics V. 5.0 software program (Applied Maths, Austin, TX). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between profiles were calculated with the band based Dice coefficient. Cluster analysis was performed with the Unweighted Pair Group Method using Arithmetic averages (UPGMA). A band matching analysis was performed and a band table

was created for polymorphism analysis. Bootstrap analysis of 1000 replicates was performed to define tree robustness. Multi-Dimensional Scaling (MDS) was completed to compare the clusters generated over different crop rotations.

Unique bands from each sample as well as common bands in all samples were excised and re-amplified. The PCR product was re-run on DGGE to confirm that the sample yielded a single band at the same position from which it was recovered. The samples were then sequenced as earlier described and compared to known sequences in GenBank using BLASTN. A putative identification was made for sequences matching those in GenBank with a score greater than 100 bits and an e-value lower than 0.001. Sequences matching the criteria were putatively identified to the species level for 97% or higher maximum identity and to the generic level for 75-96% maximum identity. Sequences with a 74% or lower maximum identity where considered as not significantly matching the sequences held in GenBank.

Results

Primer Specificity: PCR amplification of genomic DNA using the nem1/nem2 primer pair with GC clamp yielded products ~670 bp for all species tested (Fig 1). Detection of the two fungal species by the primer pair suggests that the primers may be universal to the small subunit ribosomal DNA gene (18S rDNA) of eukaryotic organisms, rather than specific to nematodes. Nematodes were extracted from the soil prior to DNA extraction for further testing in this study.

DGGE efficiency and sequence confirmation: DGGE analysis of the nematode DNA yielded bands at different positions or different denaturant concentrations for most samples (Fig 2). Despite different melting profiles obtained from WinMelt software (Bio-Rad, Hercules, CA), *M.*

arenaria and *M. incognita* yielded bands at the same location. *Rhabditis* sp. and *Tylenchorhynchus* sp. samples yielded two separate bands. *Aspergillus flavus* and *S. rolfsii* did not yield a band at this denaturing concentration range.

Sequence of the partial 18S rDNA was obtained from the three standard samples excised from the DGGE gel. The *R. reniformis* sample was closely matched to other *R. reniformis* sequences with a maximum identity of 98%. *Meloidogyne arenaria* and *M. incognita* samples were matched with other *Meloidogyne* sp. sequences with a maximum identity of 95% and 96%, respectively. The two bands retrieved from the *Rhabditis* sp. sample were most closely matched to *Rhabdias bufonis* and *Rhabdolaimus* sp. with 97% maximum identity for both samples. The two bands retrieved from the *Tylenchorhynchus* sp. sample were matched to *Tylenchorhynchus claytoni* with 96% maximum identity and *Tylenchorhynchus dubius* with 94% maximum identity.

DGGE analysis of nematode community from peanut rotation soils: The DGGE profile of nematode populations from different peanut crop rotations showed similarities among community profiles of replicated plot samples from two separate cropping sequences (Fig 3). Three distinct groups were defined at 50% or greater similarity with one outlier. Some common bands were observed between crop rotations irrespective of cropping sequence. DGGE banding patterns from the continuous peanut cropping system indicated that there was approximately 55% similarity between these plots. In the case of continuous bahiagrass, 73% similarity was observed between the plots. The peanut/corn plots did not group together but rather grouped with continuous bahiagrass plots, except one that grouped with the continuous peanut plots. Three

peanut/cotton plots grouped together with 75% similarity while the other did not group with anything and was considered an outlier.

A total of 37 bands were observed between all of the plots. The lowest number of bands observed in a sample was nine and the highest was 17. The average number of bands in a single plot was 13. There was no single band observed across all samples. Multi-Dimensional Scaling (MSD) of the DGGE community profiles from different cropping sequences revealed that nematode communities pertaining to each crop rotation were located in different clusters with a few outliers (Fig 4).

The DGGE profile yielded 17 common or unique bands that were excised and re-amplified. Each DNA sample recovered from the original DGGE gel yielded a single band at the original position from which it was recovered when again subjected to DGGE. All 17 samples were sequenced and matched to closely related sequences in GenBank's database. There were 15 partial 18S rDNA sequences found to have sequence similarities that placed them into known nematode genera (Table 2). The remaining sequences had no significant similarities within the nucleotide collection of the GenBank database. There were six sequences aligned with 97% or higher maximum identity matching the sequences at a species level. The remaining samples had a 76-96% maximum identity matching them at a generic level to know sequences. The putative identification made of the 15 partial gene sequences represented 13 separate genera, two vertebrate parasites, one entomopathogen, one herbivore, two predators, and the remaining represented microbivores.

Discussion

Understanding the soil community and how the populations within the community interact and affect plant health is important in agriculture. Identifying the populations within the nematode community to date has been cumbersome. Using molecular fingerprinting methods such as DGGE and recovering DNA to sequence and analyze these populations has several key advantages over morphological identification including the savings in time and skill level required. The results of this current study show that DGGE can be successfully applied to analyze populations within the nematode community and to monitor shifts in the populations due to cropping rotations.

The consensus nematode primers used in this study amplified a wide trophic range of nematode DNA. The primer pair also amplified DNA from the two non-target fungal organisms. This indicates that the primers may be universal to all eukaryotic organisms rather than specific to nematodes. To ensure that other non-target eukaryotic organisms are not amplified from soil samples, nematodes must be extracted from the soil prior to extracting DNA from the nematodes.

DGGE analysis of the DNA collection separated most of the samples at different denaturant concentrations. The two species that were resolved at the same denaturant concentration, *M. arenaria* and *M. incognita*, have similar melting profiles. This indicates that DGGE may not reliably separate samples at the species level, at least species that are closely related. Other DNA samples, *Rhabditis* sp. and *Tylenchorhynchus* sp., yielded two separate denaturant concentration bands. The DNA recovered from these bands were matched to separate genera and separate species, respectively. This confirms the difficulty in correctly identifying some nematode species using morphological characteristics. The two fungal species did not yield bands between 20-50% denaturant concentrations. Other studies using DGGE to profile fungal

communities use denaturant concentration ranges between 10-60% (Buesing et al., 2009; Duong et al., 2006; Anderson et al., 2003), further demonstrating the need to eliminate other eukaryotic organisms from the sample prior to nematode DNA extraction.

DGGE profiles of nematode communities from peanut soils under different cropping sequences revealed population shifts between crop rotations. Common and unique bands can be found throughout the DGGE profile irrespective of cropping sequence. Multi-Dimensional Scaling of the DGGE profile indicated diversity of the nematode community in soils of different cropping sequences. Clusters of the profiles were observed with respect to replications and differed due to cropping sequence.

The band matching analysis revealed 37 bands across the DGGE profile, indicating that 37 different species were detected throughout the plots sampled. Only 17 bands were excised to recover DNA and match sequences to those previously identified in GenBank. High background fluorescence inhibited visualization of weaker bands and ultimately inhibited recovery of the DNA at those positions. There is a limited number of nematode 18S gene sequences compiled in the nucleotide collection of GenBank's database. This explains why only six out of 17 sequences were matched with 97% maximum identity. It has been estimated that there are possibly 500,000 different nematode species in existence, yet only approximately 12,000 have been described (Myers, 2001). As greater numbers of nematode sequences are identified and deposited in complied databases, sequence matching will become more precise.

Our results indicate that this DGGE technique combined with DNA recovery and sequencing can be used to reliably and effectively monitor nematode populations in agricultural soils. Further sampling throughout the growing season is needed to better understand the effects the nematode population may have on crop health. Effective plant management practices may be

devised with a more thorough understanding of the nematode community accomplished through constant monitoring with precise and high resolution DNA fingerprinting techniques that can analyze populations within the nematode community and detect shifts in those populations due to cultural practices.

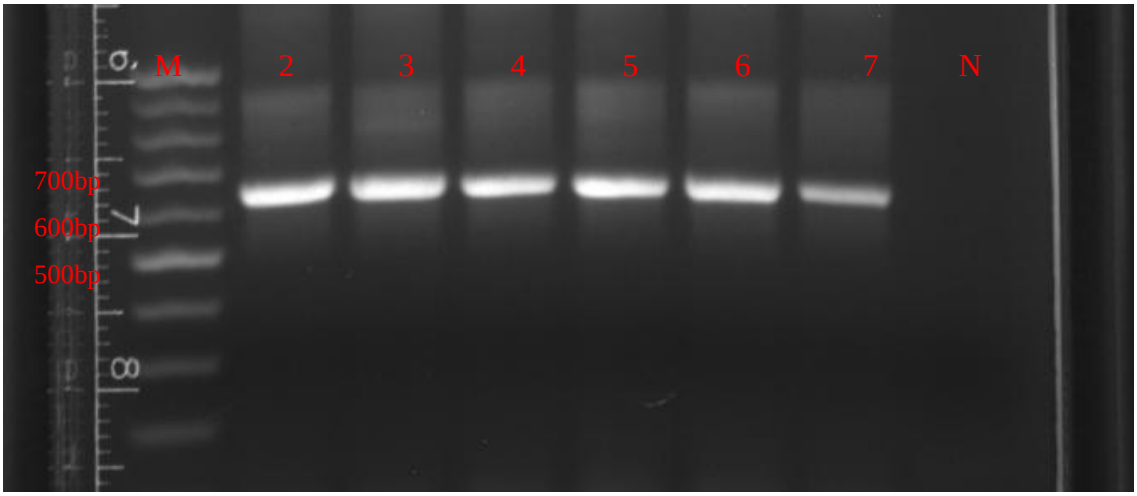
Table 1. Trophic group and species list in nematode DNA collection used to test specificity of nematode consensus primers.

Sample	Species	Trophic group
1	*Meloidogyne arenaria*	Plant-parasitic nematodes
2	*Meloidogyne incognita*	‘‘
3	*Rotylenchulus reniformis*	‘‘
4	*Tylenchorhynchus* sp.	‘‘
5	*Helicotylenchus* sp.	‘‘
6	*Mesocriconema* sp.	‘‘
7	*Mesorhabditis* sp.	Bacterial-feeding nematodes
8	*Acrobeles* sp.	‘‘
9	*Prismatolaimus* sp.	‘‘
10	*Panagrolaimus* sp.	‘‘
11	*Neoactinolaimus* sp.	Fungal-feeding nematode
12	*Monochus* sp.	Predatory nematode
13	*Sclerotium rolfsii*	Fungus
14	*Aspergillus flavus*	‘‘

Table 2. Putative identification of partial 18S rDNA sequences re-amplified from excised bands from DGGE profile.

Rotation	**Band position**	**Closest related sequence**	**Max id %**	**Trophic group**	**Nematode order**	**Putative Identification**
Cont peanut	13.7%	*Metachromadora* sp.	90%	Algivore-omnivore-predator	Chromadorida	*Metachromadora* sp.
Peanut/corn	22.8%	*Prismatolaimus dolichurus*	99%	Microbivore	Enoplida	*Prismatolaimus dolichurus*
Cont peanut	29.8%	*Panagrellus redivivus*	97%	Microbivore	Rhabditida	*Panagrellus redivivus*
Peanut/cotton	36.6%	*Panagrolaimus rigidus*	98%	Microbivore	Rhabditida	*Panagrolaimus rigidus*
Peanut/corn	36.6%	*Alaimus* sp.	92%	Microbivore	Enoplida	*Alaimus* sp.
Peanut/cotton	42.8%	*Anatonchus tridentatus*	97%	Predatory	Monochida	*Anatonchus tridentatus*
Cont bahia	47.2%	*Mylonchulus brachyuris*	94%	Predatory	Monochida	*Mylonchulus* sp.
Cont peanut	49.4%	*Acrobeles ciliatus*	99%	Microbivore	Rhabditida	*Acrobeles ciliatus*
Cont peanut	51.3%	*Acrobeloides butschlii*	99%	Microbivore	Rhabditida	*Acrobeloides butschlii*
Peanut/cotton	55.6 %	*Panagrolaimus superbus*	90%	Microbivore	Rhabditida	*Panagrolaimus* sp.
Cont peanut	58.5%	*Meloidogyne javanica*	95%	Herbivore	Tylenchida	*Meloidogyne* sp.
Cont peanut	60.4%	*Caenorhabditis elegans*	96%	Microbivore	Rhabditida	No match
Cont peanut	63.2%	*Meloidogyne arenaria*	96%	Herbivore	Tylenchida	*Meloidogyne* sp.
Peanut/cotton	68.2%	*Gongylonema pulchrum*	95%	Vertebrate parasite	Spirurida	*Gongylonema* sp.
Peanut/cotton	70.2%	No significant similarity found				No match
Peanut/cotton	73.7%	*Steinernema bicornutum*	95%	Entomopathogen	Rhabditida	*Steinernema* sp.
Peanut/cotton	86.6%	*Toxocara vitulorum*	76%	Vertebrate parasite	Ascaridida	*Toxocara* sp.

Figure 1. Polymerase Chain Reaction amplified product detection of nematode 18S rDNA.

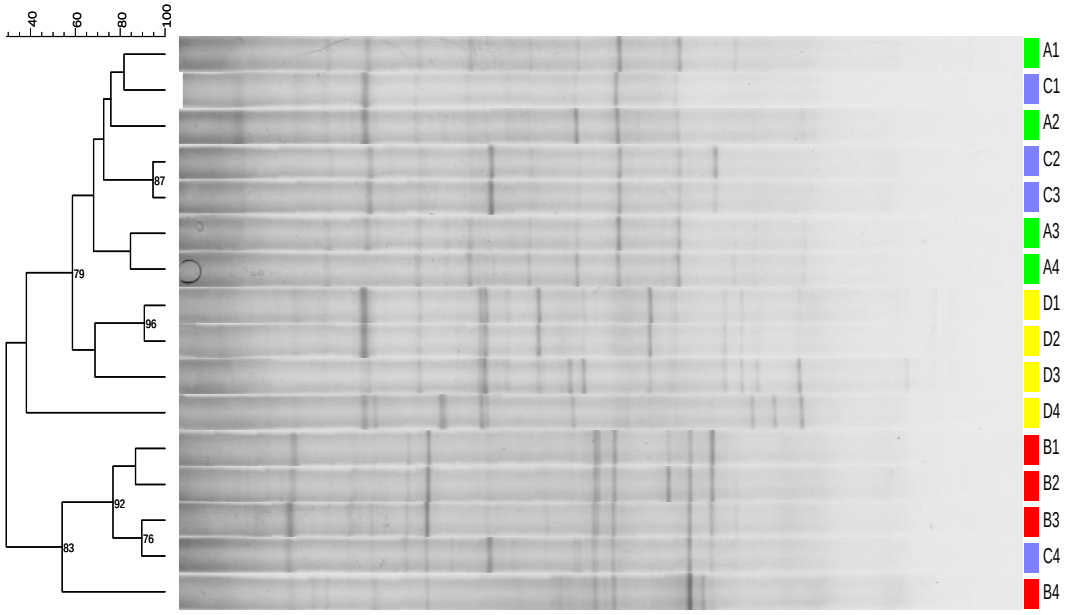


Note: M – 100 bp marker, N - negative control, Lane 2-7 - samples in nematode DNA collection: *Aspergillus flavus*, *Panagrolaimus* sp., *Helioctylenchus* sp., *Meloidogyne arenaria*, *Rotylenchulus reniformis*, *Mesocriconema* sp., and *Neoactinolaimus* sp.

Figure 2. Denaturing Gradient Gel Electrophoresis image of nematode and fungal 18S rDNA amplified products.

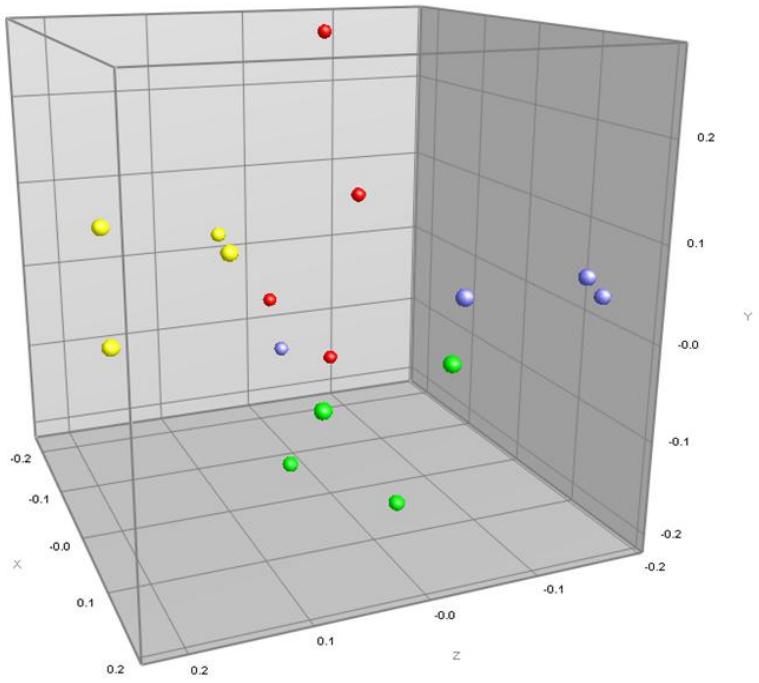
Note: Lane 1-15 – samples in DNA collection: *Aspergillus flavus*, *Panagrolaimus* sp., *Helioctylenchus* sp., *Meloidogyne arenaria*, *Rotylenchulus reniformis*, *Mesocriconema* sp., *Neoactinolaimus* sp., *Rhabditis* sp., *Prismatolaimus* sp., *Acrobeles* sp., *Monochus* sp., *Helicotylenchus* sp. (repetition), *Tylenchorhynchus* sp., *Meloidogyne incognita*, and *Sclerotium rolfsii*.

Figure 3. Nematode Denaturing Gradient Gel Electrophoresis profile obtained from different peanut cropping sequences at the Wiregrass Research and Extension Center at pre-plant 2008.



Note: The scale represents % of similarity calculated by the Dice correlation. The dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Colors are representative of crop rotations: green – continuous bahiagrass, blue – peanut/corn, yellow – peanut/cotton, and red – continuous peanut.

Figure 4. Multi-dimensional Scaling of nematode communities from peanut soil samples under various crop rotations collected at pre-plant from the Wiregrass Research and Extension Center in 2008, colored by crop rotation.



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Chapter III. DGGE Fingerprinting of Nematode Community Structure under Peanut Rotation Systems

Abstract

The nematode community within agricultural soils consists of plant-parasitic and free-living nematodes, both of which can affect plant health. Understanding the nematode community structure may be important in disease management. The objective of this study was to identify the populations present in the nematode community of peanut soils under differing crop rotations using genetic fingerprinting methods to determine if factors exist that result in nematode population shifts. Genetic profiles of the total nematode community from peanut soils were generated through extraction of nematode DNA, amplification of partial 18S rDNA using nematode consensus primers and subsequent separation by denaturing gradient gel electrophoresis (DGGE). Samples were collected from four different crop rotation patterns (continuous peanut, peanut/corn, peanut/cotton, and continuous bahiagrass) at three sampling periods (pre-plant, mid-season, and harvest) for two consecutive years (2008 and 2009). Unique and common bands in each molecular fingerprint were then excised, re-amplified and sequenced in order to identify populations within the nematode community. DGGE results indicated rotation sequence resulted in population shifts, although minimal similarities were found between replications of crop rotations (51-68%). Sampling time impacted nematode community structure also. Free-living nematodes accounted for 64% of the recovered DNA sequences, althoughplant-parasitic nematodes, animal parasitic nematodes, and entomopathogenic nematodes were present in lower populations. Trends in the data suggest that some microbivore

nematode populations may play a role in the suppression of plant-parasitic nematodes. These results indicate that crop rotation and other environmental factors affect nematode community structure and certain populations may affect disease management.

Introduction

The nematode community represents a complex structure within the soil that affects plant health. This community consists of plant-parasitic nematodes and free-living nematodes. Free-living nematodes include microbivores (bacterial-feeders), fungivores (fungal-feeders) and predatory nematodes. These free-living nematodes have direct and indirect effects on soil nutrition that can affect other soil organisms (Neher, 2001). Free-living nematodes are commonly attributed to increased plant growth, increased nitrogen (N) uptake by plants, decreased or increased bacterial populations, increased CO_2 evolution, increased N and phosphorous (P) mineralization, and increased substrate utilization (Ingham et al., 1985).

In order to understand the entire nematode community and the effects it has on crop health nematode populations need to be identified. Traditional techniques employed to describe the composition and diversity of nematode populations in the soil rely on phenotypic characteristics, which is time-consuming and requires extensive training. Limitations to nematode community analysis may be overcome by using molecular analytical tools to directly explore the composition in a soil sample based on the nucleic acids present.

Among the molecular analytical techniques available, molecular fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), can help monitor changes in microbial communities over time with a simplified representation of the community. These methods

generate population specific fingerprints that display the ribosomal polymorphism naturally present in the community.

Previous research has shown that nematode populations can be analyzed using a combination of DGGE and DNA recovery for sequencing (Conner and Huettel, unpublished). Using nematode consensus primers, nematodes that have previously been extracted from the soil can be amplified by Polymerase Chain Reaction (PCR) and the amplified products can be separated by DGGE. Bands within the genetic profiles, representing nematode genera, can be excised and DNA can be reamplified for sequencing. Sequences can then be compared to those previously identified in GenBank to putatively identify the populations present in the nematode community. Genetic profiles can also be analyzed to observe trends between samples.

The objective of the current research was to identify the populations present within the nematode community of peanut soils under differing crop rotations to determine if rotation sequence results in a shift in nematode populations. This was accomplished by generating population specific fingerprints using DGGE to monitor nematode populations throughout the growing season under different crop rotations. DNA was recovered from these fingerprints to identify nematode populations present in the peanut soils.

Materials and Methods

Soil samples: Soil samples were obtained from the Wiregrass Research and Extension Center in Headland, Alabama (31˚ 21´ N, 85˚ 20´ W) from a long term rotation study. The rotation sequences used in this study included: continuous peanuts, peanut/cotton, peanut/corn, and continuous bahiagrass. The soil is a Dothan sandy loam (OM<1%). Rotation sequences are arranged in a randomized complete block design with four replications. Each plot is 50 ft long

with 12 rows per plot and three ft between each row. Samples were collected at pre-plant, mid-season or pegging and harvest for 2 consecutive years (2008 and 2009). Seven soil cores (6 inch depth) were taken randomly across each plot from the root zone in each replication. Samples were placed in a plastic bag, mixed thoroughly and stored at 10°C until needed.

Nematode and DNA extraction: Nematodes were extracted from a 100 cm^3 sub-sample from each plot using a sieving process followed by sugar flotation (Jenkins, 1964) prior to DNA extraction. The sugar flotation process was completed twice to ensure clean specimens (Miller, 1957). Total genomic DNA was extracted using the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories Inc. Carlsbad, CA) following the manufacturer's instructions. DNA quality and quantity was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). DNA from each sample was diluted to 20 ng/µL and stored at -80°C until required for downstream applications.

PCR amplification of 18S rDNA: Nematode consensus primers designed to amplify a ~630 bp fragment of the 18S rDNA were used in this experiment: nem1 - forward (5'-GCAAGTCTGGTGCCAGCAGC-3') and nem2 - reverse (5'-CCGTGTTGAGTCAAATTAAG-3') (Foucher and Wilson, 2002). The forward primer contained a 39 bp GC clamp at the 5' end to prevent complete denaturation of the amplified product during electrophoresis (Myers et al., 1985).

DNA extracted from each sample was PCR amplified in 50 µl volumes consisting of 5 µl 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 5 µl 25mM $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl each 10 µM forward and reverse primers, 0.3 µl 5 U/µl Platinum® *Taq* DNA

Polymerase (Invitrogen, Carlsbad, CA), and 5 µl template DNA (100 ng). A negative control sample without template DNA was included in each run. The amplification process was performed in a Techne TC-312 thermocycler. The PCR program consisted of 94ºC for 5 minutes; 35 cycles of 94ºC for 45 seconds, 48ºC for 45 seconds and 72ºC for 1 minute; followed by a final extension of 72ºC for 10 minutes. PCR products were separated on a 1% agarose gel at 100 V for 1 hour and visualized on a 312 nm Variable Intensity Transilluminator (Fisher Scientific, Pittsburgh, PA) after staining with ethidium bromide (EtBr) to confirm the presence of the PCR amplified product.

DGGE analysis: DGGE analysis was performed on amplified DNA using the Dcode® Universal Mutation Detection System (Bio-Rad, Hercules, CA). PCR amplified products were separated on a 20-50% denaturant solution, 1 mm, 6% polyacrylamide gel. The polyacrylamide gel was prepared by mixing 15 ml of each denaturant solution with 81 µl 10% ammonium persulfate (APS) and 4.5 µl N,N,N',N'-tetramethylethylenediamine (TEMED). The high and low denaturant solutions were mixed using a manual gradient delivery system (Bio-Rad, Hercules, CA). Electrophoresis was run at 100 V for 16 hours in 1X TAE buffer at 60°C. After electrophoresis, the gel was stained with GelStar® Nucleic Acid Gel Stain (Lonza, Rockland, ME) for 30 minutes and rinsed with deionized water. The gel was subjected to UV illumination and photographed using an Olympus C-4000 digital camera with the GelStar® Photographic Filter (Lonza, Rockland, ME).

DNA sequencing: Unique bands from each sample and common bands in all samples were excised from the gel using a wide borer pipette tip and placed in 30 µl sterile water. The DNA

was allowed to diffuse into the water at 4˚C overnight (Ampe et al., 2001). This DNA was used as template and re-amplified using PCR conditions as described above. Electrophoresis on a 1% agarose gel was used to confirm the presence of the PCR amplified product. Prior to sequencing, purity of the re-amplified DNA was checked using another DGGE run to confirm that the sample yielded a single band at the same position it was recovered from. The samples were then sequenced by Lucigen Inc. in both directions. The partial 18S sequences were compared to know sequences in Genbank's nucleotide collection using the basic local alignment search tool (BLASTN) (Altschul et al., 1990). A putative identification was made for sequences matching those in GenBank with a score greater than 100 bits and an e-value lower than 0.001. Sequences matching the criteria were putatively identified to the species level for 97% or higher maximum identity and to the generic level for 75-96% maximum identity. Sequences with a 74% or lower maximum identity where considered as not significantly matching the sequences held in GenBank.

Phylogenetic analysis: The DGGE gel images were analyzed with BioNumerics V. 5.0 software program (Applied Maths, Austin, TX). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between profiles were calculated with the band based Dice coefficient. Cluster analysis was performed with the Unweighted Pair Group Method using Arithmetic averages (UPGMA). A band matching analysis was performed and a band table was created for polymorphism analysis. Bootstrap analysis of 1000 replicates was performed to define tree robustness. Multi-Dimensional Scaling (MDS) was completed to compare the clusters generated over different crop rotations and between different sampling periods. The band table was exported from BioNumerics and subjected to Principle Component

Analysis (PCA) using SAS version 9.1.3 (SAS Institute, Cary, NC) to determine if any relationships existed between band classes.

Results

DNA sequencing: DGGE analysis of nematode communities extracted from soil samples under different crop rotations revealed individual banding patterns with a number of distinguishable bands representing different nematode taxa. In total, 121 partial 18S rDNA sequences were recovered from the DGGE gels. There were 103 sequences that matched previously identified sequences held online at GenBank (Tables 1-2). Sequences that showed 97–100% maximum identity accounted for 41% of the total recovered DNA sequences and those that showed 75–96% maximum identity accounted for 59% of the total recovered DNA sequences. There were 18 sequences that did not meet the criteria previously described for putative identification.

A total of 93 recovered DNA sequences were matched to nematodes representing 29 genera within 10 nematode orders, and 10 sequences were matched to fungi representing 3 fungal genera (*Nematoctonus* sp., *Paeilomyces* sp. and *Fusarium* sp.). Putative identification of free-living nematodes accounted for 64%, plant-parasitic nematodes accounted for 14.5%, animal parasitic nematodes accounted for 8.7%, entomopathogenic nematodes accounted for 2.9%, and fungi accounted for 9.7% of the recovered DNA sequences.

Phylogenetic analysis: The nematode DGGE profiles showed similarities among communities of some replicates sampled from the same crop rotation (Fig 1-2). Common bands were observed among the majority of samples irrespective of sampling period and cropping sequence. Similarities within a range of 51-68% were observed among the plots of different cropping

sequences in 2008 (Fig 5) and 52-59% in 2009 (Fig 6). DGGE banding patterns in continuous peanut plots indicated that there were approximately 58% similarities in all samples that were taken at pre-plant 2008, 64% at mid-season 2008, 55% at harvest 2008 and 52% at harvest 2009. In the continuous bahiagrass rotation, 68% similarities were observed in plots that were sampled during pre-plant 2008. In the peanut/cotton rotation plots sampled at mid-season 2008, 66% similarities were observed whereas the plots sampled at harvest 2008 had 60% and harvest 2009 had 59%. The peanut/corn rotation plots sampled at harvest 2008 showed 51% similarity and those sampled at harvest 2009 showed 59% similarities. In 2009, peanut plots were divided into two varieties Florida 07 and Tifguard. DGGE profiles of nematode communities showed similarities between the varieties ranging from 51-93% similarity.

Multi-dimensional Scaling (MDS) based on DGGE community profiles of different cropping sequences revealed that nematode communities pertaining to each cropping sequence had fewer similarities in general with greater scattering in 2008 and 2009, indicating the impact of cropping sequence on nematode diversity is minimal (Fig 3a & 4a). More similarities were observed with respect to nematode composition in the plots that were sampled during identical sampling periods irrespective of the cropping sequence in practice (Fig 3b & 4b).

In 2008, there was a total of 50 bands across all samples; whereas, there were 57 in 2009. The mean number of bands from a single sample in 2008 was 17 and in 2009 the mean number of bands was 20. In both years, sampling period significantly affected total bands within a sample ($p=<0.0001$). In 2008, pre-plant samples contained fewer bands (mean=13) than mid-season samples (mean=19), and harvest samples contained the most bands (mean=21). A similar tend occurred in 2009, pre-plant samples contained the fewest bands (mean=14), mid-season

samples contained a mean of 20 bands and harvest samples contained the most bands (mean=26). Crop rotation did not affect total bands.

Principal component analysis of band table data created from nematode communities in 2008 and 2009 revealed loadings of similar value (>0.20 or <-0.30) between band classes that were putatively identified from recovered DNA from genetic profiles. The data from both years indicated that certain microbivore populations (putatively identified as *Panagrellus redivivus*, *Panagrolaimus rigidus* and *Prismatolaimus dolichurus*) consistently had positive loadings ranging from 0.20-0.29 while the putatively identified band class *Meloidogyne* sp. consistently had negative loadings ranging from -0.21 to -0.26 in the first component. The second component revealed positive loadings on band classes putatively identified as *Rhabdolaimus* sp. and *Acrobeles ciliatus* (0.21-0.27). *Anatonchus tridentatus* and *Mylonchulus* sp. putative band classes consistently had a positive load (0.22) in the third component. These three components explained only 33% of the variance in the data in both years.

Discussion

The combined use of Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing of DNA recovered from genetic profiles was applied in this study to identify the populations present within the nematode community of peanut soils under differing crop rotations to determine if rotation sequences resulted in a shift in nematode populations. Our results indicated nematode populations did shift based on peanut cropping sequence, although similarities were minimal between replications within crop rotations. Multi-dimensional scaling revealed scattering based on nematode community profiles of different crop rotations indicating the impact of cropping

sequence on nematode diversity was minimal. These results show a wide range of nematode community polymorphisms were present irrespective of crop rotation.

Similarities were also observed between plots sampled at the same period. Multi-dimensional scaling revealed clustering of nematode communities based on sampling period indicating the period when samples were taken had a greater impact on nematode composition than did cropping sequence. Since the sampling periods were set at prescribed times through the growing season based on crop age, the specific crop age and environmental factors could be playing an important role in the nematode community rather than plant species. Sampling period also significantly affected the total number of bands from a sample. Pre-plant samples contained fewer bands than mid-season samples, and harvest samples contained the most bands. Pre-plant samples were expected to support lower biodiversity because all plots were fallowed through the winter. This follows the rate of reproduction of most nematodes with populations increasing in fall.

Results of DNA recovery from genetic profiles and DNA sequencing revealed that free-living nematodes accounted for the majority of populations present in the nematode community. Plant-parasitic nematodes, animal parasitic nematodes, entomopathogenic nematodes, and nematophagus fungi were also present in the plots sampled but at much lower population levels. Sequences that showed 97-100% maximum identity with those in the nucleotide collection of the GenBank database accounted for 41% of the total recovered sequences. The GenBank database only contains approximately 20,000 nematode 18S sequences. It has been estimated that there are possibly 500,000 different nematode species in existence, yet only approximately 12,000 have been described (Myers, 2001). As more nematode sequences are identified and deposited in complied databases, sequence matching will become more precise.

There were 29 nematode genera and three fungal genera putatively identified out of the 57 total bands. High background fluorescence inhibited visualization of weaker bands and ultimately inhibited recovery of the DNA at those positions.

Previous studies have reported 100 nematode species belonging to 48 genera present per sampling site in agricultural settings (Baird and Bernard, 1984). In this study we found 29 nematode genera. It has been well documented that DGGE techniques only display populations that make up 1% or more of the total community (Murray et al., 1996; Muyzer et al., 1993; Foucher et al., 2004). It is possible that more genera were present in these samples but were omitted because they represented <1% of the total nematode biomass.

Three fungal genera were identified within the samples sequenced. These were putatively identified as *Nematoctonus* sp., *Paeilomyces* sp. and *Fusarium* sp. These fungal genera have been reported to parasitize nematodes and are classified as nematophagus fungi (Jaffee et al., 1998; Dickson et al., 1994; Olatinwo et al., 2006). When extracting nematodes from the soil, the sugar flotation process was performed twice in order to ensure specimens were clean of debris because the primers used to amplify nematode DNA also amplify fungal DNA. This indicates these three fungal organisms were most likely present inside the nematode body.

Four vertebrate parasites were identified in the samples sequenced. These were putatively identified as *Gongylonema* sp., *Thelazia* sp., *Toxocara* sp., and *Passalurus* sp. *Gongylonema* sp. is a nematode parasite of birds and other mammals transmitted by insects (Kudo et al., 2005). *Thelazia* sp. is a genus of nematodes parasitic in the eyes of mammals transmitted by species of *Diptera* (Otranto and Traversa, 2005). *Toxocara* sp. is the genera of animal parasitic nematodes that cause infections in pets known as round worms (Samuel et al., 2001). *Passalurus* sp. is a

nematode parasite of rabbits (Erickson, 1944). Juveniles of animal parasitic nematodes may be found in the soil.

Principal component analysis of putatively identified band classes revealed a trend between *Panagrellus redivivus*, *Panagrolaimus rigidus* and *Prismatolaimus dolichurus*, all of which are microbivorous nematodes, and *Meloidogyne* populations. These results suggest that the presence of microbivouous nematodes may suppress herbivorous populations.

Using DGGE techniques combined with nematode DNA recovery from genetic profiles followed by sequencing as an alternative method to monitoring nematode communities and identifying nematode populations proved beneficial in studying the impact of long term crop rotations on resident nematode communities. The four peanut cropping sequences selected in this study are widely used in agriculture for the management of several peanut diseases (Rodriguez-Kabana et al, 1991; Timper et al., 2001; Bowen et al., 1996). This research demonstrates that peanut crop rotations affect nematode community profiles and that sampling period has the greatest influence on nematode population composition. The data generated from DNA recovery and sequencing also suggested that microbivore nematode populations may play a role in the suppression of herbivore nematodes. Further sampling refinement is needed to better understand the nematode biodiversity in these soils. Plant disease management practices may only be devised through constant monitoring of factors that influence the nematode community and the impact individual populations may have on plant health.

Table 1. Putative identification of nematode partial 18S rDNA sequences re-amplified from excised bands recovered from 2008 Denaturing Gradient Gel Electrophoresis profiles of peanut soil samples under various rotations from the Wiregrass Research and Extension Center.

Putative identification of genus	**Nematode order**	**Trophic group**
Acrobeles	Rhabditida	Microbivore
Acrobeloides	Rhabditida	Microbivore
Alaimus	Enoplida	Microbivore
Anatonchus	Monochida	Predator
Aphelenchoides	Tylenchida	Fungivore
Cephalobus	Rhabditida	Microbivore
Fusarium	Ascomycete	Fungus
Gongylonema	Spirurida	Vertebrate parasite
Helicotylenchus	Tylenchida	Herbivore
Meloidogyne	Tylenchida	Herbivore
Metachromadora	Chromadorida	Algivore-omnivore-predator
Mylonchulus	Monochida	Predator
Nematoctonus	Basidiomycete	Fungus
Paeilomyces	Hypocreomycetidae	Fungus
Panagrellus	Rhabditida	Microbivore
Panagrolaimus	Rhabditida	Microbivore
Panagrolaimus	Rhabditida	Microbivore
Paratrichodorus	Triplonchida	Herbivore
Pratylenchus	Tylenchida	Herbivore
Prismatolaimus	Enoplida	Microbivore
Rhabdolaimus	Araeolaimida	Microbivore
Steinernema	Rhabditida	Entomopathogen
Thelazia	Spirurida	Vertebrate parasite
Toxocara	Ascaridida	Vertebrate parasite

Table 2. Putative identification of nematode partial 18S rDNA sequences re-amplified from excised bands recovered from 2009 Denaturing Gradient Gel Electrophoresis profiles of peanut soil samples under various rotations from the Wiregrass Research and Extension Center.

Putative identification of genus	**Nematode order**	**Trophic group**
Acrobeloides	Rhabditida	Microbivore
Alaimus	Enoplida	Microbivore
Anatonchus	Monochida	Predator
Bathyodontus	Monochida	Predator
Bunonema	Rhabditida	Microbivore
Bursaphelenchus	Tylenchida	Herbivore-fungivore
Eucephalobus	Rhabditida	Microbivore
Fusarium	Ascomycete	Nematophagus fungus
Gongylonema	Spirurida	Vertebrate parasite
Heterocephalobus	Rhabditida	Microbivore
Meloidogyne	Tylenchida	Herbivore
Mylonchulus	Monochida	Predator
Nematoctonus	Basidiomycete	Nematophagus fungus
Odontophora	Araeolaimida	Algivore-omnivore-predator
Paeilomyces	Ascomycete	Nematophagus fungus
Panagrellus	Rhabditida	Microbivore
Panagrobelus	Rhabditida	Microbivore
Panagrolaimus	Rhabditida	Microbivore
Paratrichodorus	Triplonchida	Herbivore
Passalurus	Oxyurida	Vertebrate parasite
Pratylenchus	Tylenchida	Herbivore
Prismatolaimus	Enoplida	Microbivore
Rhabdolaimus	Araeolaimida	Microbivore
Steinernema	Rhabditida	Entomopathogen
Toxocara	Ascaridida	Vertebrate parasite
Trischistoma	Enoplida	Predator

Figure 1. Denaturing Gradient Gel Electrophoresis profile of nematode communities from peanut soil samples under various crop rotations collected at pre-plant, mid-season and harvest from the Wiregrass Research and Extension Center in 2008.

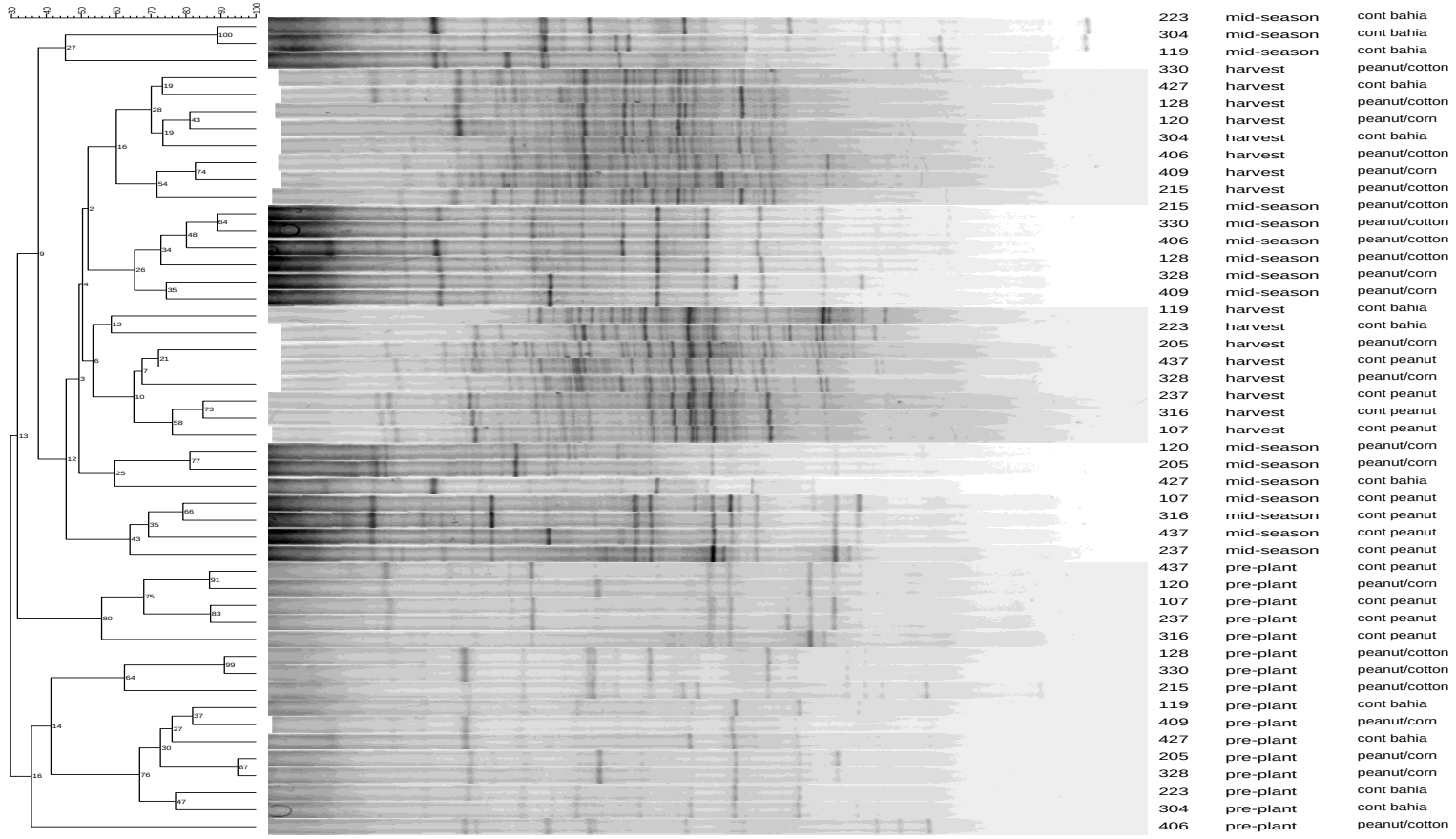


Figure 2. Denaturing Gradient Gel Electrophoresis profile of nematode communities from peanut soil samples under various crop rotations collected at pre-plant, mid-season and harvest from the Wiregrass Research and Extension Center in 2009.

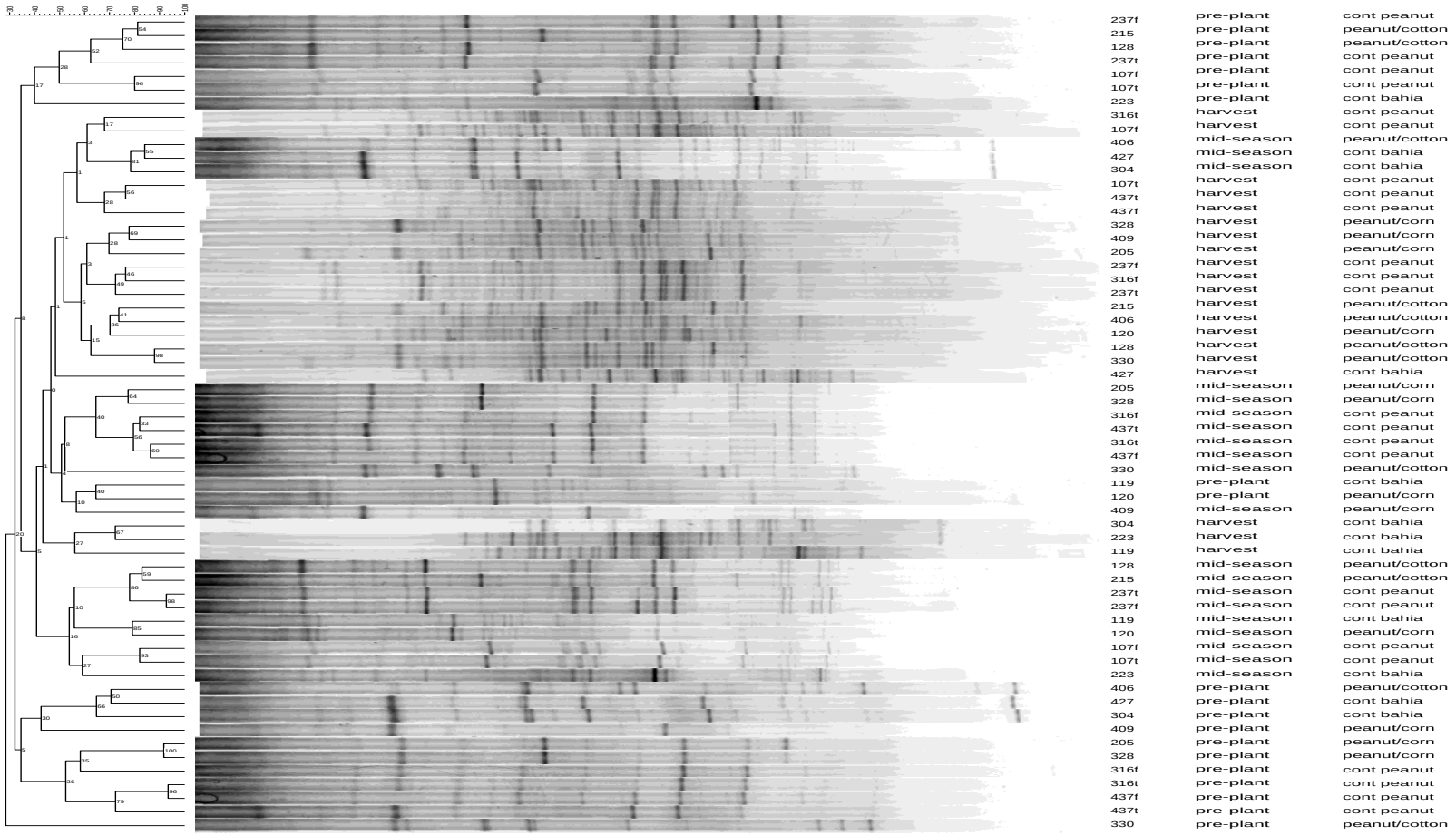
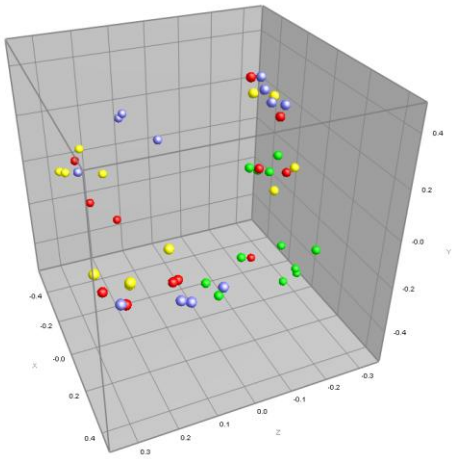


Figure 3. Multi-dimensional Scaling of nematode communities from peanut soil samples under various crop rotations collected at pre-plant, mid-season and harvest from the Wiregrass Research and Extension Center in 2008:

a) Colored by rotation



b) Colored by sampling period

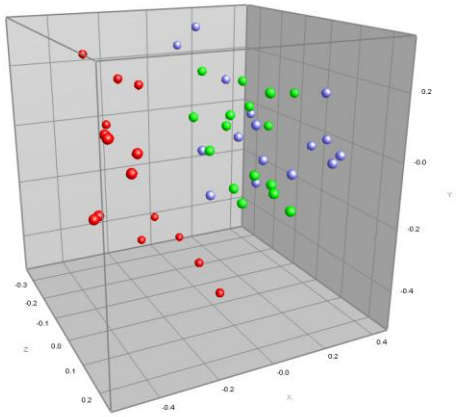
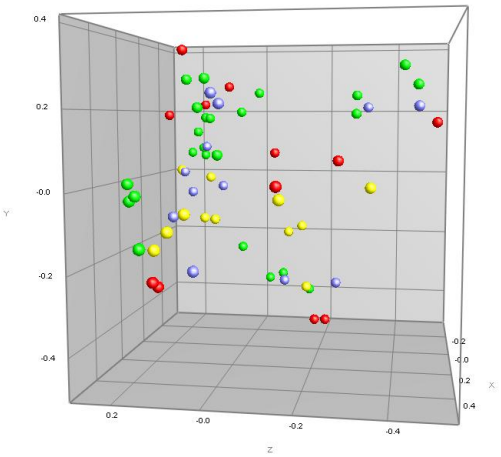


Figure 4. Multi-dimensional Scaling of nematode communities from peanut soil samples under various crop rotations collected at pre-plant, mid-season and harvest from the Wiregrass Research and Extension Center in 2009:

a) Colored by rotation



b) Colored by sampling period

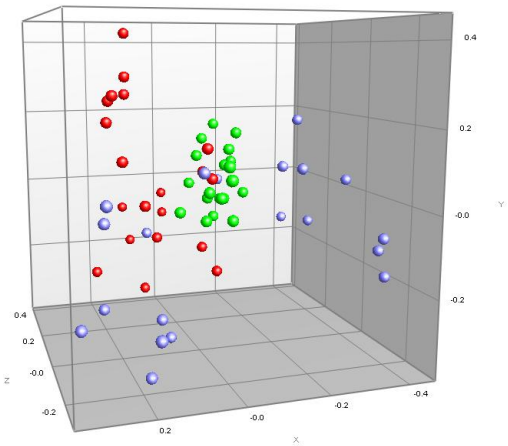


Figure 5. Dendrogram construction using the unweighted pair-group method with arithmetic mean (UPGMA) based on nematode community band table data from different peanut cropping sequences collected at pre-plant, mid-season and harvest from the Wiregrass Research and Extension Center in 2008. The scale represents % of similarity calculated by the Dice coefficient.

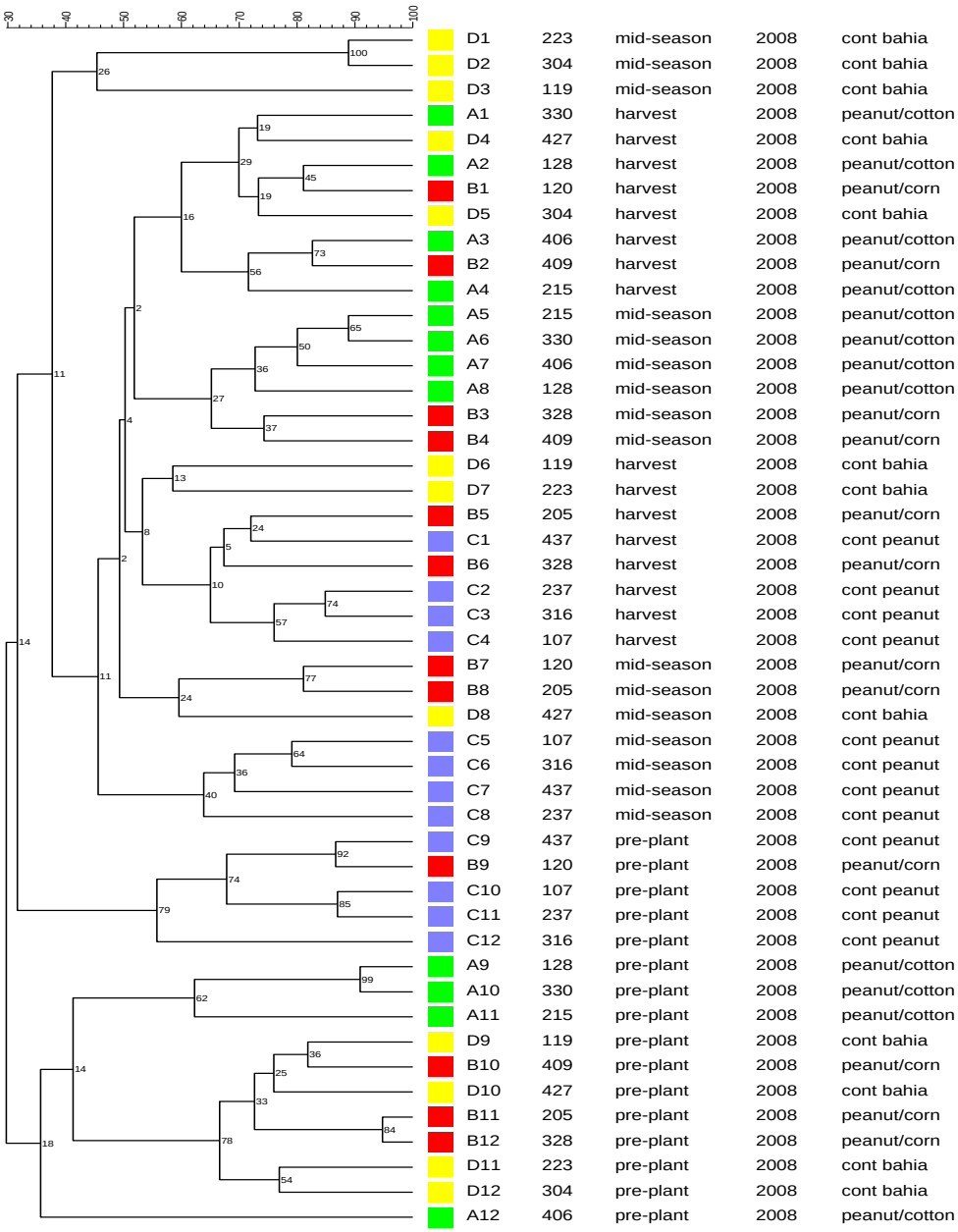
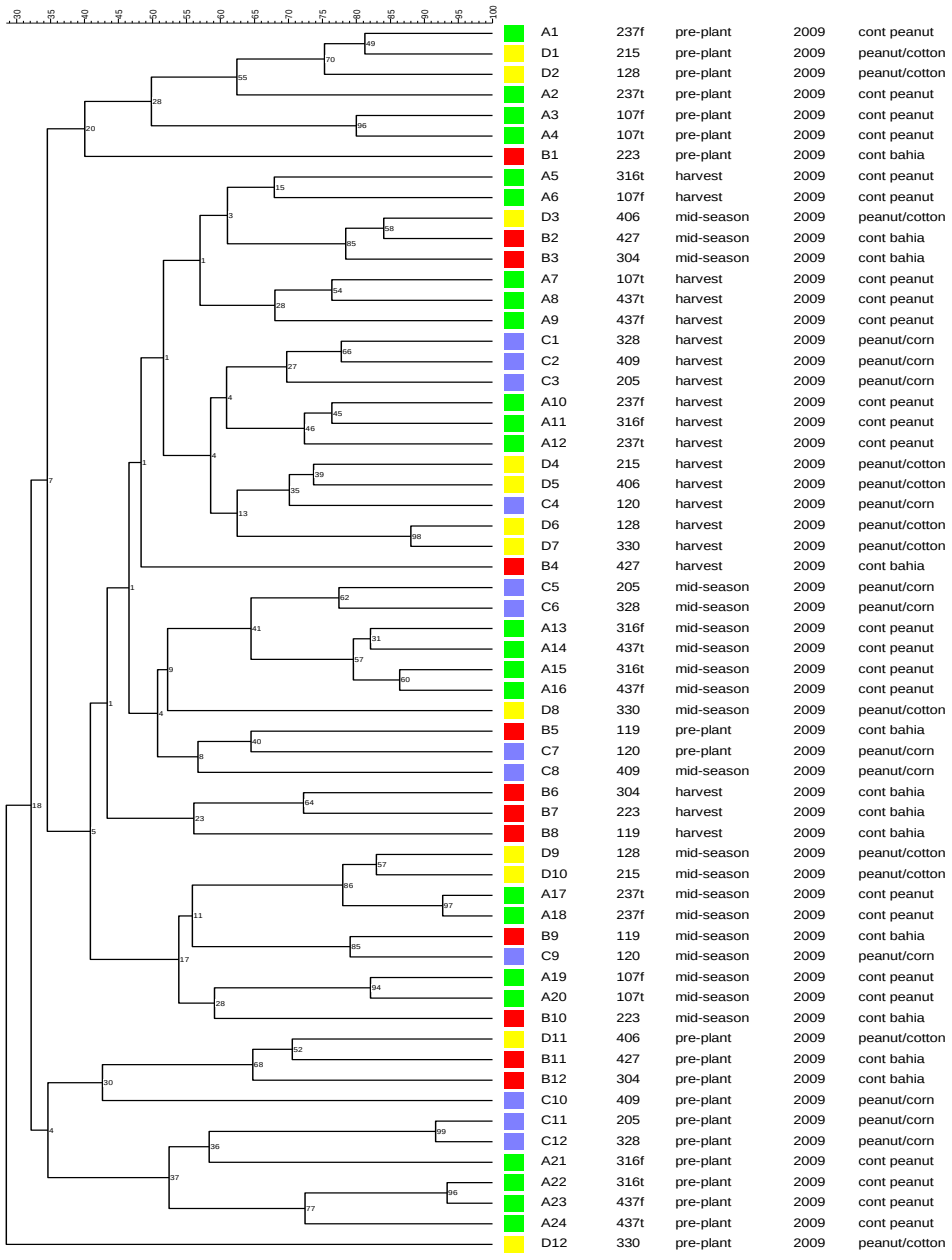


Figure 6. Dendrogram construction using the unweighted pair-group method with arithmetic mean (UPGMA) based on nematode community band table data from different peanut cropping sequences collected at pre-plant, mid-season and harvest from the Wiregrass Research and Extension Center in 2009. The scale represents % of similarity calculated by the Dice coefficient.



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Chapter IV. Influence of Nematode Community on Aflatoxin Contamination of Peanuts

Abstract

The nematode community within peanut soils, or any agricultural soils, consists of plant-parasitic and free-living nematodes, the latter of which can be attributed to increases in plant health. The peanut root-knot nematode can detrimentally affect peanut yields and may facilitate invasion by aflatoxigenic fungi. The objective of this study was to determine if beneficial free-living nematodes act to increase peanut yields or decrease aflatoxin contamination. Samples were collected from four different cropping rotations (continuous peanut, peanut/corn, peanut/cotton, and peanut/bahiagrass) at three sampling periods (pre-plant, mid-season, and harvest) for three consecutive years (2007 - 2009). Nematodes were microscopically identified, after which peanut pods were collected from each rotation for a visual examination of damage or fungi and tested for aflatoxin contamination. Bahiagrass rotations supported higher populations of microbivore nematodes and lower levels of aflatoxin contamination than continuous peanut monocropping. Significant negative correlations occurred between microbivores and total aflatoxins as well as microbivores and plant-parasitic nematodes. These results suggest that free-living nematodes may play a role in the suppression of plant-parasitic nematodes and subsequent aflatoxin contamination in peanuts.

Introduction

Peanut (*Arachis hypogaea* L.) is an important crop in Alabama and throughout the southeastern United States. In 2009, 155,000 acres of peanuts were grown for a value of $104.5 million in Alabama alone, while 1.1 million acres were grown for a value of $835 million throughout the United States (NASS, 2010). This high value crop can be detrimentally affected by a number of soil-borne organisms, including the peanut root-knot nematode *Meloidogyne arenaria* (Neil, 1889) Chitwood, 1949, race 1. The peanut root-knot nematode can reduce yields 3-15% annually (Holbrook et al., 2008). Damage from this nematode has been shown to facilitate invasion by the aflatoxigenic fungi *Aspergillus flavus* Link and *A. parasiticus* Speare (Timper et al., 2004). Aflatoxins, produced by the *A. flavus* fungal group, are highly carcinogenic, strictly regulated to ensure a safe food supply, and can decrease the economic return from a peanut crop (Dorner et al., 2003). Contamination of aflatoxins in peanut seeds results in a loss of $2.6 million per year to peanut growers (Lamb and Sternitzke, 2001). There is no highly effective control for aflatoxigenic fungi, but minimization of this problem may be possible through a greater understanding of the microbial community that influences *A. flavus* production of aflatoxins.

Management of nematodes to reduce yield loss and potential aflatoxin contamination is generally obtained with chemical control. However, few nematicides are currently available to treat peanut crops (ACES, 2010). Crop rotations with non-host crops are currently the main method used to control the peanut root-knot nematode, including corn (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* (L.) Merr.), and bahiagrass (*Paspalum notatum* Flugge) (Rodriguez-Kabana et al, 1991; Timper et al., 2001; Bowen et al., 1996).

There are some resistant peanut cultivars to the root-knot nematode, however breeding for resistance has been slowed by the occurrence of tomato spotted wilt virus (TSWV). Since 1985, TSWV has become the most important disease problem for many growers in the southern United States. Until recently, peanut cultivars were available with resistance to either the peanut root-knot nematode or TSWV but not both. In 2008, the USDA released the cultivar Tifguard, which has resistance to TSWV and the root-knot nematode (Holbrook et al., 2008). Continuous planting of this cultivar in the same fields is likely to eventually lead to resistance-breaking nematodes (Rich and Tillman, 2009).

To better maintain resistance in peanuts to root-knot nematodes, a greater understanding of the nematode community and the impact it has on plant health is needed. The nematode community consists not only of plant-parasitic nematodes but also free-living nematode (microbivores, fungivores and predators). Free-living nematodes are commonly attributed to increased plant growth, increased nitrogen (N) uptake by plants, decreased or increased bacterial populations, increased CO_2 evolution, increased N and phosphorous (P) mineralization, and increased substrate utilization (Ingham et al., 1985).

This objective of the current research is to identify the populations present within the nematode community of peanut soils under differing crop rotations to determine if nematode populations, especially beneficial free-living nematodes act to increase peanut production. This was accomplished by identifying nematode populations present in the peanut soils, collecting yield data, rating peanut pods for physical damage and testing pods for the presence of aflatoxin contamination. Nematode populations were compared to various plant health factors, including yield and aflatoxin levels, to determine if any interactions exist that increase plant health.

Materials and Methods

Soil samples and nematode identification: Soil samples were obtained from the Wiregrass Research and Extension Center in Headland, Alabama (31˚ 21´ N, 85˚ 20´ W) from a long term rotation study established in 1988. A total of 34 cropping sequences have been established at this site. The rotation sequences used in this study included: continuous peanuts, peanut/cotton, peanut/corn, and peanut/bahiagrass (Table 1). The soil is a Dothan Sandy Loam (OM<1%). Rotation sequences are arranged in a randomized complete block design with four replications. Each plot is 50 ft long with 12 rows per plot and three ft between each row. Samples were collected at pre-plant, mid-season or pegging and harvest for 3 consecutive years (2007-2009). Seven soil cores (6 inch depth) were taken randomly across each plot from the root zone in each replication. Samples were placed in a plastic bag, mixed thoroughly and stored at 10°C until needed.

Nematodes were extracted from 100 cm^3 sub-samples from each plot using a sieving process followed by sugar flotation (Jenkins, 1964). Nematodes were counted and microscopically identified to trophic level for free-living and genus level for plant parasites on a Nikon Eclipse TS100 inverted microscope for each year of the study using the Interactive Diagnostic Key to Plant Parasitic, Free-living and Predaceous Nematodes from the UNL Nematology Lab.

Peanut health assessment: Peanut pods were collected after harvest from each plot (described above) planted to peanuts for all 3 years of the study (2007-2009). After yields were determined, 150 peanut pods per plot were rated for physical damage including: small or immature pods, pod rot, discoloration, insect scars, nematode damage, insect holes, cracks, and visible fungi.

The pods were then shelled by hand, ground and tested for the presence of aflatoxins. Toxin assays were performed using High Pressure Liquid Chromatography (HPLC) methods described by Wilson and Romer (1991) with modifications. The aflatoxins were extracted from a 50 g sample of ground peanuts from each plot. Each sample was added to 100 ml of 90% acetonitrile and incubated for 12 hours at room temperature. The solution was then filtered and 5 ml filtrate was purified using a Mycosep Multifunctional Cleanup Column (Romer Labs, Inc., Washington, MO). The purified extract was added to a derivatizing solution and incubated at 55°C for 30 minutes. This purified extract was then used to determine B_1, B_2, G_1 and G_2 aflatoxin concentrations. Aflatoxin levels were recorded for each sample.

Statistical analysis: Aflatoxin levels and nematode counts were transformed in order to normalize data and eliminate zero values. Aflatoxin levels, visual peanut pod evaluation of physical damage ratings, nematode counts, rainfall observations, rotation sequences, and yields were compared for a total of 3 years. Spearman's rank correlation coefficients was calculated using SAS version 9.1.3 (SAS Institute, Cary, NC) to determine if any correlations exist between variables.

Results

Aflatoxin levels (B_1 and total aflatoxins) in 2007 were higher in the continuous peanut plots than in peanuts cropped after bahiagrass. The mean total aflatoxin and B_1 aflatoxin content in the peanut/bahiagrass rotation was 19.8 ppb and 2.0 ppb, respectively. The mean total aflatoxin and B_1 aflatoxin content of the continuous peanut rotation was 41.8 ppb and 2.8 ppb, respectively.

There were no aflatoxins detected in 2008 or 2009 for any of the cropping sequences planted to peanut.

Microbivore nematode populations were significantly affected by crop rotation at pre-plant 2007, harvest 2008 and mid-season 2009 (Fig 1). Microbivore nematode populations were similar for peanut/bahiagrass and peanut/corn rotations and significantly higher in continuous peanut and peanut/cotton rotations at pre-plant 2007. In 2008, at the harvest sampling period, peanut/bahiagrass plots supported significantly higher populations of microbivores than all other rotations. Peanut/bahiagrass, peanut/cotton and peanut /corn rotations supported higher populations of microbivore nematodes than the continuous peanut rotation in 2009 at mid-season.

Total plant parasitic populations were also significantly affected by crop rotation based on samples collected at pre-plant 2008, pre-plant 2009 and mid-season 2009 (Fig 2). The continuous peanut and peanut/bahiagrass rotations (which where both planted to peanut the previous year) supported similar and significantly higher levels of plant-parasitic nematodes than the peanut/corn and peanut/cotton rotations (which were both previously planted to cotton) at pre-plant 2008. Pre-plant 2009 total plant-parasitic nematode populations for the continuous peanut rotation were higher than all other rotations. In 2009, at mid-season, total plant-parasitic nematodes in the continuous peanut and peanut/corn rotation were significantly higher than the peanut/bahiagrass and peanut/cotton rotation.

Spearman's rank correlation coefficient revealed a significant positive correlation between total free-living nematode populations and microbivore nematodes at each sampling period (pre-plant, mid-season and harvest) for each year correlations were calculated (2007-2009) (Tables 2-10). Microbivore nematode populations were also negatively correlated to root-

knot nematodes at pre-plant and mid-season in 2007. Furthermore, microbivore nematodes were negatively correlated to G_2 aflatoxin levels at pre-plant and mid season 2007 and negatively correlated to total aflatoxins at mid-season 2007. A positive correlation also occurred between root-knot nematodes and G_2 aflatoxin levels at pre-plant and mid-season. Other factors that affected aflatoxin contamination included positive correlations between insect holes in pods and B_1 aflatoxins, and total plant-parasitic nematodes at pre-plant on B_2 aflatoxins.

Nematode damage to pods was positively correlated to discolored pods in 2007 and 2008, and positively correlated with visible fungi on pods in 2007. Visible fungi on pods were also positively correlated to cracked pods and pod rot in 2009, whereas pod rot was positively correlated to cracked pods in 2009. A negative correlation occurred between discolored pods and yield in 2007. Microbivore nematode populations, at harvest 2007, were positively and significantly correlated to total plant parasitic nematodes indicating that nematode populations late in the growing season may increase to a level at which they do not adversely affect each other. In 2007, at pre-plant, fungivore nematodes were negatively correlated to immature pods and total plant-parasitic nematodes were positively correlated to pod rot. In 2008 and 2009, fungivore nematodes were positively correlated to total free-living populations and negatively correlated to visible fungi on pods at harvest. Yield was negatively correlated to total plant-parasites at pre-plant and lesion nematodes at harvest in 2008. In 2009, lesion nematodes were negatively correlated to total free-living nematode populations at pre-plant.

Discussion

Aflatoxin contamination was only present in 2007 during the course of this study. Hill et al., 1983, reported that aflatoxins are more likely to be produced when environmental conditions are

hot and dry, three to six weeks prior to peanut maturity. In 2007, drought conditions were present with a total rainfall level during the growing season of approximately 17 inches, while rainfall levels in the last six weeks of the growing season totaled approximately 4.51 inches. In 2008 drought conditions were only present in the beginning of the season. Total rainfall levels during the 2008 growing season were 19.5 inches, although rainfall levels in the last six weeks of the growing season were 8.78 inches. In 2009 drought conditions were not present and the total rainfall level during the growing season totaled approximately 29 inches. In 2007, only two cropping rotations were planted to peanuts, continuous peanut and peanut/bahiagrass. B_1 and total aflatoxin levels were lower in the peanut/bahiagrass rotation. No inference could be made about the peanut/cotton and peanut/corn rotations and their ability to suppress aflatoxin contamination.

Relationships were observed between microbivore nematode populations and rotation sequence. The data suggests that bahiagrass planted in rotation with peanuts supported a higher population level of microbivore nematodes except following the year when peanuts were planted, than continuously planted peanuts. Bahiagrass rotations might have contributed to soil organic matter thereby increasing the food source and the population levels of microbivorous nematodes. Also, a relationship was discovered between plant-parasitic nematode populations and crop rotation. Continuously cropped peanut monocultures resulted in higher levels of plant parasitic nematode populations than the bahiagrass rotation except in the year when bahiagrass plots were planted to peanuts. It has been well documented that peanut monoculture increases *M. arenaria* populations, which are the main nematode parasites of peanuts, and decreases yields (Katsvairo et al., 2007; Rodriguez-Kabana et al., 1991; Bowen et al., 1996).

Visual evaluation of physical damage to peanut pods and microscopic identification of nematodes revealed some interesting correlations. Negative correlations occurred between microbivore nematode populations and aflatoxin contamination including G_2 and total aflatoxins, although these correlations were observed at pre-plant and mid-season. This suggests that nematode populations found earlier in the growing season have the most influence on aflatoxin contamination. In addition aflatoxin contamination was influenced by root-knot nematodes, total plant-parasitic nematode populations and insect damage to pods. Nematode damage to pods was also correlated to visible fungi on pods, although visible fungi on pods were not correlated with aflatoxin levels. Timper et al., 2004, reported that aflatoxins occurred more frequently in pods that had more nematode damage. It was believed that nematode damage to pods may have provided a site where *A. flavus* could enter the pod and subsequently lead to aflatoxin contamination. Our results indicate that a combination of factors may play a role in aflatoxin contamination including nematode damage to pods, insect holes or any other form of damage leading to entry points for the fungus during periods of drought.

Negative correlations occurred between free-living nematodes (microbivores and fungivores) and plant parasitic nematodes including root-knot nematodes. This suggests that higher levels of free-living nematode populations could lead to suppression of herbivore populations. This relationship may be due to an increase in plant health free-living nematodes are commonly attributed to, helping the plant tolerate nematode infection.

The results of this study indicate that free-living nematodes tend to have a negative effect on plant parasitic nematode populations. Decreases in B_1 and total aflatoxin levels were observed when peanut was cropped following several years of bahiagrass compared to continuously cropped peanuts. Further testing is needed to determine the effects of nematode populations in

peanut/cotton and peanut/corn rotations and confirm the effects of bahiagrass and continuous peanut rotations on nematode populations in years when environmental factors are conducive for aflatoxin contamination. Overall, when considering crops to plant in succession with peanuts to maintain crop health bahiagrass is preferable to peanut monocropping. Bahiagrass rotations in peanut fields increase microbivore nematode populations, which may in turn decrease aflatoxin levels.

Table 1. Year-wise cropping pattern in different peanut rotations sampled for this study at Wiregrass Research and Extension Center.

Crop rotation	2006	2007	2008	2009
Continuous peanut (P-P-P-P)	Peanut	Peanut	Peanut	Peanut
Peanut/bahiagrass (B-P-B-B)	Bahiagrass	Peanut	Bahiagrass	Bahiagrass
Peanut/cotton (P-Ct-P-Ct)	Peanut	Cotton	Peanut	Cotton
Peanut/corn (Cr-Ct-P-Cr)	Corn	Cotton	Peanut	Corn

Table 2. Spearman rank correlation coefficients calculated among nematode populations observed at pre-plant, aflatoxin levels detected in peanuts, yield, and visual peanut evaluations for pod damage in 2007 under various peanut rotations in Headland, AL.

	Total free-living	**Root-knot**	**Spiral**	**G2 Aflatoxins**	**B1 Aflatoxins**	**B2 Aflatoxins**	**Total Aflatoxins**	**Yield**	**Immature pods**	**Pod rot**	**Nematode damage to pods**
Microbivores	r=0.98802 p=<0.0001	r= -0.72380 p=0.0424	r=0.76509 p=0.0270	r= -0.91146 p=0.0016	-	-	-	-	-	-	-
Fungivores	-	-	-	-	-	-	-	-	r= -0.76509 p=0.0270	-	-
Total free-living	-	r= -0.72790 p=0.0406	r=0.75593 p=0.0300	r= -0.88786 p=0.0032	-	-	r= -0.70660 p=0.05	-	-	-	-
Root-knot	r= -0.72790 p=0.0406	-	-	r=0.74832 p=0.0327	-	-	-	-	-	-	-
Total plant-parasites	-	-	-	-	-	r= -0.86603 p=0.0054	-	-	-	r=0.77442 p=0.0241	-
G1 Aflatoxins	-	-	-	-	-	-	r=0.76835 p=0.0259	-	-	-	-
Discolored pods	-	-	-	-	-	-	-	r= -0.71429 p=0.0465	-	-	r=0.90476 p=0.0020
Insect holes in pods	-	-	-	-	r=0.72405 p=0.0423	-	-	-	-	-	-
Visible fungi on pods	-	-	-	-	-	-	-	-	r= -0.80608 p=0.0157	-	r=0.73055 p=0.0396

- Correlation not significant at P <0.05.

Table 3. Spearman rank correlation coefficients calculated among nematode populations observed at mid-season, aflatoxin levels detected in peanuts, yield, and visual peanut evaluations for pod damage in 2007 under various peanut rotations in Headland, AL.

	Total free-living	**Root-knot**	**G2 Aflatoxins**	**B1 Aflatoxins**	**B2 Aflatoxins**	**Total Aflatoxins**	**Yield**	**Insect holes in pods**	**Immature pods**	**Nematode damage to pods**
Microbivores	r=0.98795 p=<0.0001	r= -0.76087 p=0.0283	r= -0.75921 p=0.0289	-	-	r= -0.96386 p=0.0001	-	-	-	-
Total free-living	-	-	r= -0.83577 p=0.0098	-	-	r= -0.95181 p=0.0003	-	-	-	-
Root-knot	r= -0.83450 p=0.0100	-	r=0.85779 p=0.0064	-	-	-	-	r= -0.79768 p=0.0177	-	-
Ring	-	-	-	-	-	-	r=0.76980 p=0.0255	-	-	-
Total plant-parasitic	-	-	-	-	r= -0.92778 p=0.0009	-	-	-	-	-
G1 Aflatoxins	-	-	-	-	-	r=0.76835 p=0.0259	-	-	-	-
Discolored pods	-	-	-	-	-	-	r= -0.71429 p=0.0465	-	-	r=0.90476 p=0.0020
Insect holes in pods	-	-	-	r=0.72405 p=0.0423	-	-	-	-	-	-
Visible fungi on pods	-	-	-	-	-	-	-	-	r= -0.80608 p=0.0157	r=0.73055 p=0.0396

- Correlation not significant at P <0.05.

Table 4. Spearman rank correlation coefficients calculated among nematode populations observed at harvest, aflatoxin levels detected in peanuts, yield, and visual peanut evaluations for pod damage in 2007 under various peanut rotations in Headland, AL.

	Total free-living	Fungivores	Predators	Ring	Total plant-parasites	Total Aflatoxins	Discolored pods	Insect holes in pods	Visible fungi on pods	Insect scars on pods
Microbivores	r=0.87831 p=0.0041	-	-	r=0.93541 p=0.0006	r=0.72500 p=0.0419	-	-	-	-	-
Total free-living	-	-	-	r=0.73030 p=0.0397	-	-	-	-	-	-
Total plant-parasites	-	-	-	r=0.78842 p=0.0201	-	r= -0.72405 p=0.0423	-	-	-	-
Lesion	-	-	r=0.75593 p=0.0300	-	-	-	-	-	-	-
Root-knot	r=0.85192 p=0.0072	-	-	-	-	-	-	-	-	-
Stunt	-	r=0.71714 p=0.0453	-	r=0.71714 p=0.0453	-	-	-	-	-	-
Yield	-	-	-	r=0.73030 p=0.0397	r=0.85391 p=0.0070	-	r= -0.71429 p=0.0465	-	-	-
G1 Aflatoxins	-	-	-	-	-	r=0.76835 p=0.0259	-	-	-	r= -0.66643 p=0.0711
B1 Aflatoxins	-	-	-	-	-	-	-	r=0.72405 p=0.0423	-	-
Immature pods	-	-	-	-	-	-	-	-	r= -0.80608 p=0.0157	-
Nematode damage to pods	-	-	-	-	-	-	r=0.90476 p=0.0020	-	r=0.73055 p=0.0396	-

- Correlation not significant at P <0.05.

Table 5. Spearman rank correlation coefficients calculated among nematode populations observed at pre-plant, yield and visual peanut evaluations for pod damage in 2008 under various peanut rotations in Headland, AL.

	Total free-living	**Total plant-parasite**	**Immature pods**	**Discolored pods**	**Cracks in pods**
Microbivores	r=0.96085 p=<0.0001	r=0.67204 p=0.0167	-	-	-
Root-knot	-	r=0.81942 p=0.0011	-	-	-
Reniform	-	-	r=0.66058 p=0.0194	-	-
Yield	-	r= -0.61231 p=0.0343	-	-	-
Insect scars on pods	-	-	r= -0.59716 p=0.0403	-	-
Cracks in pods	-	-	r= -0.65368 p=0.0211	r=0.68366 p=0.0142	-
Nematode damage to pods	-	-	-	r=0.88908 p=0.0001	r=0.76802 p=0.0035

- Correlation not significant at P <0.05.

Table 6. Spearman rank correlation coefficients calculated among nematode populations observed at mid-season, yield and visual peanut evaluations for pod damage in 2008 under various peanut rotations in Headland, AL.

	Total free-living	Total plant-parasites	Lesion	Reniform	Spiral	Immature pods	Visible fungi on pods	Discolored pods	Cracks in pods
Microbivores	r=0.95775 p=<0.0001	-	-	-	-	-	-	-	-
Fungivores	r=0.61379 p=0.0338	-	-	-	-	-	-	-	-
Ring	-	-	-	-	r=0.67420 p=0.0162	-	r= -0.64775 p=0.0228	-	-
Root-knot	-	r=0.70438 p=0.0105	-	r=0.62253 p=0.0306	-	-	-	-	-
Stubbyroot	-	-	r=0.57735 p=0.0493	-	-	-	-	-	-
Stunt	-	r=0.64826 p=0.0226	-	-	-	-	-	-	-
Insect scars on pods	-	-	-	-	-	r= -0.59716 p=0.0403	-	-	-
Cracks in pods	-	-	-	-	-	r= -0.65368 p=0.0211	-	r=0.68366 p=0.0142	-
Nematode damage to pods	-	-	-	-	-	-	-	r=0.88908 p=0.0001	r=0.76802 p=0.0035

- Correlation not significant at P <0.05.

Table 7. Spearman rank correlation coefficients calculated among nematode populations observed at harvest, yield and visual peanut evaluations for pod damage in 2008 under various peanut rotations in Headland, AL.

	Total free-living	Total plant-parasites	Lesion	Stunt	Immature pods	Visible fungi on pods	Discolored pods	Cracks in pods
Microbivores	r=0.92933 p=<0.0001	-	r=0.59882 p=0.0397	-	-	-	-	-
Fungivores	r=0.69113 p=0.0128	-	-	-	-	r=0.61043 p=0.0350	-	-
Reniform	-	-	r=0.58596 p=0.0453	r=0.62313 p=0.0304	-	-	-	-
Root-knot	-	r=0.86620 p=0.0003	-	-	-	-	-	-
Ring	-	-	-	r=0.68442 p=0.0141	r=0.65057 p=0.0220	-	-	-
Yield	-	-	r= -0.61461 p=0.0335	-	-	-	-	-
Insect scars on pods	-	-	-	-	r= -0.59716 p=0.0403	-	-	-
Cracks in pods	-	-	-	-	r= -0.65368 p=0.0211	-	r=0.68366 p=0.0142	-
Nematode damage to pods	-	-	-	-	-	-	r=0.88908 p=0.0001	r=0.76802 p=0.0035

- Correlation not significant at P <0.05.

Table 8. Spearman rank correlation coefficients calculated among nematode populations observed at pre-plant, yield and visual peanut evaluations for pod damage in 2009 under various peanut rotations in Headland, AL.

	Total free-living	**Fungivores**	**Reniform**	**Root-knot**	**Yield**	**Pod rot**	**Discolored pods**	**Cracks in pods**
Microbivores	r=0.91566 p=0.0014	-	-	-	-	-	-	-
Lesion	r= -0.76047 p=0.0285	-	-	-	-	-	-	-
Spiral	-	-	r=1.00000 p=<0.0001	-	-	-	-	-
Total plant parasites	-	-	-	-	-	r= -0.72123 p=0.0435	-	-
Immature pods	-	-	-	-	r=0.71199 p=0.0476	-	r= -0.70820 p=0.0493	-
Pod rot	-	-	-	-	-	-	-	r=0.95759 p=0.0002
Visible fungi on pods	-	r=0.75955 p=0.0288	-	-	-	r=0.79768 p=0.0177	-	r=0.82722 p=0.0113

- Correlation not significant at P <0.05.

Table 9. Spearman rank correlation coefficients calculated among nematode populations observed at mid-season, yield and visual peanut evaluations for pod damage in 2009 under various peanut rotations in Headland, AL.

	Total free-living	**Predators**	**Root-knot**	**Yield**	**Pod rot**	**Discolored pods**	**Cracks in pods**
Microbivores	r=0.89822 p=0.0024	-	-	-	-	-	-
Fungivores	-	r=0.79286 p=0.0189	-	-	-	-	-
Total plant-parasites	-	-	r=0.80013 p=0.0171	-	-	-	-
Immature pods	-	-	-	r=0.71199 p=0.0476	-	r= -0.70820 p=0.0493	-
Pod rot	-	-	-	-	-	-	r=0.95759 p=0.0002
Visible fungi on pods	-	-	-	-	r=0.79768 p=0.0177	-	r=0.82722 p=0.0113

- Correlation not significant at P <0.05.

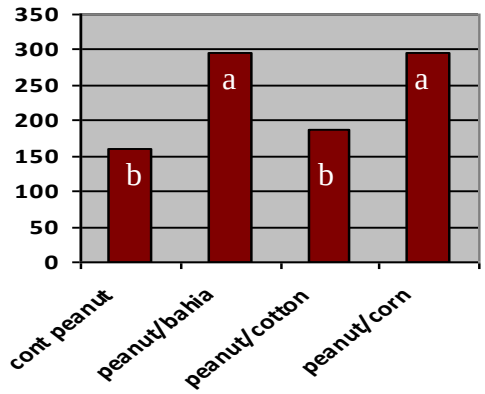
Table 10. Spearman rank correlation coefficients calculated among nematode populations observed at harvest, yield and visual peanut evaluations for pod damage in 2009 under various peanut rotations in Headland, AL.

	Total free-living	**Root-knot**	**Yield**	**Pod rot**	**Discolored pods**	**Cracks in pods**
Microbivores	r=0.93415 p=0.0007	-	-	-	-	-
Fungivores	r=0.77801 p=0.0230	-	-	-	-	-
Predators	-	-	r= -0.91287 p=0.0015	-	-	-
Dagger	-	r= -0.87149 p=0.0048	-	-	-	-
Immature pods	-	-	r=0.71199 p=0.0476	-	r= -0.70820 p=0.0493	-
Pod rot	-	-	-	-	-	r=0.95759 p=0.0002
Visible fungi on pods	-	-	-	r=0.79768 p=0.0177	-	r=0.82722 p=0.0113

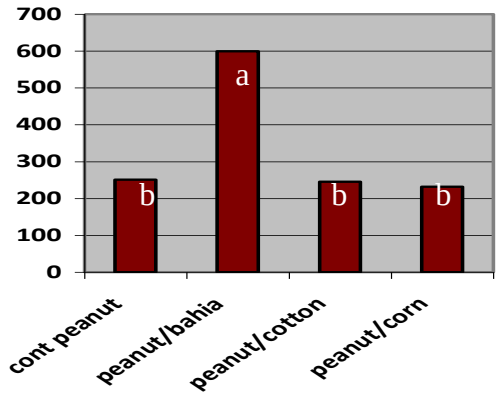
- Correlation not significant at P <0.05.

Figure 1. Mean microbivore nematode counts observed under various peanut cropping rotations from the Wiregrass Research and Extension Center sampled at:

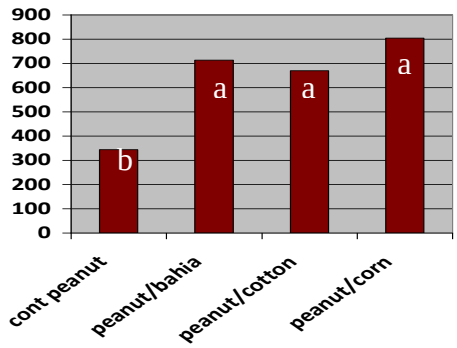
a) Pre-plant 2007



b) Harvest 2008



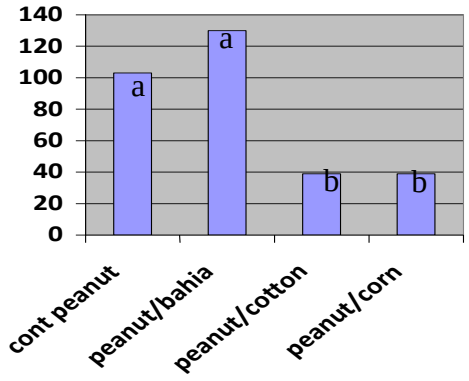
c) Mid-season 2009



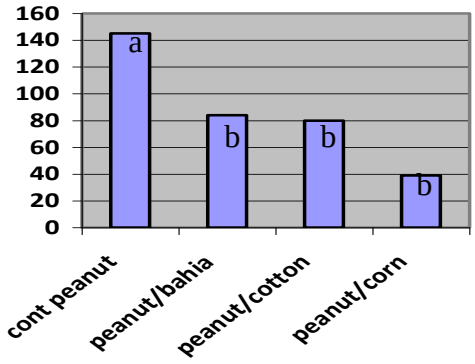
Columns with the same letter are not significantly different at P ≤0.05 according to Fisher's LSD Test.

Figure 2. Mean total plant-parasitic nematode counts observed under various peanut cropping rotations from the Wiregrass Research and Extension Center sampled at:

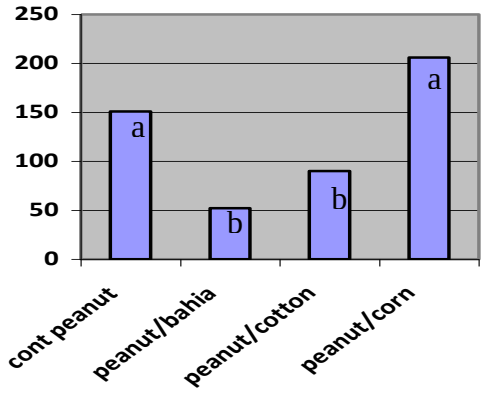
a) Pre-plant 2008



b) Pre-plant 2009



c) Mid-season 2009



Columns with the same letter are not significantly different at P ≤0.05 according to Fisher's LSD Test.

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Summary

A Denaturing Gradient Gel Electrophoresis (DGGE) technique was adapted for identifying nematode populations and monitoring shifts in those populations. Nematode consensus primers were evaluated for specificity based on a DNA collection containing a wide range of nematode trophic groups and non-target fungal organisms. The amplified 18S rDNA from all species in the DNA collection was confirmed indicating that the nematode consensus primers may be universal to all eukaryotic organisms and not specific to nematodes. To ensure other non-target eukaryotic organisms were not amplified from soil samples, nematodes were extracted from the soil prior to extracting DNA from the nematodes for the remainder of this study. DGGE successfully separated all nematodes in the DNA collection at the generic level indicating this molecular fingerprinting technique is sensitive enough to separate nematode populations in soil samples. This genetic profiling technique was then applied to peanut soil samples to determine if individual nematode populations can be identified and if profiles can reveal individual banding patterns for samples under different rotations. Through a band matching analysis, 37 different band classes were observed, although only 17 bands were recovered and sequenced. High background fluorescence inhibited recovery of DNA from weak bands. These results demonstrate that the populations with the highest level of DNA can successfully be recovered and putatively identified. The genetic profile also revealed similarities between replications of the same crop rotation indicating that rotation causes a shift in nematode populations.

Nematode community structure was evaluated using the DGGE technique adapted for identifying nematode populations and monitoring shifts in those populations in combination with DNA recovery from genetic profiles followed by sequence identification. Important peanut cropping sequences in the southeastern United States were chosen for this study including: continuous peanuts, continuous bahiagrass, peanut/corn, and peanut/cotton. Nematode DGGE profiles indicated that up to 68% similarities were observed among the replicated plots of the same peanut cropping sequences. Although these results were not consistent among all rotations and sampling periods, similarities could be the result of plant species effect on nematode communities. Results show a wide range of nematode community polymorphisms were present irrespective of crop rotation indicating the impact of cropping sequence on nematode diversity was minimal. Multi-dimensional scaling of DGGE profiles indicated closer clustering or less scattering among nematode communities with respect to sampling period rather than cropping sequence. Since the sampling periods were set at prescribed times through the growing season based on crop age, the specific crop age and environmental factors could be playing an important role in the nematode community rather than plant species.

Nematode DNA was recovered from genetic profiles by excising bands, re-amplifying the DNA and sequencing. Results from DNA sequencing revealed that free-living nematodes accounted for the majority of populations present in the nematode community. Plant-parasitic nematodes, animal parasitic nematodes, entomopathogenic nematodes, and nematophagus fungi were also present in the plots sampled in much lower proportions. Only 41% of the sequences were identified to species level with a maximum identity of 97-100% based on those in the nucleotide collection of the GenBank database, which only contains

approximately 20,000 nematode 18S sequences. The more nematode sequences identified and deposited in complied databases, the more precise sequence matching will become. There were 29 nematode genera and three fungal genera putatively identified, which may be an underestimation of the biodiversity. DGGE techniques only display populations that make up 1% or more of the total community. It is possible that more genera were present but were omitted because they represented <1% of the total nematode biomass.

Aflatoxins were only present in one year of this three year study. Results from this year (2007) showed that planting peanut following several years of bahiagrass significantly reduced B_1 and total aflatoxin levels compared to continuously planted peanuts. No inference could be made about the peanut/cotton and peanut/corn rotations because they were not planted to peanut the year aflatoxins were present. Microbivore nematode populations were negatively correlated to aflatoxin contamination. A negative correlation also occurred between free-living nematode populations and plant-parasitic nematodes. Bahiagrass rotations supported significantly higher levels of microbivore nematodes than did continuously planted peanuts, while continuously planted peanuts supported significantly higher levels of plant-parasitic nematodes than peanuts planted in rotation with bahiagrass. These results suggest that microbivore nematodes may suppress plant-parasitic nematodes, possibly through increases in plant health, and may play an important role in the suppression of aflatoxin contamination in peanuts. In order to increase microbivore populations, peanuts can be planted in rotation with bahiagrass.

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