SOIL MICROBIAL COMMUNITY STRUCTURE AND AFLATOXIN

CONTAMINATION OF PEANUTS

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SOIL MICROBIAL COMMUNITY STRUCTURE AND AFLATOXIN CONTAMINATION OF PEANUTS

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DISSERTATION ABSTRACT

SOIL MICROBIAL COMMUNITY STRUCTURE AND AFLATOXIN CONTAMINATION OF PEANUTS

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Doctor of Philosophy, August 10, 2009
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Potential management of the soilborne *Aspergillus flavus* and *A. parasiticus* fungi, a food contaminate that causes human and animal health problem, may be possible through maintaining an appropriate soil microbial diversity. The overall approach is to suppress aflatoxin producing fungi in soils through better understanding and manipulation of soil microbial populations. The objectives of investigation are 1) To develop bacterial profiles of selected soil samples from different rotational sequences: continuous peanut, continuous bahiagrass, peanut-cotton and peanut-corn; 2) To develop fungal community profiles of peanut soils and determine their relationship with *A. flavus* populations; and 3) To determine the effect of different cropping sequences on peanut aflatoxin contamination. The methodology included 1) determining the soil bacterial and fungal communities of different peanut cropping

sequences utilizing a high resolution DNA fingerprinting technique, Automated Ribosomal Intergenic Spacer Analysis (ARISA); 2) 16S rRNA gene cloning and sequencing of bacteria populations from peanut soils; 3) the enumeration of *A. flavus* population levels and estimating the minimum detectable limits of these pathogens in soils; and 4) evaluating the aflatoxin content in peanut pod samples from different cropping sequences. The results indicated that cropping sequences and time of soil sampling have considerable effect on soil microbial community structure. Microbial diversity was higher in peanut soils with bahiagrass and cotton rotations over continuous peanuts. Rarefaction curves of 16S rRNA gene sequence data for all cropping sequences further showed bacterial diversity at species and genus level. The predominant bacterial divisions included Proteobacteria, Acidobacteria, Firmicutes, Bacteroidetes and Actinomycetes. The Proteobacteria populations have significant negative correlation with Firmicutes and are positively correlated with Gemmatimonadetes. The Actinomycetes division showed significant negative correlation with Verrucomicrobia. These relationships may indicate competition among bacterial species in agronomic soils. The minimum threshold limit at which *A. flavus* can be detected in peanut soils directly from soil genomic DNA with *A. flavus* specific primers was found to be 2.6 X 10^6 CFU g^{-1}. Pod aflatoxin content was found to be less in pod samples when peanuts are rotated with bahiagrass and cotton over continuous peanuts. Our research results suggest these interactions between soil microbial communities in peanut soils may be manageable thus for suppressing peanut aflatoxin problem through different cropping sequences.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

II. EXPLORING SOIL BACTERIAL COMMUNITIES IN DIFFERENT PEANUT CROPPING SEQUENCES

III. COMPARISON OF SOIL FUNGAL COMMUNITY STRUCTURE IN DIFFERENT PEANUT ROTATION SEQUENCES USING ARISA

I. INTRODUCTION AND LITERATURE REVIEW

Microbial communities in agricultural field soils are subjected to dynamic change due to the influence of agricultural operations. These communities are further influenced by physicochemical properties of soil (53), soil particle size distribution (68), the presence and age of specific plant species (41, 44) and crop rotation (76). Growing concerns over depletion of natural resources, challenges from new pest and diseases, and the phasing out of environmentally hazardous agricultural chemicals are forcing agrarians and researchers to adapt alternative measures in developing sustainable agriculture (26).

Understanding soil microbial communities and maintaining a healthy microbial community may be of prime importance in combating soilborne pathogens in many field crops. The quantum of shift in microbial community structure towards a beneficial equilibrium with respect to crop health is desirable in checking the soilborne diseases from reaching damaging levels. Soilborne plant pathogens are major production constraints in agriculture and cause economically significant yield losses. Among different soil microbial communities, bacteria and fungi are the predominant in determining soil and crop health. The antagonism that the beneficial microbes in these groups offer toward soilborne plant pathogens could suppress plant diseases from reaching devastating levels.

Soil microbial communities are the key determinants for crop health and can influence crop yields. Monitoring of soil microbes is important and recently microbial ecology has been the focal point for many researchers in sustainable and organic agriculture. Desirable changes in soil microbial communities can be brought about only when these communities are monitored and timely ameliorative measures are initiated in order to avoid beneficial microbe population decline. Thus, a standard, reliable and highly reproducible method of profiling the microbial populations in crop soils is needed. Culture independent methods for monitoring microbial communities that are based on nucleic acids are more reliable and these include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), automated ribosomal intergenic spacer analysis (ARISA) and cloning, all of which are sometimes coupled with DNA sequencing (12, 13, 35, 38, 64, 67, 68). These molecular techniques provide a rapid and highly reproducible means of estimating the microbial diversity in soils compared to culture dependent methods. Combinations of these molecular techniques can be employed for analyzing soil microbial communities and their relationships in agricultural soils. The insight thus gained in understanding microbial communities may aid in designing the methodologies which can allow manipulation of existing microbial communities in soil and reduce the impact of soil borne pathogens as well as in stimulating healthy microbial communities for better crop health.

Peanut is an important cash crop in the southeastern US and can be affected by many soilborne pathogens (14). Aflatoxin contamination caused by the ubiquitous *A.*

flavus group of fungi is a major production constraint and is responsible for huge economic losses to the farming community and trade (37). Determination of soil microbial community structures in peanut soils may be an important step in aflatoxin management since these communities greatly influence aflatoxin contamination (39, 55, 71). The relative abundance of these soil borne pathogens is subject to change with changes in cropping sequences. This research is aimed at studying the diversity among microbial populations in different peanut cropping sequences using a DNA fingerprinting tool, automated ribosomal intergenic spacer analysis (ARISA). This technique relies on the length polymorphism of the intergenic spacer region between the small (16S) and large (23S) subunit rRNA genes (38).

Factors influencing soil microbial community structure and diversity

Microbial diversity in soils is dependent on several factors like soil physico-chemical properties, the nature of plant species grown, and soil nutritional status. It is also dependent on general management practices that are adopted during the cropping period. Several studies have shown the significance of plant type, soil type and edaphic factors on soil microbial diversity. In this section, a brief account is given on different parameters by which soil microbial diversity is subject to influence. Detailed investigations on the structure of soil microbial communities through monitoring the relative abundance of seven of the most common bacterial groups (alpha and beta Proteobacteria, Actinobacteria, Cytophagales, Planctomycetes, Verrucomicrobia and the Acidobacteria) and Eukarya have been carried out using various molecular techniques (12, 13, 19, 20, 21).

Buckley and Schmidt (19), while studying soil microbial communities revealed similarities in microbial community structure among plots that share a long-term history of agricultural management despite differences in plant community composition and land management. In another study (20), they reported that environmental factors such as soil moisture, sampling time, soil depth, and soil management history have greater influence on the distribution of the bacterial division Verrucomicrobia in the soil. Although no significant differences were found among different sampled fields with respect to plant composition, significant differences in the Verrucomicrobial rRNA abundance over different sampling times were observed. In addition, the same researchers conducted experiments in cultivated fields, fields abandoned from cultivation and fields with no history of cultivation, which revealed that these microbial communities are dynamic and exhibit significant change at temporal scales relative to seasonal events. However, the relative abundance of the rRNA of a particular microbial group is affected by the local environment in relation to the field management practices despite temporal changes in microbial community structure (21). They concluded that though there was an effect of plant community composition on the soil microbial communities, it might be masked by the impact of long-term agricultural management practices or the changes were at taxonomic levels which were ignored by determining the whole microbial group's abundance.

Greenhouse studies relating to the impact of plant and chemical factors on bacterial community structure associated with perennial plant species showed that lime and nitrogen amendments have more pronounced effects on microbial activity, biomass and bacterial ribotype number than plant species. Terminal restriction fragment length

polymorphism (T-RFLP) analysis indicated lime and nitrogen amendments altered soil bacterial community structure whereas plant species hardly had any effect (54).

Contrary to the above reports, Zak *et al* (80) concluded that microbial biomass, respiration, fungal abundance and N mineralization rates are significantly enhanced with increases in plant diversity. A positive relationship was shown between plant diversity and productivity in N-limited soils thus suggesting that the plant-microbe interactions in soil are integral components of plant diversity's impact on the functioning of an ecosystem. Collins and Cavigelli (28) investigated soil microbial community characteristics at four sites in Laguna Mountains (Southern California) that differed in elevation, soil type, plant community composition and percent plant cover. From this study they concluded that the Gram-negative bacterial community was proportionally more abundant between plants at the lowest elevation. Small differences in fatty acid methyl ester (FAME) profiles under plants at four sites suggest the importance of plant influence. Greatest differences in microbial substrate utilization profiles among sampling locations was noticed between samples taken under vs. between plants at lower elevation sites and also seemed to be more influenced by presence of plants than by plant specificity.

Studies on microbial community structure and its response to four different plant species such as maize (*Zea mays* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and commercial grass mix, showed that both plant species and land use history had significant effects on the microbial structure and diversity. This was determined by PCR-DGGE fingerprinting with universal and group-specific bacterial primers. These studies revealed that both the abundance and taxonomic composition of bacterial antagonists in soil are affected by the rhizosphere of these plants.

Comparisons of antagonistic *Pseudomonads* revealed that highest populations were present in barley and oat rhizospheres than in maize and grass rhizospheres (40). Recent studies on three different vegetation types, i.e., deciduous forest, shrubs and pastures, with the help of T-RFLP and 16S rRNA gene cloning and sequencing, indicated that the vegetation cover has a significant impact on the soil microbial community structure. Further, vegetation cover influence is greater on soil microbial community structure than climate and soil chemical properties (23).

Cropping system diversification in traditional peanut (*Arachis hypogea* L) and cotton (*Gossypium hirsutum* L) by including perennial grasses such as bahiagrass (*Paspalum notatum* Fluegge) and bermudagrass (*Cynodon dactylon* (L) Pers) in the presence of grazing with cattle (*Bos taurus*) yielded significant beneficial effects on the microbial communities. Both peanut and cotton crops, when grown after perennial grasses, were found to be more deeply rooted and exhibited more vigorous plant growth and yield coupled with the ability to endure pest pressure and environmental stress (52).

The effects of agricultural management practices over the long-term on active soil organic matter (SOM) and short-term microbial C and N dynamics were studied in detail by incorporating rye into soils. It was observed that SOM (ratio of microbial biomass C or N to total soil C or N) appeared to be related to long-term management. These ratios increased in proportion to increased organic inputs and reduced tillage or periods of fallow. Further, in all the investigated soils, MBC (Microbial Biomass C) increased and decreased rapidly following rye incorporation, but MBN (Microbial Biomass N) was found to be fairly constant. A lower ratio of bacterial to fungal biomass and lower ratio of respiration to MBC were observed in organic soils of

sustainable agricultural farming systems (SAFS) when compared to SAFS conventional soils. The rye incorporation into soils had a short-term burst of microbial growth and activity of similar magnitude in all three soils although similarities existed in the initial MB contents in all the soils (56).

Zwolinski (81) reported DNA sequencing as a potent tool for developing strategies relating to soil microbes and their environment. By utilizing 16S rRNA as the preferred gene target, soil microbial diversity and phylogenetic relationships between unknown and uncultivated microbes were established. Comparisons on the diversity and community structure of microbes in sandy and organic soils revealed that organic soils have higher diversity of cultivable bacteria when compared to sandy soils (64). This was determined at phenotypic, phylogenetic and genetic levels by combined use of molecular ecology tools like amplified rDNA restriction analysis, hybridization to oligonucleotide probes, REP-PCR, and DNA reassociation kinetics and PCR-DGGE analysis. A significant difference in these two soils was noticed in total bacterial populations compared to cultivable populations thus indicating that diversity in entire microbial community DNA is appropriate over diversity of DNA of cultivable bacteria in determining microbial diversity of soils.

Brussard *et al* (18) reported that biodiversity in soils confers stability to stress and disturbance and also protects against soilborne diseases. Further, mycorrhizal diversity in soil contributes positively to nutrient and water use efficiency. The effects of soil fauna on nutrient and water use efficiency are also apparent, but the diversity effects may be indirect, through effects on soil structure. In a recent study conducted on the effect of land use intensification on soil microbial diversity and thus soil health and quality, similar population levels of *Bacillus* spp. (log 5.87-6.01 CFU/g dw soil)

was obtained from the field soils of three different land use types in China's Yangtze River Delta. Further, population counts of *Pseudomonas* spp. (log 5.44 CFU/g dw soil) were lower in the polytunnel greenhouse vegetable land than open vegetable land and traditional rice-wheat rotation land. Cucumber wilt, caused by *Fusarium oxysporum*, pathogen populations (log 3.21 CFU/g dw soil) were significantly lower in the traditional rice-wheat rotation land compared to the other two land use types suggesting that the level of soil suppressiveness to this pathogen depends on the functional diversity of the soil microbes (78). Wertz *et al* (79) studied the impact of decline in biodiversity on ecosystem sustainability, functioning and stability with regard to bacterial groups like denitrifiers and nitrite oxidizers. They reported that resistance and resilience to disturbance differed between these two communities with the nitrite oxidizers group being most affected. Further, it was observed that a reduction in biodiversity of the two microbial functional groups did not impair either their resistance or their resilience following disturbance.

The effect of different agricultural management practices on *Burkholderia* community, based on a *Burkholderia*-specific polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), was investigated in three agricultural management regimes of crop rotation, maize monoculture and grassland. These results indicated a conversion of *Burkholderia* communities typical of permanent grassland to those of an arable land after four years of cropping. However, the period for reverse transition of these communities was beyond the duration of field experiment. Further, cultural practices such as fertilization and tillage have more effect on the change of *Burkholderia* community structure than agricultural management regime. It was also observed that in areas under permanent grassland and

grassland converted to maize monoculture, highest percentage of *Burkholderia* strains (mainly *B. pyrrocinia* and *Burkholderia* spp. LMG 22929) with strong antagonistic activity against *Rhizoctonia solani* AG-3 were observed (69).

Among the different management practices that were followed in cultivating crops, tillage is an important agricultural operation, which basically prepares the land for seeding. Depending on the type of tillage, it brings various levels of disturbance to the soil and thereby influences the resident soil microbial communities. Lupwayi *et al* (57), while studying the microbial diversity and community structure in wheat cropping systems as influenced by crop rotation and tillage practices, revealed that tillage significantly reduced bacterial diversity in the soil due to substrate reduction. Further, tillage has a more profound effect on microbial diversity in bulk soils than in the rhizosphere at the flag-leaf stage of the crop. Conventional tillage resulted in more similar structures of bacterial community assemblages or substrate utilization patterns than zero tillage. The results suggest that both conservation tillage and legume-based crop rotations support diversity of the soil microbial community and will affect sustainability of agricultural ecosystems. However, the depth of soil subjected to disturbance with tillage is also an important factor in influencing the microbial communities. Drijber *et al* (32) reported that cropped plots had higher microbial biomass than their fallowed counterparts but did not differ significantly with tillage for the 0-15 cm depth. The fatty acid methyl ester (FAME) profiles from the fallow plow were most dissimilar from cropped soils thus suggesting a mutual relation between tillage management and the long-term resiliency of the microbial community in wheat cropping systems.

Keeping in view of all the above studies, various edaphic factors along with plant community composition had shown significant affect on the soil microbial community structure and diversity. In addition, few methodological variations could also affect the results obtained while studying the diversity of microbial communities. For example sampling strategies, DNA extraction methods, PCR amplification biases, and finally the resolution of the DNA fingerprinting method, selected for analysis influence the outcome (59, 67, 68). A number of studies have been conducted on soil microbial communities and their changing patterns with respect to various biotic and abiotic factors but how the specific microbial communities change the fate of soilborne pathogens occurrence is still in its infant stage. Better understanding of resident soil microbial communities in cropping soils might be helpful while studying the soil saprophytic fungi such as *A. flavus* as it is posing a serious threat to human and animal life.

Influence of crop rotations and cultural practices on soil health

Crop rotation is one of the important cultural practices followed in agriculture that can protect soil health and fertility. The role of crop rotations and cultural practices on the occurrence of soilborne pathogens and thus the impact on soil ecosystem is well documented. Horn *et al* (49) studied the effect of peanut and corn cultivation on aflatoxin producing *A. flavus* and *A. parasiticus* as affected by drought stress. Corn was shown to have higher populations of *A. flavus* infection and subsequent aflatoxin contamination when the plots were subjected to drought stress. Further, a higher

population of *A. parasiticus* in soil was due to the colonization of corn debris despite a lower infection on the crop itself.

Different agricultural management practices have the tendency to bring changes in the soil microflora and thereby increase the natural antagonistic populations in cropping soils. Studies on the impact of different crop management practices in peanut on soilborne diseases revealed that when corn was grown as a preceding crop to peanut rather than soybean under conservation tillage, population densities of fungal antagonists, such as Actinomycetes, *Trichoderma* spp. and *Gliocladium* spp. were higher at harvest (74). Moreover, the population levels of these three antagonists were the highest in peanut under no tillage conditions than in reduced tillage. Using low tillage with corn as a preceding crop, the incidences of peanut blight by *Sclerotinia minor* and root rot by *Fusarium solani* were very low indicating a possible suppression of these pathogens by soil antagonists (75). Jaime-Garcia and Cotty (51) reported that corn-cotton rotations harbor more aflatoxin producing *A. flavus* due to colonization of the fungus on corn cobs that reside on the soil surface. Further, it was reported that corn cobs from the previous season contained over 190 times more *A. flavus* propagules than soil collected from the same field. Bowen *et al* (14) reported that southern stem rot (SSR) incidence caused by *Sclerotium rolfsii* was higher in fields where peanut was consistently grown with fewer rotations and the incidence was inversely related to number of years between peanut crops.

Soil nutritional status was also affected with the crop rotations through the addition of certain nutrients to the soil and thereby improves the soil health. One such investigation revealed an increase in soil organic carbon, nitrogen and phosphorus in a rotation of wheat and subterranean clover with direct drill and mulching, while

stubble burning in wheat-lupin and wheat-wheat rotations led to soil organic matter losses. Further, the biomass of microbes was highest when organic matter was added to soil (22). Another study with organic and conventional farming systems with different crop rotations revealed that organic matter content was higher in organic farming system than in conventional farming system. However, the conventional system had more N in the mineral pools as indicated by higher NO_3-N; whereas the organic system had higher N in the microbial biomass thus indicating shifts in N pools between the two systems. Organic farming systems had more bacteriovore nematodes than in the conventional system. On the other hand, the conventional system had significantly higher populations of *Pratylenchus crenatus*, the lesion nematode than the organic system. The populations of *P. crenatus* were lowest in organic hay plots compared to corn, soybean and oats (17).

Factors affecting *A. flavus* populations, infection and subsequent aflatoxin contamination

Aspergillus group of fungi are ubiquitous in nature and found most commonly in soils (45). Pre-harvest aflatoxin contamination in peanuts is majorly influenced by various factors such as soil moisture, soil temperature, soil calcium levels, prevailing drought conditions 3-4 weeks before harvest, and physical damage caused by nematode and insect pests in addition to the population load of *A. flavus* group toxigenic strains (16, 58, 70). Further, a positive correlation was obtained between soil calcium content, nematode populations and to that of aflatoxin contamination.

Laboratory experiments using artificial inoculation to study the effect of soil density of *Flavi* group (*A. flavus, A. parasiticus, A. caelatus* and *A. tamari*) section *Nigri*, and *A. terreus*, on the incidence of peanut seed colonization, revealed that percentage seed colonization was higher with *Flavi* species, but well below 100% despite high species densities in some soils. Competition among *Flavi* species was the primary reason of reduced seed colonization. Further, an average of two or fewer propagules of each *Aspergillus* spp. in soil is required at the wound site for colonization of 20% of peanut seeds and the invasion by other *Aspergillus* spp. occurs only when densities of *Flavi* and *Nigri* species are low (46).

Studies on the population dynamics of *Aspergillus* species (section *Flavi*), the aflatoxin producing fungi, from soils of three peanut-growing regions of Cordoba Province, Argentina, revealed no significant differences in pathogen population between planting and harvest time in two regions (7). However, significant differences in CFU g^{-1} of the total fungal population and *Aspergillus* species from section *Flavi* were noticed in the other region. Among the soilborne fungi, *A. flavus* was found to be the dominant species. Significant differences were noticed in the composition of toxigenic and atoxigenic strains with respect to period and the regions evaluated, and one of the evaluated regions had higher frequencies of toxigenic strains of *A. flavus* and *A. parasiticus* than atoxigenic strains and a concomitantly high level of aflatoxins in peanuts.

A study was conducted along a transect from eastern New Mexico through Georgia to eastern Virginia, USA, on *A. flavus* isolates for aflatoxin B1 and cyclopiazonic acid production. It was observed that the S- strains (n=309, with small sclerotia of < 400 µm in diameter) produced higher levels of aflatoxin B1 whereas L strains (n=774,

with sclerotia > 400 µm) were more variable for aflatoxin B1 and cyclopiazonic acid production compared to S- strains. Further, a positive correlation was noticed between aflatoxin B1 production and cyclopiazonic acid production in both the strains although 12% of the L-strains produced only cyclopiazonic acid. The western half of Texas and peanut growing regions of Georgia and Alabama had 62 to 94% *A. flavus* isolates which produced >10 µg/ml of aflatoxin B1. However, half of the L-isolates were found to be atoxigenic and also did not produce cyclopiazonic acid (48).

Studies on sclerotial production and toxigenicity of *A. flavus* proved that the L phenotype of the pathogen was isolated more frequently than the S phenotype and represented 59% of the total isolates (369 strains). In addition, significant differences were observed between L-, S-, and non-sclerotial strains with regard to aflatoxin and cyclopiazonic acid (CPA) production. Notably, the S strains produced higher levels of mycotoxins than the L-, and non-sclerotial strains and about 10% of these S strains had an unusual pattern of mycotoxin production due to simultaneous production of aflatoxins B and G and also CPA (6).

Sanders *et al* (70) reported that the mean soil temperatures in irrigated, drought, drought-heated soil and drought-cooled soil treatments at the end of growing season for peanuts were about 21.5, 25.5, 30 and 20^0 C respectively. The plant stem temperatures in all drought treatments reached a maximum of 40^0 C for 6-7 hr each day, which is as much as 10^0 C higher than those of irrigated peanut stems. The pod temperatures were 34 and 30^0 C in drought-heated soil and drought treatments respectively, and at optimum pod temperatures (for *A. flavus* growth, 35^0 C), the colonization of kernels with the pathogen and subsequent aflatoxin concentration increased. However, increased plant temperatures without an increase in pod

temperatures (drought-cooled soils) resulted in only slightly higher colonization percentages by the fungi and aflatoxin concentrations.

Field screening of peanut genotypes that were either resistant, susceptible or highly susceptible to in-vitro colonization by *A. flavus* proved that resistant genotypes had significantly lower levels of seed infection than susceptible genotypes and the genotypic differences in seed infection levels by *A. flavus* were consistent over seasons from 1983-84. Further, the peanut resistant cultivar, 'Junagadh-11' had lower aflatoxin content compared to other susceptible genotypes and drought stress during the year 1984 increased the susceptibility to *A. flavus* seed infection and other fungi and also to aflatoxin contamination (60).

Greenhouse studies on the effect of drought stress on peanut genotypes proved that low soil moisture tension increased colonization of shells and kernels with *Aspergillus* spp. Kernels of all examined genotypes were susceptible to *A. flavus* and *A. parasiticus* colonization under both long and short drought stress conditions compared to non-stressed conditions. Further, no significant differences were found between kernels of the genotypes PI 337409, Starr and J-11 with respect to degree of *Aspergillus* colonization. Screening under microplot conditions revealed that kernels from TX811956 and TX798736 (short stress treatments) had significantly lower *Aspergillus* infestations and the genotypes PI 337409 and TX811956 and TX798736 had significantly lower levels of aflatoxins (4).

A standard greenhouse screening method of peanut plants for resistance to *A. parasiticus* infection and preharvest aflatoxin contamination was developed by Anderson *et al* (2). Pods were completely isolated from the root zone and drought

stress was imposed only on pegs and pods to develop high levels of fungal infection. High levels of preharvest aflatoxin accumulation could be obtained by completely isolating the pods from the root zone. The peanut genotypes that were previously identified as being partially resistant were screened with this technique and the results indicated variability in terms of aflatoxin contamination. No tested cultivars were found to be significantly lower than the standard cultivars relative to aflatoxin accumulation.

In a recent study, Boken *et al* (11) carried out prediction studies by using Normalized Difference Vegetation Index (NDVI) derived Advanced Very High Resolution Radiometer (AVHRR) satellite data at the reproductive phase of the crop. They correlated annual peanut yield to drought and aflatoxin contamination in peanut and reported that a moderate relationship existed between these variables (R^2 =0.56). Further, the aflatoxins in peanut samples were measured and were found to be linked to the NDVI, total precipitation and maximum temperature averaged over the reproductive phase of the crop.

Among different factors influencing peanut aflatoxin contamination, physical damage of pods by nematodes and other insects is important. Pod cracks and wounds due to these biotic agents provide entry points to *A. flavus* group of fungi before subsequent toxin contamination. According to Timper *et al* (73), pod invasion by nematodes and other soil insect pests can increase the chances of infection by aflatoxigenic fungi. Higher aflatoxin levels (1190 ppb) were reported in pods collected from nematode infected plants that had more nematode galls than those with fewer galls.

Lower aflatoxin levels and higher peanut yields were observed in peanut plots with low incidence of lesser cornstalk borer, *Elasmopalpus lignosellus* (15) compared to higher infestation of plots with this soil insect pest. In a separate study by Bowen *et al* (14), pod invasion by *A. flavus* group of fungi was positively correlated with damage by lesser cornstalk borer. External scarification of seeds due to larval feeding had significantly increased *A. flavus* group fungal infection in seeds thereby indicating that contamination of seeds with aflatoxigenic fungi could be increased by external injury of lesser cornstalk borer (58).

Contrary to the above reports, Bell *et al* (8) reported that nematode damage did not affect the incidence of *A. flavus* or aflatoxin contamination. Further, the nematode damage hardly contributed to aflatoxin problem in peanut belt of Georgia, USA.

Soil *A. flavus* determination and aflatoxin estimation

Of different mycotoxins produced by various fungi such as *Penicillium* spp., *Fusarium* spp., and *Aspergillus* spp., aflatoxins are most potent and highly toxic to human and animal life (33). These aflatoxins are produced by *A. flavus* group of fungi and consist of B1, B2, G1 and G2 which are highly carcinogenic, teratogenic and hepatotoxic in nature (29). Detection and quantification of the *A. flavus* group of organisms from soil as well as from foods is, generally carried out on specific media such as *Aspergillus flavus* and *parasiticus* agar (AFPA) and modified dichloran-rose bengal medium (mDRB), based on traditional isolation, culturing, analytical and morphological methods (1, 47, 66). But these are laborious and time consuming ways of detection in addition to the technical expertise needed for identification (34).

Keeping in view of these mycotoxins importance in food and feed stuffs, there is a need for rapid detection of aflatoxins and their source fungi. Hence many biochemical and molecular techniques are available today. The literature in this section covers the molecular and biochemical techniques that are adopted for detection and estimation of aflatoxins and its source fungi.

The polymerase chain reaction is a powerful tool in the molecular biology since its inception into scientific world by Mullis *et al* (63). In fungi the preferred target genes for amplification are ribosomal DNA genes with two internally transcribed spacer regions (18S - ITS 1 - 5.8S - ITS 2 – 28S) which have conserved and highly variable regions for distinguishing closely related species. For PCR based aflatoxigenic fungi detection, various genes involved in aflatoxin biosynthetic pathway are evolved as easy targets with the help of specific primers (9, 36, 42, 72). Though there are many highly sensitive and specific PCR methods available for detecting these fungi from food commodities (43), their use in detecting the *Aspergillus* group of fungi directly from the soil is limited.

Earlier detection of aflatoxins was based on toxigenicity tests of *A. flavus* strains that exhibit fluorescence at 365 nm under UV light when grown in coconut milk agar medium (65). Highly accurate, precise and easy to use sensitive techniques of estimating these toxins are available today. Among them, enzyme linked immunosorbent assay (ELISA) is an accurate, sensitive and rapid method that can be used even for grading farmer’s stock peanuts (27). This method can serve as an alternative to other advanced chromatographic methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS) (5). However, high correlation between

TLC and HPLC techniques is reported with regard to both *A. flavus and A. parasiticus* contamination (3). An improved method of HPLC for aflatoxin estimation is an immunoaffinity column-HPLC wherein the recovery of aflatoxins from corn and peanuts were 72.88% and 87-104% with a relative standard deviation (RSD) of 0.5-3.5% and 2.7-7.4% respectively (62). Other methods of toxin estimation that are commonly used include matrix solid-phase dispersion and liquid chromatography, and delayed luminescence spectra (10, 24).

Aflatoxin Management

Management of the aflatoxin problem in peanuts is generally achievable through biological, cultural, chemical control and host plant resistance. Biological control is the most widely used method wherein antagonistic bacteria and fungi are used. An economically viable integrated management strategy involving host plant resistance, amending the soil with lime and organic supplements for enhancing water holding capacity, plant vigor and seed health, use of biocontrol agents such as *Trichoderma* spp. and *Pseudomonas* spp. is however an ideal option. It is also important to use timely operations of harvesting and postharvest drying as well as bringing awareness and conducting training courses for disseminating technology to the end-users (77). Biological control with atoxigenic strains of *A. flavus* and *A. parasiticus* that were applied in different formulations in the preceding cropping season can result in significant reduction (92%) in peanut aflatoxin concentrations. This method was found effective in delivering competitive levels of atoxigenic strains of *A. flavus* and *A. parasiticus* to soil and also in reducing subsequent aflatoxin contamination (30).

However, reports indicate that application of atoxigenic *A. flavus* strain alone was found to be more effective than the non-toxigenic strain of *A. parasiticus*. Combined applications of atoxigenic strains of both *A. flavus* and *A. parasiticus* were also proved to be effective (31). The mechanism by which aflatoxin management can be achieved is through competitive exclusion between the strains of these *A. flavus* group of fungi (25). The conidia of these atoxigenic fungi remained near the soil surface in spite of heavy rains and varying amounts of water through irrigation. Further, it was observed that rainfall could wash the conidia along the furrows and in directions perpendicular to peanut rows up to 100 meters. The retention of conidia of these aflatoxigenic fungi in upper soil layers is vital to reducing aflatoxin contamination of peanuts, maize and cottonseed (50).

Chemical control of aflatoxins is through application of gypsum either to soil or seed alone or in combination. Gypsum application results in reduced colonization by *A. flavus* and *A. parasiticus*. Further, gypsum also enhances the control of seed colonization when applied in conjunction with the bioagent, *Trichoderma harzianum*, PCNB (Quintozene)-fensulfothion or CGA 64250. No aflatoxins were detected in peanuts harvested from gypsum-treated plots (61). Although, several management options are available against pre-harvest aflatoxin contamination of peanuts, the field results are not consistent. Toxins continue to enter the food chain persistently since the fungi are ubiquitous in nature. Successful management of aflatoxin problem in peanuts may be brought about by manipulating soil microbial communities that can be achieved through better understanding of their changing profiles towards the concept of disease suppressive soils.

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II. EXPLORING SOIL BACTERIAL COMMUNITIES IN DIFFERENT PEANUT CROPPING SEQUENCES

ABSTRACT

Soil bacterial communities have significant influence on soil borne plant pathogens and thus crop health. The present study focuses on profiling soil bacterial communities and their profiles with respect to different peanut cropping sequences in Alabama. The objective is to explore these community profiles for their role in managing soilborne plant pathogens in peanut. Four cropping sequences of peanut at the Wiregrass Research Station, Headland (continuous peanuts, continuous bahiagrass, peanut-cotton and peanut-corn), were sampled in 2006 and 2007. Soil sampling was done early-, mid-season and at harvest. Bacterial community fingerprints were developed from soils using a high resolution DNA fingerprinting technique, Automated Ribosomal Intergenic Spacer Analysis (ARISA) combined with 16S rRNA cloning and sequencing. Rarefaction curves were generated from 16S rRNA gene libraries for all the cropping sequences. ARISA results indicated more than 70% dissimilarities among different cropping sequences. However, 90% similarities were noticed among replicated plots of same cropping sequences. Cropping sequences and time of soil sampling had considerable effect on soil microbial community structure. Bahiagrass rotation with peanuts was found to

have the highest bacterial diversity as indicated by high Shannon Weaver Diversity index. Overall, higher bacterial diversity was noticed with bahiagrass and corn rotations compared to continuous peanuts. Rarefaction curves of 16S rRNA gene sequence data indicated that bacterial diversity at species and genus levels in all the four cropping sequences was far from complete. Proteobacteria, Acidobacteria, Firmicutes, Bacteroidetes and Actinomycetes were the predominant bacterial divisions found in all peanut cropping sequences. The Proteobacteria in these soils were negatively correlated with Firmicutes ($r=-0.65$, $p=0.006$) but have significant positive correlation with Gemmatimonadetes ($r=0.64$, $p=0.007$). The Actinomycetes group is negatively correlated with Verrucomicrobia ($r=-0.51$, $p=0.04$). These results indicate complex interactions among soil bacterial communities in peanut soils that may be useful further to determine the functional differences.

INTRODUCTION

Soil microbial communities are dynamic systems that can be influenced by several edaphic factors, including the type of plant species grown, agronomic practices and chemicals used to control major biotic stresses. Within the soil microbial community, bacteria are the dominant organisms in all ecosystems (31). While some bacteria can cause plant diseases, most are beneficial (21, 30). The bacterial populations in plant rhizospheres contribute to crop health either by direct and/or indirect influences. Bacterial influence on plant pathogens can be due to antibiosis, competition for nutrients, contribution towards induction of systemic resistance in hosts, and siderophore production (24, 26).

Peanut is an important cash crop in the southeastern United States and is affected by many soilborne and foliar diseases. However, aflatoxin contamination is a major threat to the peanut industry since this problem affects the quality of the crop and thus trade and crop profitability (17). Aflatoxins are produced by the *Aspergillus flavus* group of fungi, i.e., *A. flavus* and *A. parasiticus*, following invasion of peanut pods during pre-harvest and post-harvest stages (11, 13). Aflatoxins are secondary metabolites which are carcinogenic, teratogenic and hepatotoxic. The distribution of these aflatoxigenic fungi in peanut soils is largely dependent on the prevalence of antagonistic microflora especially the bacterial community (23).

Bacterial communities in the soil can be monitored through culture dependent and independent techniques. The culture dependent methods include enumeration of bacterial cells on media followed by identification. These methods are biased in estimating the diversity and richness of soil microbial communities due to varied reasons such as the representativeness of a few bacterial divisions that are cosmopolitan and the remainder that are habitat specific (28). On the other hand, the culture independent methods use nucleic acids-based identification and characterization that gives more resolution such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), automated ribosomal intergenic spacer analysis (ARISA) and cloning, all of which are sometimes coupled with DNA sequencing (6, 16, 18, 28). These molecular techniques provide a rapid and highly reproducible means of estimating the microbial diversity in soils over culture dependent methods.

Diversity among bacterial populations in different peanut cropping sequences was investigated through a combination of culture independent techniques. The objective was to determine the microbial communities' distinctions among four different peanut cropping sequences as well as within each cropping sequence over different sampling times using Automated Ribosomal Intergenic Spacer Analysis (ARISA). To further refine the fingerprint information obtained by ARISA, 16S rRNA gene cloning and sequencing was also conducted on the total genomic DNA extracted for the ARISA study.

MATERIALS AND METHODS

Site history and soil sampling. Soil samples were obtained from the Wiregrass Research and Extension Center, Headland, AL from a long term rotation study. The rotation sequences used in this study were: continuous peanuts (P-P-P-P); peanut-cotton (Ct-P-Ct-P), peanut-corn (P-Cn-Ct-P), and continuous bahiagrass for 4 years followed by peanuts (B-B-B-B-P). Rotation sequences were arranged in a randomized complete block design with four replications. Soil sampling was conducted early-season (June), mid-season (August) and at harvest (October) during each cropping season of 2006, 2007 and 2008. Five soil cores were taken randomly across each plot from the root zone in each replication. Samples were placed in a plastic bag and transferred in a cooler.

DNA extraction. Total genomic DNA was extracted from 10 grams of soil using the Power Mac Soil kit from MoBio (MoBio Laboratories Inc. Carlsbad, CA) following manufacturer's instructions. The DNA was extracted within 24 hours after sampling

in order to maintain uniformity for comparing the bacterial communities. The quality and quantity of extracted DNA was checked by the NanoDrop Spectrophotometer (Thermo Scientific, USA). DNA stock solutions of 5 ng/ µl were prepared and stored at -80^0 C for downstream application.

ARISA-PCR. The bacterial ribosomal intergenic spacers were amplified using the primers ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (9). Reaction mixture (50µl) for PCR consisted of 5 µl of 10X dilution buffer (20 mM Tris-HCl, pH 7.5, 100mM KCL, 15mM $MgCl_2$, 1mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween 20 (vol/vol), 0.5% Nonidet P40 (vol/vol), 50% glycerol (vol/vol)), 2 µl of dNTPs, 2 µl of 10µM concentrated ITSF primer, 1µl of 10µM concentrated ITSReub primer, 10 µl of 1µM concentrated ITSReub primer labeled with IRD700 fluorochrome at 5' end from LI-COR (Lincoln, NE, USA), 0.2 µl of Taq polymerase (Promega, Wisconsin, USA), 10 µl of 25µM $MgCl_2$ and 20 µl of template DNA (100ng). Amplification was performed in Peltier thermal cycler (PTC 200, MJ Research) after a hot start at 94^0 C for 3 min, followed by 30 cycles consisting of 94^{0}C for 45 s, 55^0 C for 1 min, and 68^0 C for 2 min, and final extension of 7 min at 68^0 C. The polyacrylamide gel was prepared by mixing 30 ml of 5.5% LI-COR poly-acrylamide (KB plus- LI-COR), 200 µl of 10% ammonium persulphate (APS) and 20 µl of N, N, N',N'-tetramethylethylenediamine (TEMED). Five µl of each PCR product was transferred to new tubes and 2.5 µl of stop buffer was added (LI-COR, Blue Stop Solution). Amplified products were denatured at 98^0 C for 5 min. The PCR products (0.6-0.8 µl) were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions. ARISA

fragments were resolved on 5.5% poly-acrylamide gels and run under denaturing conditions for 8 hr at 1,500 V on LI-COR 4300 DNA sequencer.

The ARISA gel images were analyzed by the BIONUMERICS V. 5.0 software program. Levels of similarity between fingerprints were calculated with the Pearson product-moment correlation coefficient (*r*) after the banding patterns were subjected to conversion, normalization, and background subtraction with mathematical algorithms. Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA) (2). Multi-dimensional Scaling Analysis (MDSA) was done to compare the clusters generated over different crop rotations as well as different sampling times.

16S rRNA Cloning and Sequencing. The genomic DNA of June, 2006 samples were PCR amplified using universal bacterial primers 27F (5'-AGA GTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGHTACCTTGTTACGACTT-3') targeting the 16S rRNA gene. PCR reaction components contained 25 µl of 2x Go Green (Promega), 1 µl of (20uM concentration) each primer, and 50 ng of template DNA in a final volume of 50 µl. To improve PCR, 0.5 µl of 100X bovine serum albumin was added to the reaction mixture. Temperature cycling was performed in Eppendorf Thermal Cycler®. DNA template was added after the thermal cycler reached to 95^0 C (hot start). Reaction mixtures were held at 30 cycles of amplification at 95^0 C 30s, 55^0 C for 1 min, 72^0 C for 2 min and a final extension of 72^0 C for 5 min. The amplification product was verified on 1% agarose gel followed by ethidium bromide staining and a clear amplification band of 1500 bp was observed from all the samples. The PCR product was purified by

centrifugation with the help of Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions.

The PCR product was ligated into a TOPO-TA pCR2.1 vector (Invitrogen, Carlsbad, CA). After transforming into *E. coli*, the transformants were grown on Luria Bertani (LB) agar plates containing 25 µg/ml kanamycin. A total of 288 different rRNA clones from each soil sample were sequenced using Rolling Circle Amplification of plasmid DNA and unidirectional sequencing with a vector primer (SymBio Corp., CA). The sequences were trimmed at both ends using Sequencher. The trimmed sequences were subjected to BT- BATCHBLAST, a high throughput BLAST search for large sets of small queries. Divisional level affiliation was annotated manually based upon BLASTn analysis with the nr/nt GenBank database.

Rarefaction curves and Diversity indices. Rarefaction curves were constructed for all 16S rRNA gene libraries generated from four different peanut cropping sequences at different distant matrices levels. The trimmed sequences after using Sequencher were further fine trimmed with Chromas Pro Version 1.41 before Clustal X alignment was done. DOTUR (Distance-based OTU and Richness) analysis was performed (34) to construct the rarefaction curves. Distance matrices generated from Clustal X were directly used as input files for DOTUR analysis. Sequence similarities of 97% (0.03 distant matrices) and 80% (0.20 distance matrices) were considered to distinguish the bacterial communities at species and divisional levels while constructing rarefaction curves (22, 32).

The Shannon-Weaver diversity index (H) for relative abundance of 16S rRNA at the divisional level was calculated based on

$$H = -\sum p_i \ ln \ (pi)$$

and the Simpson Index (1- *D*) was also calculated by

$$1\text{-}D = 1\text{-} \sum p_i^{\ 2}$$

Where p_i = the relative abundance of each group of organisms.

Statistical analysis. Correlations were calculated among the abundance of bacterial groups using SAS 9.1.3 (SAS Institute, Cary, NC) version statistical package.

RESULTS

Bacterial ARISA profiles. The ARISA profiles indicated significant similarities among community profiles of replicated plots sampled from same crop rotation (Fig 1). Some common bands were observed among all crop rotations irrespective of time of sampling and type of cropping sequence. In general, similarities within a range of 60-80% were observed among the plots of different cropping sequences from 2006 to 2007. ARISA banding patterns from continuous peanuts cropping system (P-P-P-P) indicated that there was approximately 95% similarity in the plots that were sampled in October 2007. However, approximately 89% similarity was observed among the plots that were sampled during August and October 2006 and also in August 2007. In case of continuous bahiagrass (B-B-B-B-P) similarities of 88% were observed in plots that were sampled during August 2007. The banding pattern of the same rotation plots showed a marginal increase in similarity levels when sampled during October 2007 (90% similarity). In the peanut-cotton rotation (Ct-P-Ct-P), for plots sampled in August 2006, the similarities were 92% when plots were sampled in August, and plots

sampled in October 2006 had a similarity of 89%. The peanut-corn (P-Cn-Ct-P) rotation plots sampled in 2006 August clustered at 85% similarity but showed 93% similarity when sampled during October 2007.

Multi-dimensional Scaling Analysis (MDSA) of ARISA community profiles of different cropping sequences revealed that bacterial communities pertaining to each cropping system are located at generally different clusters with a few outliers indicating the impact of cropping system on soil bacterial diversity (Fig. 2a). However, greater similarities were observed according to sampling time than to cropping sequences in practice (Fig 2b).

Relative abundance of bacterial groups through 16S rRNA gene survey. BLASTn analysis of 16S rRNA gene sequences of 288 colonies from each library were sequenced and approximately 240 sequences from each sample were found to have sequence similarities that placed them into known bacterial divisions. The remainder of the sequences did not have a significant match with the cultured bacterial species (unknown or unculturable bacteria). Even though the sequences were allocated to many more divisions, five were found to be predominant among all the peanut cropping sequences and these were: Proteobacteria, Acidobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Table 1).

Among the bacterial divisions that were analyzed by the 16S rRNA gene library, the predominant gram-negative bacterial populations belong to the division Proteobacteria (32%) followed by Acidobacteria (23.3%) and Bacteroidetes (7.7%) (Table 1). Other gram-negative bacterial divisions, namely Planctomycetes, Chloroflexi, Gemmatimonadetes, Nitrospirae, and Verrucomicrobia were found in the

range of 1.5 to 3.2% of the total clones sequenced. Among the gram-positive bacterial divisions that were detected, Firmicutes (low G+C ratio) and Actinomycetes (high G+C ratio) account for 13.8 and 7.5% of total clones, respectively. There was no significant difference (p>0.05) among crop rotations with respect to the rRNA abundance in bacterial divisions. However, correlation analysis indicated that Proteobacteria populations were negatively correlated with Firmicutes populations (r = -0.65 and p=0.006) and positively correlated with Gemmatimonadetes (r = 0.64 and p=0.007). A similar negative correlation existed between Actinomycetes and Verrucomicrobia (r = -0.51 and p=0.04).

Diversity indices and Rarefaction curves. Among the cropping systems, diversity was slightly greater from rotation involving bahiagrass followed by peanuts (B-B-B-B-P) as indicated by highest H value (5.42±0.33) over other cropping systems (Table 2). The remaining cropping systems are also rich in microbial diversity with high H values (H>5.2). Richness and diversity of all these peanut soils is also indicated using Simpson's diversity index which falls near zero (1-D ~0.24) and is an indication of a more heterogeneous nature with respect to microbial communities (Table 2).

The rarefaction curves were constructed by comparing 16S rRNA sequences to determine the distances between known and unknown sequences. These curves arbitrarily compare the bacterial diversity with 3% assumed to differentiate to the species level, 5% to the genus level, 15% and 20% to the divisional level. It is evident that at 0.15 and 0.20 level, the curves have started to level off, indicating that the sampling is sufficient for determining the bacterial diversity at divisional levels for all the cropping systems under study (Fig 5a-5d). However, the curves at 0.03 and 0.05 level have a steep slope for all the cropping sequences thus indicating that sampling

was inadequate for assessing the bacterial diversity to species and genus level, respectively.

DISCUSSION

The combined use of high resolution, culture independent techniques like Automated Ribosomal Intergenic Spacer Analysis (ARISA) and 16S rRNA gene cloning and sequencing was applied to study the impact of long term crop rotations on the diversity and richness of resident soil bacterial communities. ARISA profiles of bacterial populations in this study revealed more than 70% dissimilarities among cropping sequences. However, greater similarities in bacterial profiles (approximately 90%) among replicated plots of same cropping sequences were observed even though these replications in the field studies are located 150 m apart. These similarities among replicated plots of the same cropping sequence and dissimilarities among different cropping sequences are attributed to the diversity of crops grown in each cropping sequence along with peanuts. The interaction of different plant species have been shown to influence the bacterial diversity that exists in soil especially in the rhizosphere (27). The bacterial community structure in the rhizospheres of chickpea, rape and sudangrass is a result of complex interaction between the soil type, plant species and root zone location of these crops as determined through PCR-DGGE (Denaturing Gradient Gel Electrophoresis) (27). Plant type had relatively greater impact over soil type and plant age in determining the soil microbial community structure in clover, bean and alfalfa (36). However, contradictory reports on the impact of plant and soil types on bacterial diversity have been documented in the past.

For example plant and soil effects are masked by the strong influence of other important practices that are followed in agricultural soils where bacterial community structure is mostly dictated by the agronomic practices in cultivated fields, abandoned fields from cultivation and fields with no history of cultivation (7, 8).

Multi-dimensional scaling analysis of ARISA profiles indicated diversity of bacterial communities in soils of different cropping sequences. Close clusters of communities were observed with respect to sampling time and differed due to the cropping sequence. Since planting time denotes the age of crop in cultivation in a particular field, diversity in the present study with respect to sampling time is attributed to the crop age as well as the weather at that particular time. Since chemical fertilizer applications contribute to nutrient availability to crops due to their influence of nutrient status of field soils, the population levels of bacterial communities could be undergoing a dynamic shift (29). In our findings, sampling time had more impact on the clustering of community profiles and this change is attributed to the influence of soil environment factors such as soil moisture, soil temperature, soil nutrient status and plant age at that particular point of time during a cropping season.

The results of cloning and sequencing revealed that the relative abundance of bacterial divisions based on 16S rRNA sequences is not significantly different among peanut cropping sequences. However, in all rotations there was an abundance of Proteobacteria, Acidobacteria, Firmicutes, Bacteroidetes, and Actinomycetes over other bacterial divisions. This is in agreement with the earlier studies on soil agro-ecosystems (7, 8). The important bacterial genera that fall in these divisions include *Pseudomonas*, *Rhizobium* (Proteobacteria) and *Bacillus* spp. (Firmicutes). Both *Pseudomonas* and *Bacillus* spp. include major plant growth promoting rhizobacteria

(PGPR) that contribute may suppress many soil borne diseases in crops (24, 25, 35). Interestingly, correlations that existed between the abundance of certain bacterial communities seem to be of more importance than the presence of various bacterial divisions. The relative abundance of Proteobacteria and Firmicutes in all the cropping sequences had a significant negative correlation. Similarly, the population levels of Actinomycetes and Verrucomicrobia were negatively correlated. However, the populations of Proteobacteria had significant positive correlation with Gemmatimonadetes. Interactions between rhizosphere bacteria and other microbes is a dynamic phenomenon in agricultural field soils, and such interactions can ultimately have a significant impact on crop health through alterations that are brought about in the population levels of soil borne plant pathogens (1, 4, 5, 19). For example, the antagonistic effect of the Firmicute bacterium, *Pasteuria penetrans* on root knot nematode could alter plant parasitic nematode populations (10, 33). In a long term rotation study, the effect of cropping sequences might be masked by the dominant effect of management practices which includes structural changes and depletion of soil nutrients (14).

The four peanut cropping sequences selected for this investigation are under the influence of intensive agronomic practices. Continuous monitoring of these bacterial communities at the divisional level may help in determining the microbial interactions in peanut soils under different cropping sequences. These 16S rRNA gene clone libraries provide the ability to critically examine the relative abundance of bacterial populations in different peanut cropping sequences. However, construction of these gene libraries is still expensive particularly with a larger sample size (3).

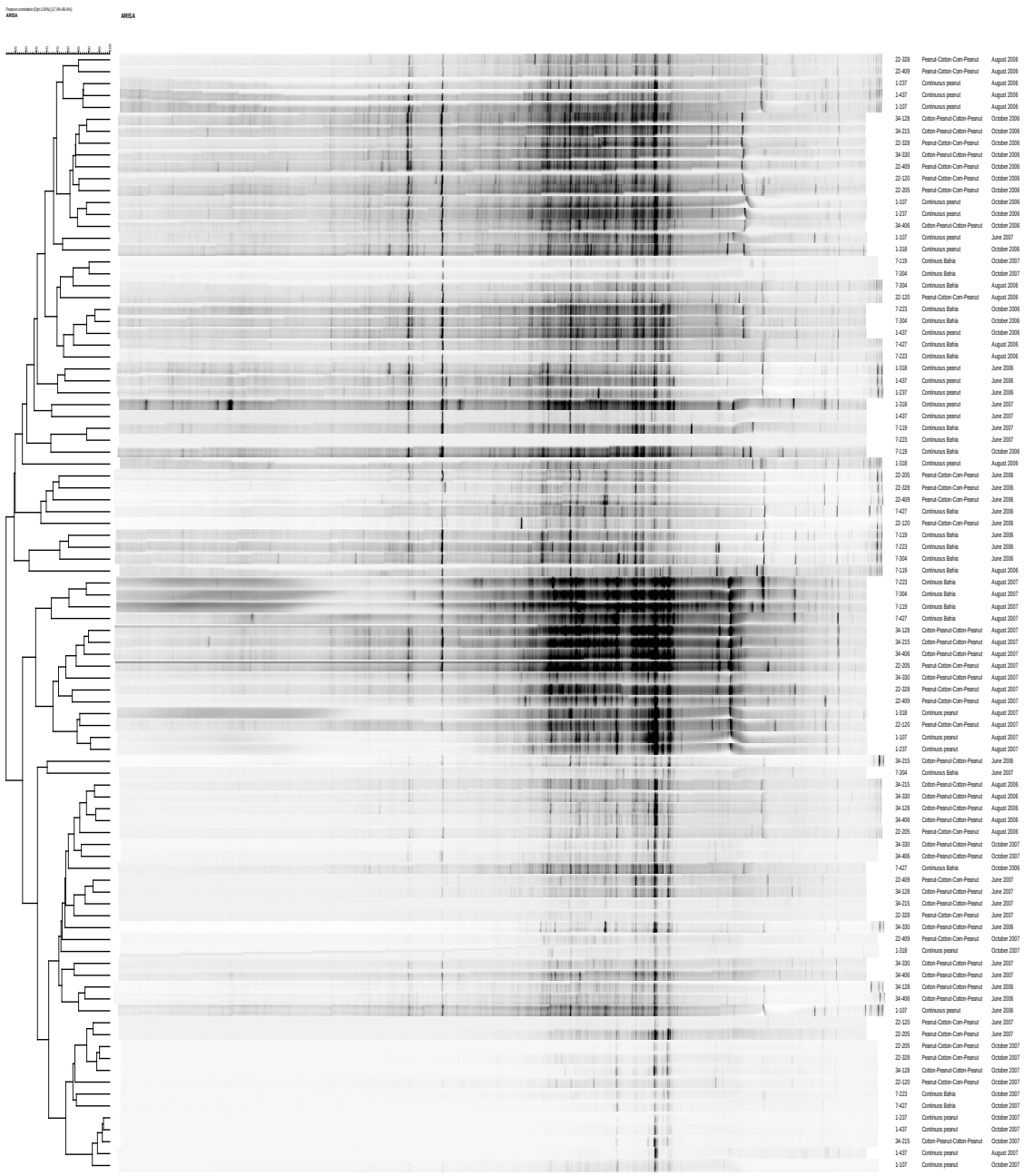
The bacterial diversity in all cropping sequences as indicated by Shannon-Weaver (H) and Simpson indices (1-D) revealed high diversity and heterogenous nature of resident soil bacterial communities of different peanut ecosystems. While the Simpson Index represented heterogeneity among these cropping sequences, the Shannon-Weaver index projected no significant differences with respect to bacterial communities' diversity among the four cropping sequences under study. However, continuous bahiagrass rotation followed by peanuts had slightly higher heterogeneity over other cropping sequences thus indicating the role of bahiagrass in increasing the soil microbial diversity. Bahiagrass when used as a long term rotation has the ability to improve soil microbial community profiles and may reduce the impact of soil borne diseases in peanut among different rotations practiced with corn and cotton (20).

Rarefaction curves for all the cropping sequences denoted the inadequacy of sampling to determine bacterial diversity and richness to their species and genus levels as indicated by the steep slope at 3% and 5% distant matrices respectively. Soil bacterial diversity at the species level could not be determined completely in the present study with few thousand 16S rRNA sequences. The reason could be the richness of bacterial species in soil environment that is approximated to be about 10^7 to 10^9 per every gram of soil (12, 15). On the other hand, bacterial diversity at the divisional level, as indicated by the rarefaction curves that started leveling off at 15% and 20% distant matrices depicted the adequacy of present sampling procedures in determining bacterial divisions in peanut soils. Divisional level determination of bacterial communities is an initial step towards monitoring the functionally important organisms. Our results indicated that these rarefaction curves on the whole, give a general opinion that different cropping rotations would contribute to a more

diversified bacterial community in peanut soils as explained by the steep slope obtained at 0.03 and 0.05 levels.

Further sampling refinement and intensity are needed to better understand the bacterial diversity in these soils. Effective plant disease management practices may be devised only through constant monitoring and understanding of extant bacterial populations through precise and high resolution DNA fingerprinting techniques that can detect the microbial shifts at divisional, genus and species level. Based on the community profiles we obtained through ARISA, cropping sequences may be determined to ameliorate the soil microbial community structures in favor of beneficial bacteria in peanut soils for successful management of soilborne pathogens in peanuts.

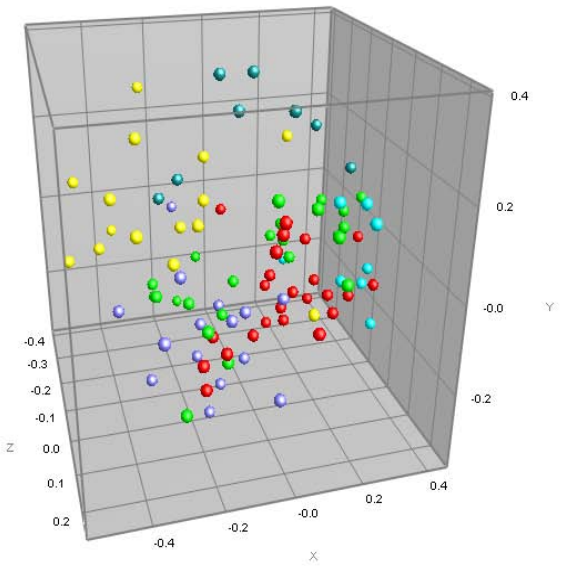
Fig. 1. Bacterial-ARISA patterns obtained from different peanut cropping sequences.



Note: The scale represents % of similarity calculated by the Pearson's product correlation. The dendrogram was constructed by using unweighted pair-group method with arithmetic mean (UPGMA).

Fig. 2. Multi-dimensional Scaling Analysis of soil bacterial communities:

a) Colored by rotation



b) Colored by sampling time

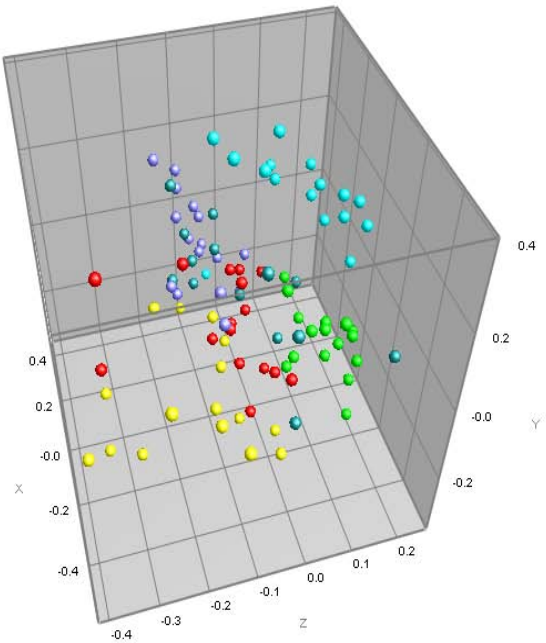


Fig. 3. Dendrogram constructed based on bacterial-ARISA similarity matrix from different peanut cropping sequences by sampling time.

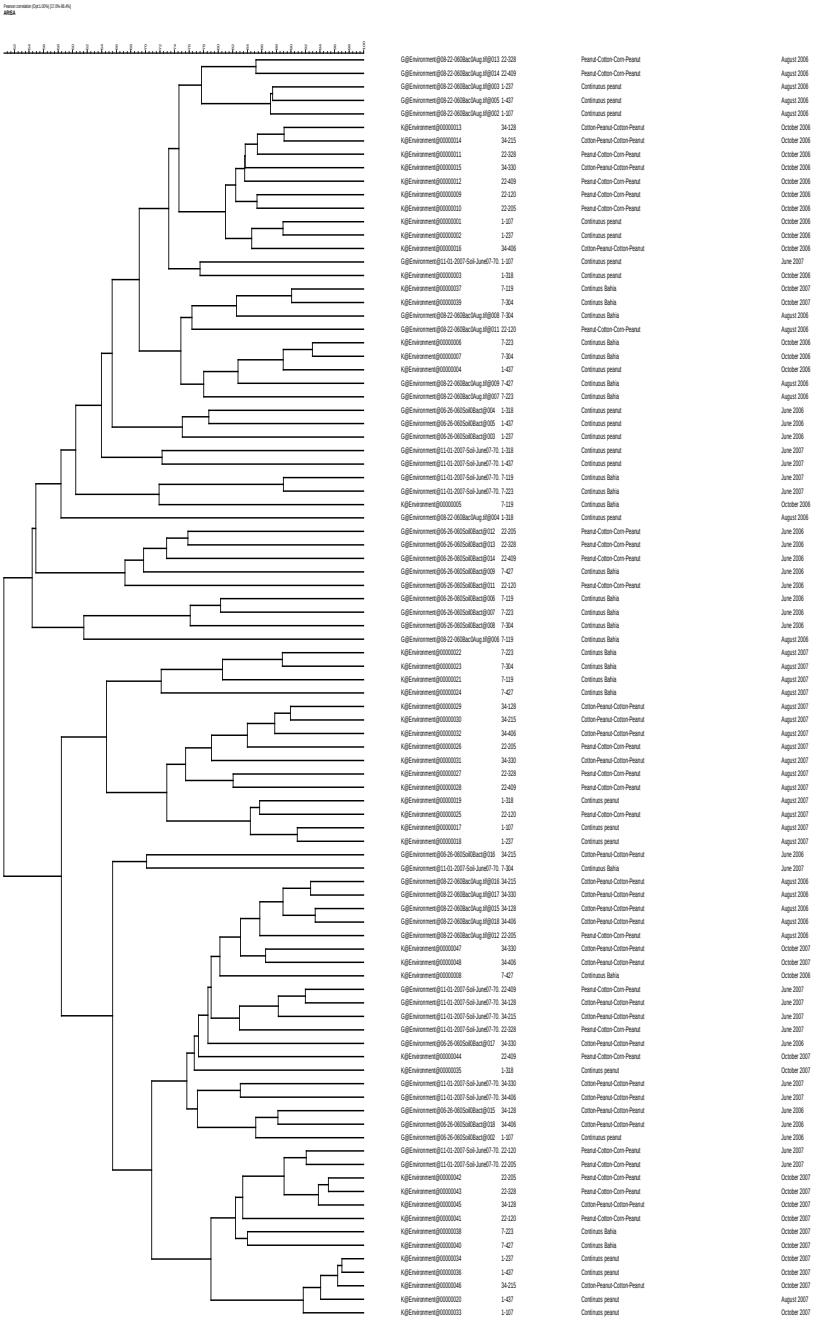


Fig. 4. Dendrogram constructed based on bacterial-ARISA similarity matrix from different crop rotations.

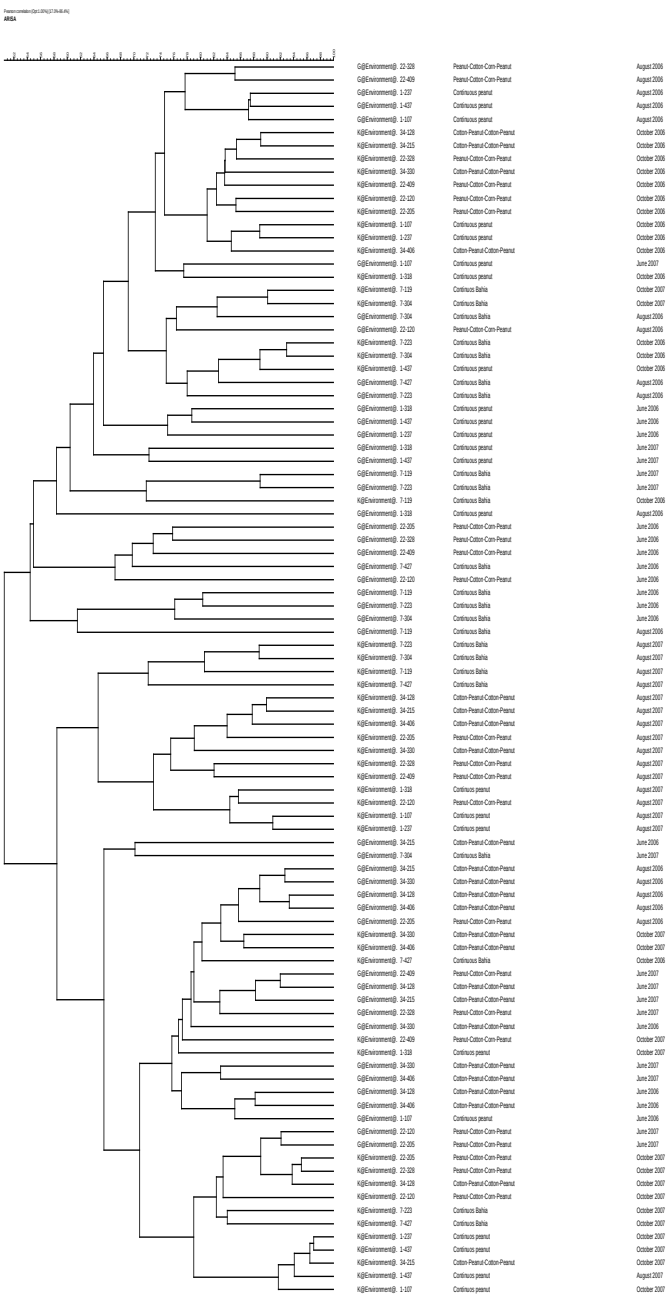
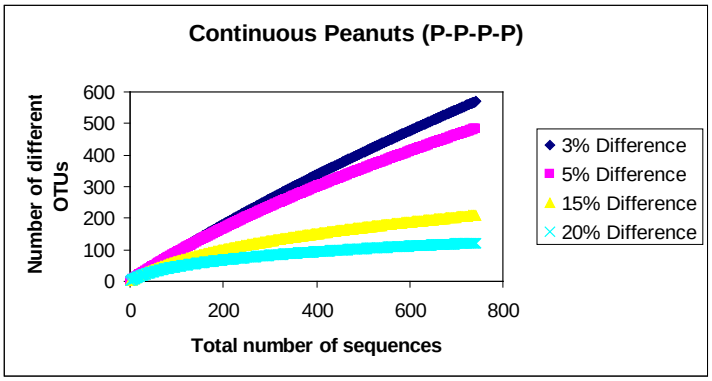
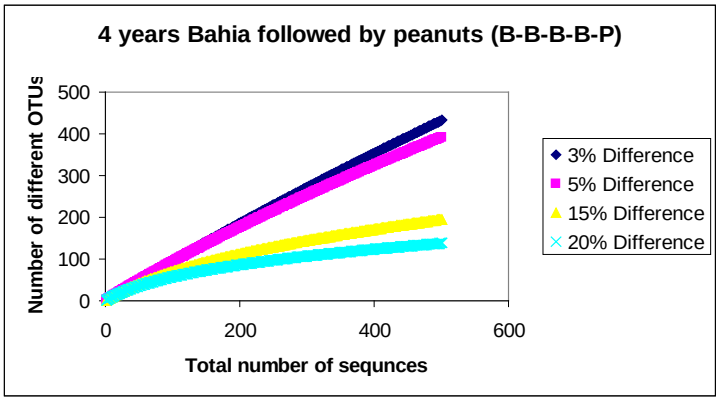


Fig. 5. Rarefaction curves constructed with the sequences generated from 16S rRNA libraries of different peanut cropping sequences at various distances.

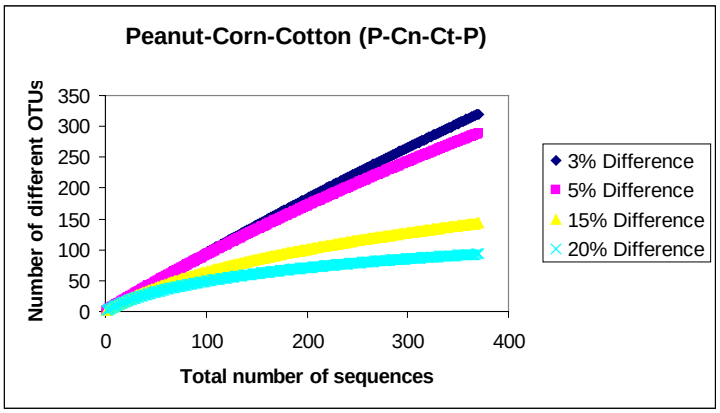
a)



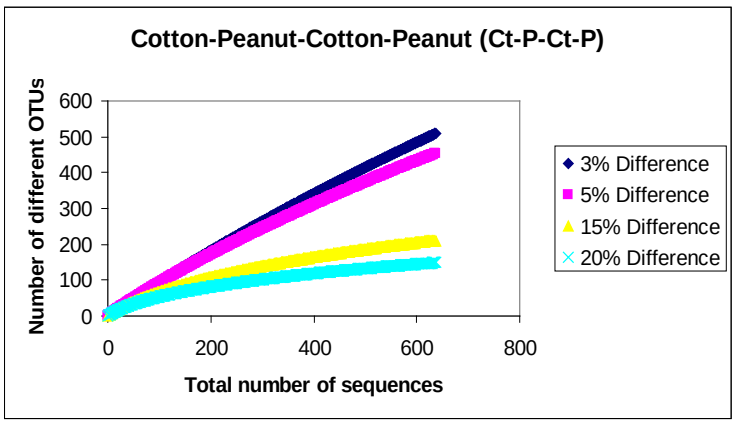
b)



c)



d)



Note: The distance in each case represent the maximum allowable difference for DOTUR for considering a group of sequences to be in the same OTU (Operational Taxonomic Unit)

Table 1. Bacterial composition in different peanut cropping systems as determined by 16S rRNA library sequencing

Bacterial Division	Total (%)	Continuous Peanuts	Continuous Bahiagrass	Peanut-Cotton	Peanut-Corn
Proteobacteria	32.0	75	75	70	77
Acidobacteria	23.3	65	50	49	59
Firmicutes	13.8	29	29	38	36
Bacteroidetes	7.7	15	21	19	19
Actinobacteria	7.5	18	20	17	17
Planctomycetes	3.2	7	9	7	8
Chloroflexi	3.0	7	6	8	8
Gemmatimonadetes	2.4	8	5	5	5
Nitrospirae	1.8	6	1	6	5
Verrucomicrobia	1.5	6	3	2	4
Others	4.1	12	7	9	12

Values represent sequenced clones that correspond to a particular bacterial division

Table 2. Values for ecological diversity indices of Shannon-Weaver (H) and Simpson (1-D) obtained using 16S rRNA gene relative abundance in different peanut cropping sequences.

	Diversity Index Value (Avg ± SD) for each cropping sequence*			
Diversity Index	**P-P-P-P**	**B-B-B-B-P**	**Ct-P-Ct-P**	**P-Cn-Ct-P**
H	5.24±0.19	5.42±0.33	5.27±0.11	5.20±0.24
1-D	0.24±0.01	0.23±0.02	0.23±0.01	0.23±0.01

- Data were calculated from 4 replicated plots of each cropping sequence.

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III. COMPARISON OF SOIL FUNGAL COMMUNITY STRUCTURE IN DIFFERENT PEANUT ROTATION SEQUENCES USING ARISA IN RELATION TO AFLATOXIN PRODUCING FUNGI

ABSTRACT

The present study focuses on determining soil fungal community structure in different peanut cropping sequences by using a high resolution DNA fingerprinting technique, Automated Ribosomal Intergenic Spacer Analysis (ARISA). The objective of the study is to develop fungal community profiles in four peanut cropping sequences (continuous peanuts, continuous bahiagrass, peanut-corn and peanut-cotton rotations) with an emphasis on the aflatoxin-producing fungi, the *Aspergillus flavus* group. Fungal profiles were developed based on different times of sampling soil and from the four cropping sequences. Results indicated 75% similarities among fungal communities from the same cropping sequences as well as with similar times of sampling. PCR-based detection of *A. flavus* directly from these soils was carried out using *A. flavus* specific primers (FLA1 and FLA2) and also through quantitative estimation on *Aspergillus flavus* and *A. parasiticus* agar medium (AFPA). Though the population levels of *A. flavus* in soil samples ranged from zero to 1.2 x 10^3 CFU g^{-1} soil (based on culturable methods), the fungus was not detected with *A. flavus* specific primers. The minimum threshold limit at which these aflatoxin-producing fungi could be detected from the total soil genomic DNA was determined through artificial inoculation of samples with 10-fold increase concentrations. The results indicated

that a minimum population density of 2.6 x 10^6 CFU g^{-1} soil is required. These results are useful in further determining the relative population levels of these fungi in peanut soils with other soil fungi.

INTRODUCTION

The relative abundance of soilborne fungi in a given soil microbial community can play a defining role in economic losses to the farming community and thus trade (6, 8). Determining soil microbial community composition in peanut soils might be an important step in understanding not only disease complexes but in the management of aflatoxigenic fungi in the *Aspergillus flavus* group. Peanut is an important cash crop in the southeastern US and is affected by many soilborne diseases as well as aflatoxin contamination. Aflatoxins, produced by *A. flavus* group fungi, are known to be carcinogenic, hepatotoxic and teratogenic (25). In peanut production, resident soil fungal communities vary in location and abundance (10, 19, 24). The relative abundance of these soilborne pathogens is subject to change with different cropping histories (4). Peanut in the southeastern US is usually grown as continuous crop or in rotations. These rotation sequences affect the relative abundance of *A. flavus* group of fungi in peanut soils also (1). The particular cropping sequence may result either in increased relative soilborne pathogen abundance or may lessen its population level in the soil (20). However, short term manipulation of plant community composition through cropping sequences may not have a significant impact on soil microbial diversity or to what extent in time and space needed to expect the changes is still under study (11).

Recently, soil microbial community structure has been characterized using culture independent techniques. These culture independent techniques include DNA fingerprinting techniques like DGGE (Denaturing Gradient Gel Electrophoresis), T-RFLP (Terminal Restriction Fragment Length Polymorphism), LH-PCR (Length Heterogeneity PCR), ARISA (Automated Ribosomal Intergenic Spacer Analysis), cloning and sequencing in addition to conventional microbial estimation through quantitative methods (9, 21, 26). These techniques provide rapid, lucid, reliable and highly reproducible results for determining soil fungal community structures. Of these molecular techniques, ARISA provides greater resolution and analysis of soil microbial diversity over other techniques (9). Since soils are characterized by greater microbial diversity than aquatic and other ecosystems, the ARISA can compare and differentiate these microbial communities at a finer scale. Use of ARISA in determining fungal diversity in soils exploits the length polymorphism of the nuclear ribosomal DNA (rDNA) region that contains two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2) (23). A better insight into these community structures with an efficient molecular technique like ARISA with emphasis on aflatoxin-producing fungi may provide a better understanding and subsequent management approach to the aflatoxin problem.

In this study, comparisons of the fungal community from cropping sequences of peanuts (P-P-P-P), continuous bahiagrass followed by peanut (B-B-B-B-P), peanut-corn (P-Cn-Ct-P) and peanut-cotton (P-Ct-P-Ct) were examined to determine the fungal complexes as well as density of *A. flavus* aflatoxin producing fungi. Detection of the *A. flavus* population load in soils was carried out using *A. flavus* specific primers and through quantitative estimation on AFPA (*Aspergillus flavus* and

parasiticus agar) medium (22). Quantification of minimum population loads of aflatoxin-producing fungi in soils, to determine levels needed for analysis of soil genomic DNA through molecular methods, was conducted.

MATERIALS AND METHODS

Field site history and description. The experimental site was located at Wiregrass Research and Extension Center (WGREC), Headland, Alabama. The site was established in 1988 with an objective of managing peanut diseases such as early leaf spot, late leaf spot, southern stem rot and peanut root knot diseases (15). Studies on peanut cropping sequences have been conducted at this site with a total of 34 cropping sequences of which the selected ones are: continuous peanuts (P-P-P-P), continuous bahiagrass followed by peanut (B-B-B-B-P), peanut-cotton (Ct-P-Ct-P) and peanut-corn (P-Cn-Ct-P) (Table 1). These cropping sequences were selected for the present study. The soil is Dothan fine sandy-loam (fine-loamy, siliceous, thermic Plinthic Palendults) type (<1% Organic Matter). The crops were supplemented with side-roll irrigation system on an as needed basis.

Soil sampling. For each sample, five soil cores were collected from the pegging zone (up to 10 cm depth) during the years 2006, 2007 and 2008 from each plot and these were bulked and mixed for a representative sample. Samples were collected 3 times: early-season (June), mid-season (August) and at harvest (October). The samples were processed within 24 h for total soil genomic DNA extraction and preserved genomic material at -20^0 C for further downstream applications. The samples collected at the time of harvest were subjected to enumeration of *A. flavus* group populations as well

as determining the minimum threshold population limit of *A. flavus* group of fungi at which they are detectable in soils.

DNA extraction and quantification. Total genomic DNA was extracted from 10 grams of each soil sample using the Power Mac Soil kit from MoBio (MoBio Laboratories Inc. Carlsbad, CA) following manufacturer's instructions. Extracted DNA quality and quantity was assessed by Nano Drop Spectrophotometer (Thermo Scientific, USA). DNA dilutions were carried out to 5 ng μl^{-1} and stored at -80^0 C for further downstream applications.

Fungal Community Fingerprinting by ARISA (Automated Ribosomal Intergenic Spacer Analysis). The length heterogeneity of the ITS1-5.8S-ITS2 region was exploited to characterize the fungal community. The primers used to amplify this region represent consensus sequences found at the 3' end of the 18S genes in fungi (Primer 2234C, 5'-GTTTCCGTAGGTGAACCTGC-3') and at the 5' end of the 28S genes (Primer 3126T, 5'-ATATGCTTAAGTTCAGCGGGT-3') (23). The reverse primer was labeled with IRD700 fluorochrome from LI-COR (Lincoln, NE, USA) at 5' end. Reaction mixtures (50µl) for PCR contain 5 µl of 10X dilution buffer (20 mM Tris-Hcl, pH 7.5, 100mM KCL, 15mM $MgCl_2$, 1mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween 20 (vol/vol), 0.5% Nonidet P40 (vol/vol), 50% glycerol (vol/vol)), 2 µl of 10 mM dNTPs, 2 µl of 10 µM Primer 2234C, 1 µl of 10 µM Primer 3126T, 10 µl of 1 µM 2234C primer labeled with IRD700 fluorochrome from LI-COR (Lincoln, NE, USA) at 5' end, 0.2 µl of Taq polymerase (Promega, USA), 10 µl of 25 µM $MgCl_2$ and 20 µl of template DNA (100 ng). PCR amplification was carried out in a Peltier Thermal Cycler (PTC-200, MJ Research) after a hot start at 94^0C for 3 min, followed by 30 cycles consisting of 94^0C for 45s, 55^0C for 1 min, 68^0C for 2

min, and final extension for 7 min at 68^0C. Polyacrylamide gel was then prepared by mixing 30 ml of 5.5% LI-COR poly-acrylamide (KB plus-LI-COR), 200 µl of 10% ammonium persulphate (APS) and 20 µl of N, N, N' N'-tetramethylethylenediamine (TEMED). The PCR product (5 µl) was then transferred to new tubes and to this 2.5 µl of stop buffer (LI-COR, Blue Stop Solution) was added. The contents were thoroughly mixed and denatured at 98^0C for 5 min before loading onto the polyacrylamide gel. The denatured PCR products (0.6-0.8 µl) were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) under denaturing conditions for 8 hr at 1,500 V following manufacturer's instructions.

The ARISA gel images were analyzed by the BIONUMERICS V. 5.0 software program. Levels of similarity between fingerprints were calculated with the Pearson product-moment correlation coefficient (*r*) after the banding patterns were subjected to conversion, normalization, and background subtraction with mathematical algorithms. Multi-dimensional Scaling Analysis (MDSA) tool in BioNumerics software was used to evaluate the similarities/dissimilarities between soil fungal communities. Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA) to determine the relatedness among the fungal communities of different peanut cropping sequences (3).

Enumeration and determination of minimum threshold population limit of *A. flavus* fungi in soils. Initially the soil population levels of *A. flavus* and *A. parasiticus* were enumerated on *Aspergillus flavus* and *parasiticus* agar (AFPA) according to Pitt *et al* (22). Soil samples with no traces of *A. flavus* group, as determined by quantitative enumeration on selective media were selected for this experiment. These *A. flavus* free samples were augmented with *A. flavus* spore suspensions at different

concentrations ranging from 2 X 10^4 to 2 X 10^9 CFU ml^{-1}. Ten grams of soil was weighed for each replicate of each of the six spore concentrations; 1 ml spore suspension was added to each for *A. flavus* concentrations ranging from 2 X 10^3 to 2 X 10^8 CFU g^{-1}. The total genomic DNA was later extracted from these samples using the Power Mac Soil kit from MoBio (MoBio Laboratories Inc. Carlsbad, CA) following manufacturer's instructions. DNA was purified using the protocol suggested by Liles (personal communication) for the determination of minimum threshold level for *A. flavus* detection directly from the soil.

PCR-based detection for *A. flavus* in soil. The purified total soil genomic DNA was PCR amplified using the *A. flavus* specific primers FLA1 (5'-GTAGGGTTCCTAGCGAGCC-3') and FLA2 (5'-GGAAAAAGATTGATTTGCGTTC-3') (13). The concentration of *A. flavus* spore suspension that was added to the soil at which the fungal pathogen was detectable through PCR using *A. flavus* specific primers was determined sequentially by increasing or decreasing the spore concentration as needed. PCR reactions were performed in the Peltier Thermal Cycler (PTC-200, MJ Research). The PCR amplification protocol for *A. flavus* detection was as follows: 1 cycle of 5 min at 95^0C, followed by 26 cycles of 30s at 95^0C, 30s at 58^0C, 45s at 72^0C and a final extension of 5 min at 72^0C. The PCR products were run on 1% agarose gel electrophoresis and subjected to ethidium bromide staining.

RESULTS

ARISA analysis of fungal communities. The fungal ARISA profiles showed similarities among communities of replicates sampled from the same crop rotation (Fig 1). Common bands were observed among all samples irrespective of sampling time and cropping sequence. In general, similarities within a range of 55-75% were observed among the plots of different cropping sequences from 2006 to 2007. ARISA banding patterns in continuous peanuts (P-P-P-P) indicated that there were approximately 75% similarities in all plots that were sampled in October 2006. In the case of continuous bahiagrass (B-B-B-B-P), 70% similarities were observed in plots that were sampled during June 2006. The banding pattern of the same plots showed a marginal increase when sampled during October 2006 (75% similarity). In the peanut-cotton rotation (Ct-P-Ct-P), for the plots sampled in August 2007, about 80% similarities were noticed whereas the plots sampled in August and October 2006, had about 75%. On the other hand, peanut-corn (P-Cn-Ct-P) rotation plots sampled in 2006 August clustered at 85% similarity and it was about 80% when sampled during August 2007.

Multi-Dimensional Scaling Analysis (MDSA) of ARISA community profiles of different cropping sequences revealed that fungal communities pertaining to each cropping sequence had fewer similarities in general with greater scattering indicating the impact of cropping sequence on soil fungal diversity is minimal (Fig. 2a). Also, fewer similarities were observed with respect to fungal composition in the plots that were sampled during identical time periods irrespective of the cropping sequences in practice (Fig 2b).

Enumeration and PCR-based detection of soil *A. flavus*. Results on soil *A. flavus* populations as determined at the time of harvest from each cropping sequence indicated a marginal increase in population levels of *A. flavus* within cropping sequences of continuous peanuts and in peanut-cotton rotation. The population levels in these cropping sequences ranged from 1 x 10^3 CFU g^{-1} to 1.2 x 10^3 CFU g^{-1} soil through 2006 to 2008. On the other hand, the populations remained constant in the peanut-corn rotation through 2006 to 2008. A substantial decline in soil *A. flavus* populations was observed in continuous bahiagrass cropping sequence from 2006 to 2008 (0.7 x 10^3 to 0.2 x 10^3 CFU g^{-1} soil) (Table 1).

Comparisons on mean *A. flavus* population levels across treatments over three years indicated that among the four cropping sequences under study, *A. flavus* populations were significantly less in soils of continuous bahiagrass sequence (0.2 x 10^3 CFU g^{-1} soil) compared to other treatments. The population levels in continuous peanut, peanut-cotton and peanut-corn sequences were 1.2 x 10^3, 1.2 x 10^3 and 1.0 x 10^3 CFU g^{-1} soil, respectively; and no significant differences were observed among these three cropping sequences (Table 2). The minimum threshold limit at which *A. flavus* can be detected in peanut soils directly from soil genomic DNA with *A. flavus* specific primers was found to be 2.6 X 10^6 CFU g^{-1} (Fig. 5).

DISCUSSION

Fungal ARISA profiles with common bands in all the cropping sequences in the present study may be attributed to the presence of common saprophytes in these soils. However, the phylogeny of these saprophytes in these soils could not be determined. The choice of a particular plant species on the selection of resident soil microbial

communities is well documented (11, 12, 14, 28). The influence is through different root exudates into the rhizosphere. For this reason, the microbial diversity of a rhizosphere and non-rhizosphere soil differ greatly (17). In our study, however, sampling time had a more profound effect on grouping of these communities rather than crop rotation influence. One probable explanation for this is that the experimental site is in long-term rotation, and crop management practices such as tillage, irrigation, and soil nutrient application year after year might be masking the effect of plant type on the soil microbial communities (7). Multi-Dimensional Scaling Analysis (MDSA) also revealed that there was no selection of fungal communities based on plant type as this analysis enables grouping of fungal communities based on presence or absence of bands and their intensity.

Overall, our results suggest that though different ARISA groups were detected based on sampling times and cropping sequences with peanut, a specific fungal community associated with these sequences could not be confirmed concretely. To ascertain the phylogeny of these fungal communities in different cropping sequences, 18S rDNA sequencing of fungi from the soil of these cropping sequences is an alternative for better understanding of the fungal communities associated with these crops in peanut eco-system.

Studies on the detection of aflatoxin-producing fungal populations in soils of four cropping sequences revealed that PCR–based methods using *A. flavus* specific primers could not detect these fungi even at a concentration of 10^3 cfu g^{-1} soil. There are several possible reasons for this. The first reason might be due to the presence of high amounts of PCR inhibitors in the soil metagenomic DNA that include humic acids. Separation of humic substances from DNA sample is always a critical phase

since both of these are acid macromolecules. The second could be the DNA extraction method that was used in the present study might affect the yields of DNA pertaining to *A. flavus* and subsequently the obtained DNA might be less than the primer's sensitivity limits. However, other extraction methods that might yield more *A. flavus* DNA can be more specific in identifying these populations from soils. Further studies in these directions can yield positive results in soil identification of these organisms.

Soil enumeration studies indicated that using bahiagrass in rotation with peanuts resulted in significant reduction in *A. flavus* population levels compared to other cropping sequences. Bahiagrass rotations might have contributed to soil organic matter thereby increasing the antagonistic microflora causing significant reductions in *A. flavus* population levels. An increase in soil microbial diversity is another contributing factor with bahiagrass rotation. Earlier studies indicated that bahiagrass rotation can be an alternate disease management strategy to a peanut-cotton rotation against *Cercospora* leaf spot, southern stem rot diseases and peanut root knot nematode infestations in peanut (27). Further, the peanut yields were enhanced in Ct-B-B-P (Cotton-Bahia-Bahia-Peanut) rotation compared to P-Ct-Ct-P (Peanut-Cotton-Cotton-Peanut).

High population levels of *A. flavus* in continuous peanuts, peanut-cotton and peanut-corn rotations could be attributed to continuous availability of host crops that favor multiplication of these fungi and subsequent aflatoxin contamination. In addition, corn and cotton are also prone to aflatoxin contamination (2, 5). The population levels of *A. flavus, A. parasiticus, A. terreus, A. nomius, A. tamari, A. niger,* and *A. fumigatus* are reported to be high among common soil inhabitants of soils with peanut, corn, and cotton cultivation (5, 16). High population levels in continuous

peanut, peanut-corn and peanut-cotton rotations in the present study can be attributed to continuous cultivation of crops that are hosts to this *Aspergillus* group of fungi. On the other hand, bahiagrass rotation was found to reduce significantly the *A. flavus* population levels. Sod-based rotations help to improve soil organic matter and thereby reduce the incidence of soilborne pest and diseases (18). Bahiagrass rotation with peanuts in the present study might have improved the beneficial microflora through addition of soil organic matter and thus reducing the soil resident *A. flavus* population levels.

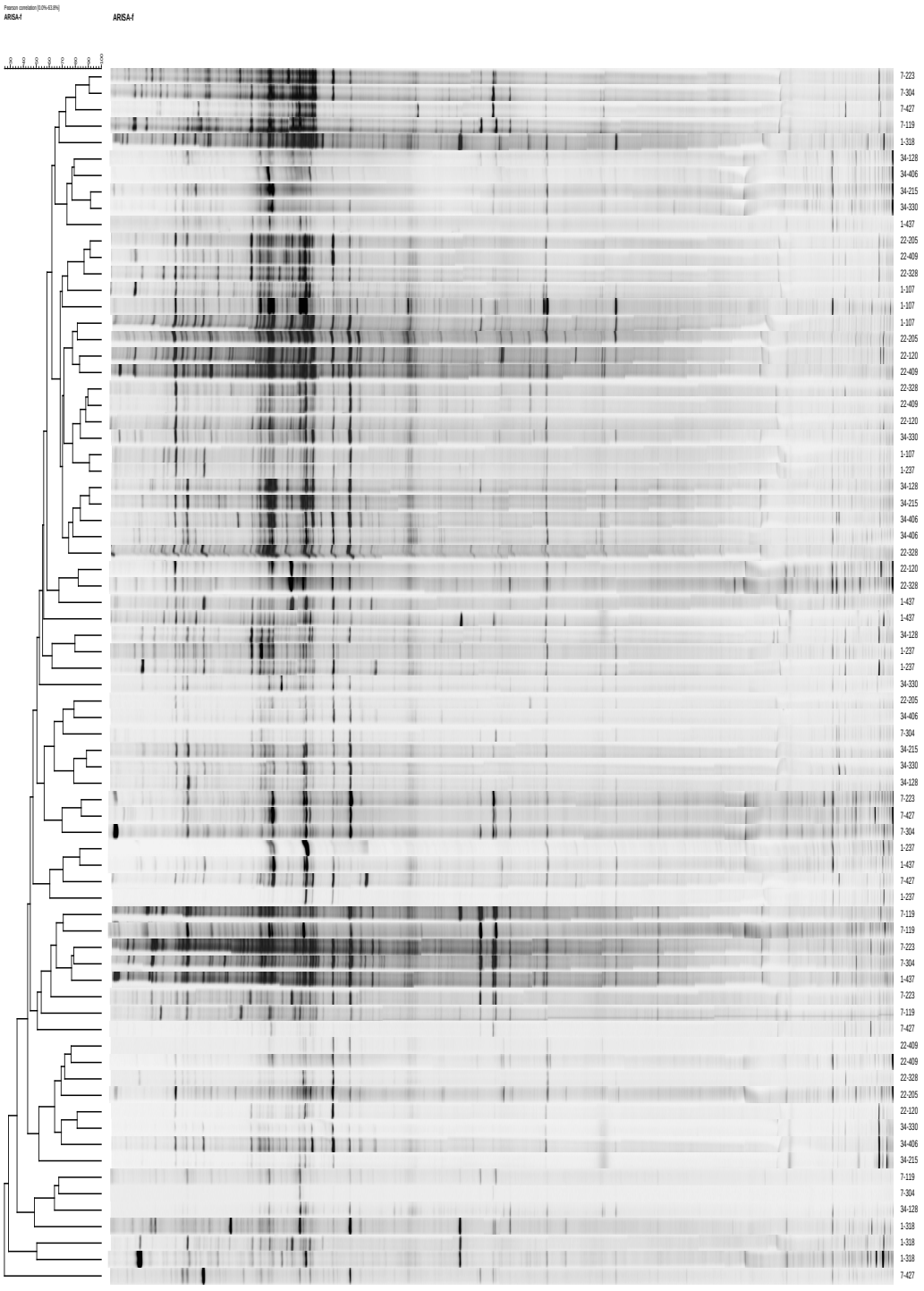
Table 1. Year-wise cropping pattern in different peanut rotations at Wiregrass Research and Extension Center (WGREC)

Crop rotation	2005	2006	2007	2008
Continuous Peanuts (P-P-P-P)	Peanut	Peanut	Peanut	Peanut
Continuous Bahiagrass (B-B-B-B-P)	Bahiagrass	Bahiagrass	Peanut	Bahiagrass
Peanut-Cotton (Ct-P-Ct-P)	Cotton	Peanut	Cotton	Peanut
Peanut-Corn (P-Cn-Ct-P)	Peanut	Corn	Cotton	Peanut

Table 2. Population levels of *Aspergillus flavus* group in soils from different peanut cropping sequences

Rotation	Soil *Aspergillus flavus* population at harvest ($x10^3$ cfu g^{-1} soil)			
	2006	2007	2008	Mean over three years
Continuous peanuts	1.0	1.0	1.2	1.1[a]
Continuous bahiagrass	0.7	0.0	0.2	0.3[b]
Peanut-Cotton	1.0	1.1	1.2	1.1[a]
Peanut-Corn	1.0	0.8	1.0	0.9[a]

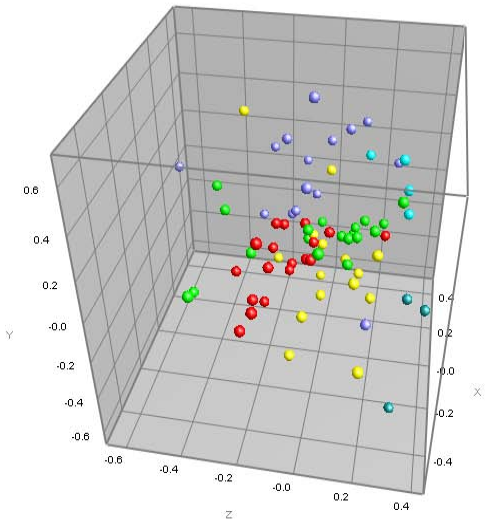
Fig. 1. Fungal ARISA profiles obtained from different peanut cropping sequences.



Note: The scale represents % of similarity calculated by the Pearson's product correlation. The dendrogram was constructed by using unweighted pair-group method with arithmetic mean (UPGMA).

Fig. 2. Multi-dimensional Scaling Analysis of soil fungal communities:

a) colored by sampling time



b) colored by crop rotation

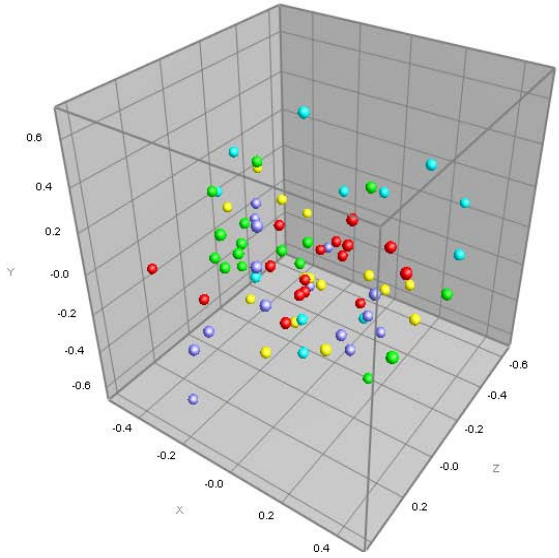


Fig. 3. Dendrogram constructed based on fungal-ARISA similarity matrix from different peanut cropping sequences by sampling time

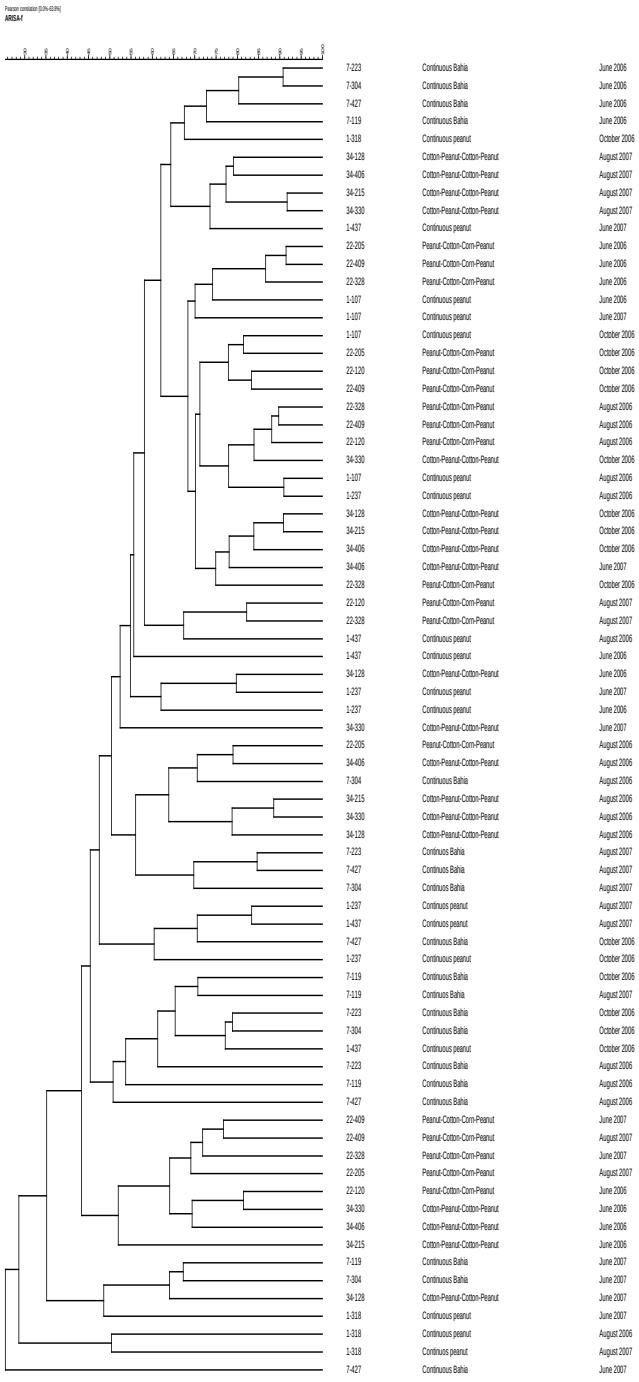


Fig. 4. Dendrogram constructed based on fungal-ARISA similarity matrix from different crop rotations

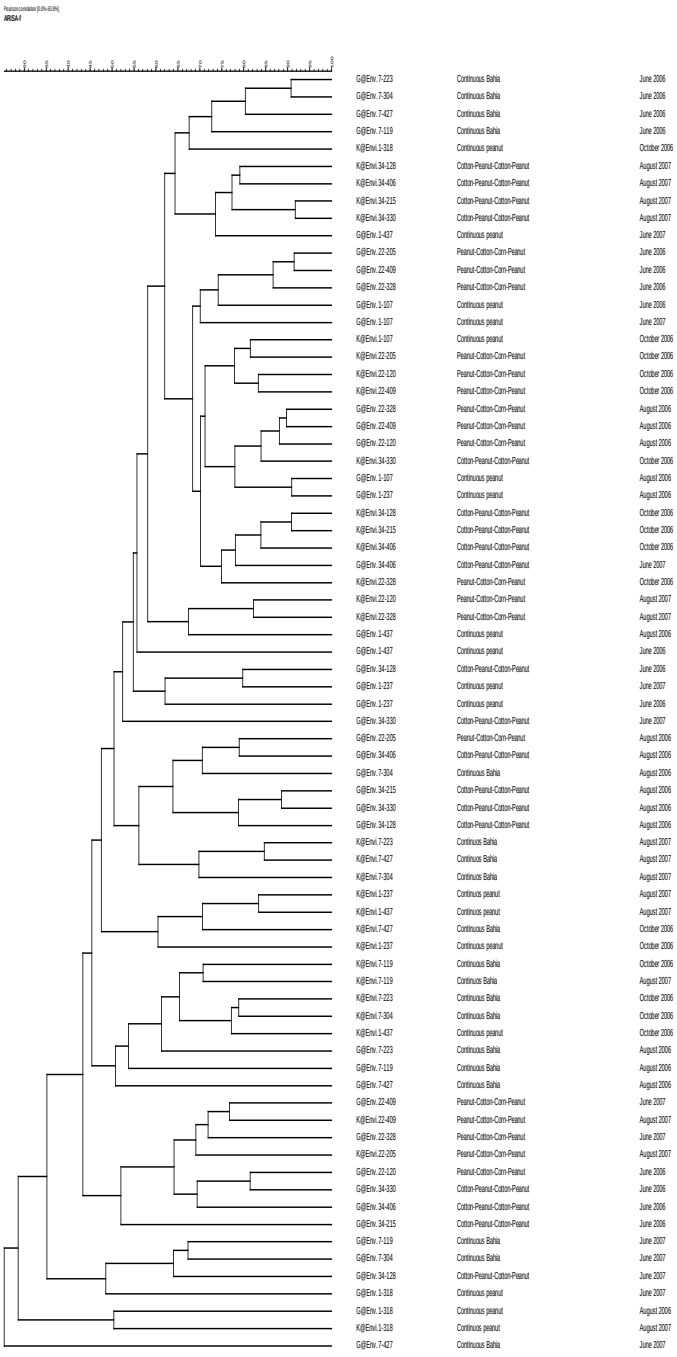
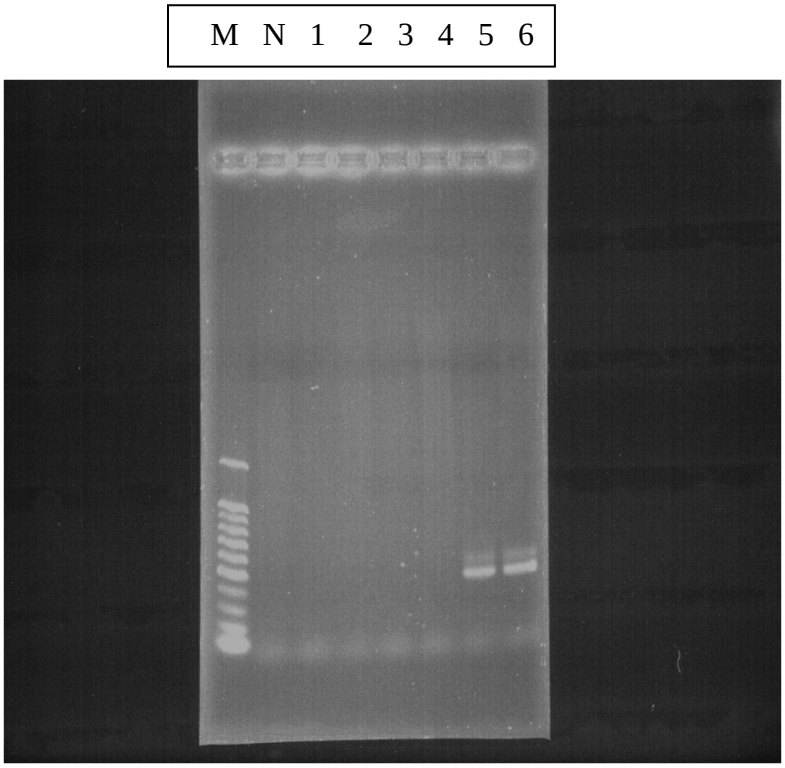


Fig. 5. PCR with *A. flavus* specific primers



M- 100bp marker

N- Negative control & 1-6 lanes are soil samples inoculated with *Aspergillus flavus* spores @ 10^2 - 10^7 cfu/g soil.

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IV. INFLUENCE OF SOIL MICROBIAL COMMUNITIES, CROP ROTATIONS AND OTHER FIELD FACTORS ON AFLATOXIN CONTAMINATION OF PEANUTS

ABSTRACT

Aflatoxin contamination of peanuts is a common problem affecting peanut trade worldwide. The present study is aimed at investigating the role of soil microbial community structure, crop rotations and other field factors on aflatoxin contamination. A long-term peanut rotation experiment involving continuous peanuts, continuous bahiagrass, peanut-cotton and peanut-corn rotation was considered for this study. DNA fingerprints of these soils indicated that higher microbial diversity tends to improve the soil health and thereby substantial reduction in aflatoxin contamination of peanuts. The harvested pods from these plots were categorized into immature, rotten, discolored, nematode damaged, having insect scars and holes, having cracks and for visible *A. flavus* contamination and were further correlated with pod aflatoxin content. Our results indicated that nematode damage and pod discoloration were significantly correlated to total aflatoxin content of pods. Further, nematode damage to pods was significantly correlated to visible fungal contamination and pod discoloration. However, no significant correlations existed between visual contaminations of pods to that of total aflatoxin content. Scars, holes and cracks on pods due to insect pests are the contributing factors to pod aflatoxin contamination.

Results on pod aflatoxin content in different cropping sequences over different years indicated that these toxin levels were greater in 2006 compared to 2007 in continuous peanuts. However, aflatoxin B1 was found to be less in 2006 over 2007. For continuous bahiagrass, the mean aflatoxin content and B1 was found to be 19.7 and 1.9 ppb respectively during 2007 and the toxin content decreased with bahiagrass rotation over peanut monoculture. Conclusively, the toxin levels were comparatively higher in fields with peanut monocropping over fields that were practiced with rotations. Overall, our results indicate the role of different factors in peanut aflatoxin contamination and suggest that practicing bahiagrass rotation in peanut fields contributes peanut crop health with reduced aflatoxin contamination.

INTRODUCTION

Aflatoxin contamination in peanuts is a serious concern because of the potential health problems associated with consumption of aflatoxin contaminated commodities. These toxins are secondary metabolites produced by the *Aspergillus flavus* group of fungi. Aflatoxins are potentially carcinogenic, teratogenic and hepatotoxic in nature (15). These fungi are ubiquitous soil inhabitants and include *A. flavus* and *A. parasiticus*. Pre-harvest aflatoxin contamination usually is a result of fungal invasion into pods during pod formation. Pod invasion by *A. flavus*-type fungi is also influenced by several climatic and edaphic factors such as relative humidity, precipitation, soil temperature and soil calcium content (3, 12). Physical damage of pods by soil insect pests like the lesser cornstalk borer (*Elasmopalpus lignosellus*) and nematodes allow *A. flavus* invasion (2, 8, 16). Current management strategies to

minimize aflatoxin contamination of peanut include timely irrigation, soil amendments with lime and gypsum, use of biological control agents, and agronomic and cultural practices (5, 9). However, aflatoxins continue to occur in food and animal feed usually above acceptable limits as most of the available management options were either inconsistent under field conditions or are inadequate relative to wholly affecting this system.

In addition to peanuts, aflatoxin contamination is also reported on other crops such as cotton, corn, and tree nuts (14). Successful management of aflatoxin contamination in peanut may include devising biocontrol strategies that are compatible with other options in the integrated control. For exploring the specific microbial community that is associated with peanut soils, a thorough understanding of those is essential. A desirable shift in the microbial community structure towards the dense beneficial community might be helpful for managing soil *A. flavus* population levels. Crop rotation with peanut production is one viable option to bring changes in soil microbial communities in order to manage aflatoxin-producing fungi (6). For this, an economically viable rotation strategy with peanut that is deleterious to soilborne peanut pathogens, as well as to *A. flavus*, is desirable.

The present investigation is aimed at studying the soil microbial community structure and diversity among different peanut cropping sequences through a culture independent automated ribosomal intergenic spacer analysis (ARISA). This high resolution DNA fingerprinting technique develops fungal (F-ARISA) as well as bacterial (B-ARISA) profiles for soils. In a broader perspective the specific objective of this study is to observe the role of various interdependent factors such as microbial communities, cropping sequences, and edaphic factors like soil moisture and soil

temperature on the epidemiology of *A. flavus* populations and subsequent aflatoxin contamination.

MATERIALS AND METHODS

Soil sampling. Soil samples were collected at the Wiregrass Research and Extension Center, Headland, AL from a long term rotation study. The rotation sequences used in this study were continuous peanuts, continuous bahiagrass, peanut-cotton and peanut-corn rotation. The experimental plots were arranged in a randomized complete block design with four replications. From each replicated plot, five samples of 250 gm each were collected from the rhizosphere (up to 10 cm depth) and bulked to get a representative sample. Sampling was done during early-planting (June), mid-season (August) and at harvest (October) of 2006, 2007, and 2008 cropping seasons.

DNA extraction. Total genomic DNA was extracted from 10 grams of soil using the Power Mac Soil kit from MoBio Labs (MoBio Laboratories Inc. Carlsbad, CA) following manufacturer's instructions. The DNA was extracted from each of the samples within 24 hours after sampling. The quality and quantity of extracted DNA was checked by the NanoDrop Spectrophotometer (Thermo Scientific, USA). DNA stock solutions of 5 ng/ µl were prepared and stored at -80^0 C for downstream application.

ARISA fingerprinting. The bacterial ribosomal intergenic spacers were amplified using the primers ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (4). For fungal community fingerprinting, the length

heterogeneity of the ITS1-5.8S-ITS2 region was exploited. The primers used to amplify this region were 2234C (5'-GTTTCCGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3') (11). Amplified products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions. ARISA images were processed with BioNumerics (Applied Maths, Austin, TX). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints was calculated with the Pearson product-moment correlation coefficient (r). Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA) (1)

Physical evaluation of pods and aflatoxin estimation. Harvested pods were bulked and later sorted into different categories by visual examination for each replicated plot. A total of 150 pods were visually evaluated from each sample as being small/immature, rotten, discolored, nematode damaged, having insect scars and holes, having cracks and having visible *A. flavus*. Aflatoxin estimation for pod samples was carried out using Mycosep Multifunctional Cleanup Columns (Romer Labs, Inc., Washington, MO) in HPLC according to Wilson and Romer (17).

Statistical analysis. Data were analyzed using SAS 9.1.3 version statistical software (SAS Institute, Cary, NC). As aflatoxins were not detected from any sample in 2008, these were not included in data comparisons.

RESULTS

Physical evaluation of pods and aflatoxin estimation. Results indicated that a significant positive correlation between nematode damage and total aflatoxin contamination of pods (r=0.5663, *p*=0.0277). Pod discoloration also had a significant positive correlation to total aflatoxin content of pods (r=0.6613, *p*=0.0073). In addition, nematode damage to pods was significantly correlated to visible fungal contamination (r=0.8042, *p*=0.0003) and to pod discoloration (r=0.8878, *p*=$\leq$ 0.0001). However, no significant correlation existed between visual fungal contamination and total aflatoxin content in pods (r=0.2396, *p*=0.3896). Other contributing factors to either total aflatoxin content or aflatoxin B1 content included insect scars on pods (r=0.3528 , *p*=0.1971 with B1), insect holes (r=0.2015, *p*=0.4714 with total aflatoxins) and insect pod cracks (r=0.1794, *p*=0.5223 with total aflatoxins), and each of these were not significant.

Total aflatoxin levels were found to be greater in 2006 compared to 2007 in continuous peanut cropping systems (Table 2). However, the predominant aflatoxin, B1 was found to be lower in 2006 than in 2007. The mean total aflatoxin content and B1 aflatoxin in continuous bahiagrass during 2007 was found to be 19.75 ppb and 1.95 ppb respectively. The total aflatoxin content in peanut-cotton rotation during 2006 was 11.73 ppb and B1 content was about 2.53 ppb during 2006, whereas no aflatoxins were detected in 2008. The total and B1 type aflatoxins were found to be zero in 2008 for all the cropping sequences.

Aflatoxin levels (both B1 and total aflatoxins) are comparatively higher in fields where peanuts are grown as monoculture over other peanut fields that were rotated

alternatively with cotton (Fig 1). Both B1 and total aflatoxin contents decreased in bahiagrass rotated fields of peanuts when compared to fields where peanuts were grown as monoculture (Fig 2).

DISCUSSION

Based on our results, it can be inferred that bahiagrass cropping contributed significantly to reducing the *A. flavus* populations in soils when compared to other rotations in general and with peanut monocropping in particular. In an earlier study, bahiagrass, when used as a component in peanut-cotton rotations, was reported to enhance root biomass of cotton and peanut crops in subsequent years thus increasing yields (7). Further, bahiagrass cultivation also is beneficial in reducing population levels of deleterious soil fauna like *Meloidogyne arenaria*, the peanut root knot nematode, when included in a cropping sequence at least once every two years (7).

Monitoring of these *A. flavus* populations in different peanut cropping sequences and determining microbial community structure in these soils must be done at regular intervals so as to determine means whether these can be manipulated to reduce aflatoxin contamination in peanuts. For that reason a precise and high resolution DNA fingerprinting methods were useful for constant monitoring of changes in microbial groups at finer scale. Based on these community profiles obtained through molecular fingerprinting methods, cropping sequences can be determined to ameliorate the soil microbial community structures in favor of beneficial microflora in peanut soils for successful management of aflatoxin problem in peanuts.

Arbitrary evaluation of pods from sampled plots for estimating the influence of different factors on aflatoxin contamination yielded interesting results. In the present study, a significant positive correlation was obtained between nematode infestation of pods with visual fungal contamination as well as with total aflatoxin contamination. Both of these factors were found to influence the total aflatoxin content of pods directly. In addition, the total aflatoxin content is indirectly being influenced by both pod discoloration and visual fungal contamination on pod surfaces. Pod discoloration has direct influence on aflatoxin contamination as well as on nematode damage which further has direct influence on total aflatoxins. None of the other factors that are visualized have significant influence on pod aflatoxin contents. Pod damage due to nematode infestation might have prone them for fungal attack and subsequent aflatoxin contamination. Timper *et al.* (16), while working on the relationship between peanut aflatoxins and nematode damage, reported that aflatoxins occurred more frequently in pods that had more nematode galls. Nematode infestation of pods may have provided sites by which the *A. flavus* in the soil gained entry and this subsequently led to toxin contamination. However, a combination of factors may play a role in aflatoxin contamination such as nematode infestation, insect damage and pod cracks that are induced by drought stress during the maturity periods (16). Nematode infestation of pods may also lead to impaired root functioning thereby making the plants predisposed to drought stress. Sanders *et al.* (13) reported that drought stress around the pod zone predisposes peanuts to aflatoxin contamination.

Although we did not detect substantial differences in aflatoxin contamination levels between rotation sequences, similar trends were seen. Specifically, decreases in B1 and total aflatoxin levels were consistently noted when peanut was cropped following

several years of bahiagrass. However, population dynamics of both *A. flavus* and *A. parasiticus* should be worked out in parallel. In addition, it needs to be kept in mind that results will vary depending upon the population levels of toxigenic and atoxigenic strains of these fungi. Trends from both experiments showed that bahiagrass rotation in peanut fields substantially reduced aflatoxin levels compared to peanut fields that are rotated with cotton which is the most frequently used rotation system in the south eastern US. In order to maintain crop health as well as soil health, sod-based crop rotation is always preferable instead of monocropping of peanuts which in turn mitigate the effect of aflatoxin contamination in peanuts.

Table 1. Population levels of *Aspergillus flavus* group in soils from different peanut cropping sequences

Year of study	Soil *Aspergillus flavus* population at harvest ($x10^3$ cfu g^{-1} soil)			
	Continuous peanuts (P-P-P-P)	Continuous Bahiagrass (B-B-B-B-P)	Peanut-Cotton (Ct-P-Ct-P)	Peanut-Corn (P-Cn-Ct-P)
2006	1.0	0.7	1.0	1.0
2007	1.0	0.0	1.1	0.8
2008	1.2	0.2	1.2	1.0

Table 2. Aflatoxin levels (ppb) in 2006 and 2007 from different peanut cropping sequences

Aflatoxin Type	2006		2007	
	P-P-P-P	P-Ct-P-Ct	P-P-P-P	B-B-B-B-P
B1	6.9	2.533	2.8282	1.9527
B2	4.375	3.444	26.3397	2.0845
G1	0	5.747	5.137	9.72
G2	8.7513	0	7.469	5.8822
Total	20.026	11.72	41.75	19.75

Fig. 1. Effect of cotton rotation in peanut fields on aflatoxin contamination (in ppb) during 2006

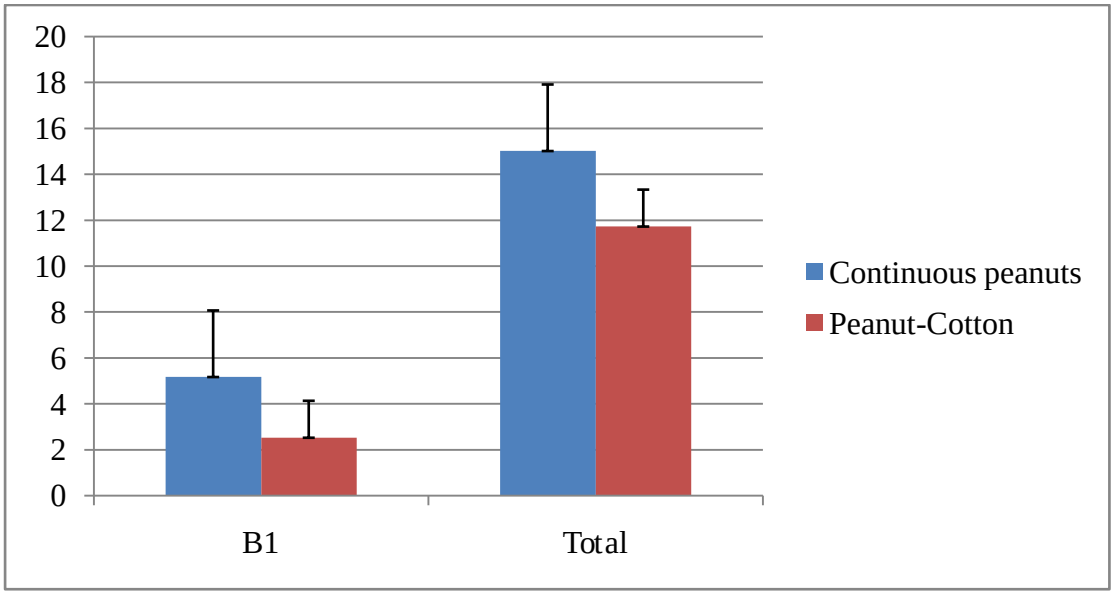
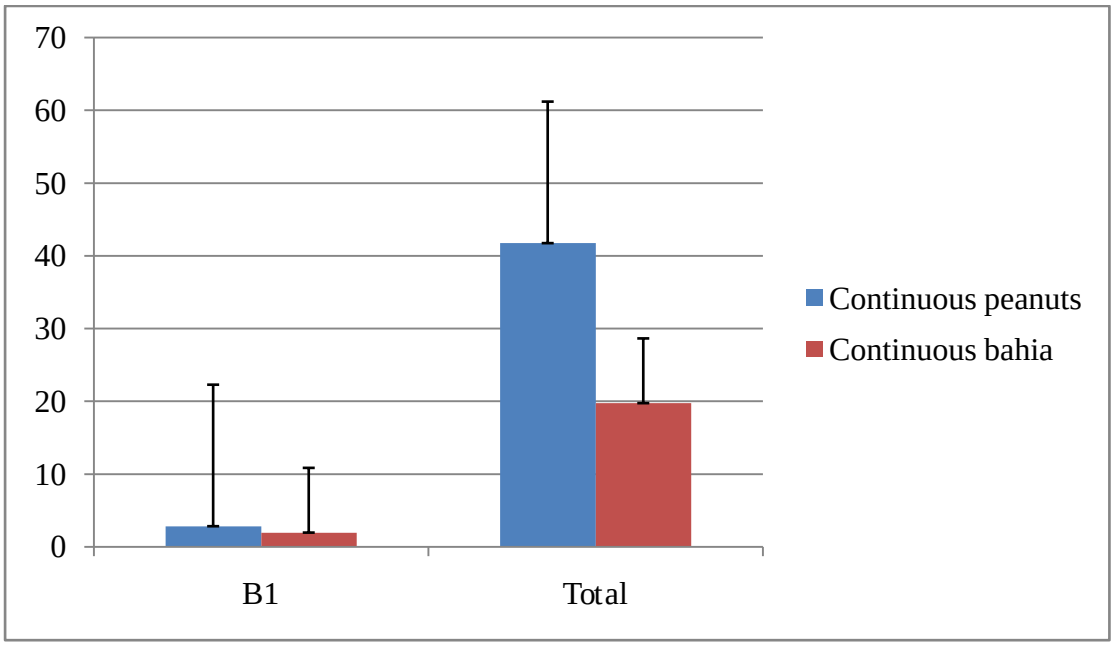


Fig. 2. Effect of bahiagrass rotation in peanut fields on pod aflatoxin contamination (in ppb) during 2007



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SUMMARY

Soil microbial community structure and diversity in peanut soils was evaluated with the help of culture independent techniques such as automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA gene cloning and sequencing. Four important peanut cropping sequences in the southeastern US were chosen for the present study including continuous peanuts (P-P-P-P), continuous bahiagrass followed by peanuts (B-B-B-B-P), peanut-cotton (P-Ct-P-Ct), and peanut-corn (P-Cn-Ct-P). Bacterial-ARISA profiles indicate that up to 90% similarities were observed among the replicated plots of same peanut cropping sequences. Though these results were not consistent among all the sampling times, the existing similarities could be the result of plant species effect on the selection of resident soil bacterial communities. Multi-dimensional scaling analysis of ARISA profiles indicated close clustering among the bacterial communities with respect to sampling time rather than cropping sequence. Since this sampling time is almost same in both the years of study (2006 & 2007), the age of crop in cultivation at that particular point of time in the field shows with change in microbial community structure. Therefore crop age as well as environmental parameters could be playing more important role in the selection of bacterial communities than plant type effect. The relative abundance of bacterial divisions is not significantly different among different peanut cropping

sequences. This was confirmed with 16S rRNA gene cloning and sequencing experiment from 2006, June sampling. However, in all cropping sequences there was an abundance of Proteobacteria, Acidobacteria, Firmicutes, Bacteroidetes, and Actinomycetes over other bacterial divisions. Interestingly, correlations that existed among various bacterial groups were of more importance than the mere presence of these populations. For example, Proteobacteria had significant positive correlation with Gemmatimonadetes. Though such interactions between different bacterial groups in the crop soils were common, some specific interactions might be helpful in altering the soilborne plant pathogen population levels as well as common soil saprophytes. High bacterial diversity and heterogeneous nature of these bacterial communities in all peanut soils was also confirmed with ecological diversity indices such as Shannon-Weaver (H) and Simpson index (1-D). However, continuous bahiagrass followed by peanuts cropping sequence had slightly higher heterogeneity over other cropping sequences thus indicating the role of bahiagrass in increasing the soil microbial diversity. Rarefaction curves of 16S rRNA gene sequences indicate the inadequacy of sampling to estimate the complete diversity of soil bacterial groups.

On the other hand fungal diversity was much lower as compared to the bacterial diversity in these crop soils. This was evident from the presence of fewer numbers of bands in the fungal-ARISA fingerprints. There were many common bands in all the cropping sequences in the present study which could be attributed to the presence of common saprophytes in these soils. However, the phylogeny of these saprophytes in these soils could not be determined. In this study, sampling time had a more profound effect on grouping of fungal communities than cropping sequence. However, no specific fungal communities were associated with particular cropping sequence.

Probable explanation for this is that the experimental site is in long-term rotation, two years of bacterial and fungal fingerprints could unable to give specific information regarding the associated resident soil microbial communities. Continuous monitoring of soil microbial communities taking into consideration of environmental parameters might be helpful to come to a conclusion about specific beneficial microbial communities associated with these crop soils.

An attempt was made to monitor the fingerprints of two important soilborne fungi *Sclerotium rolfsii* (causal agent of southern stem rot) and *Aspergillus flavus* along with the other fungal-ARISA fingerprints. These fungi could not definitively be compared because of bands generated by the other fungi in the profiles. Therefore an experiment was conducted for PCR-based detection of *A. flavus* directly from soil with specific primers (FLA-1 & FLA-2). Though the primers had the detection limit of 10^2 spores of *A. flavus* in food commodities, the primers could only detect *A. flavus* at $2.6x10^6$ cfu g^{-1} concentrations in the total soil genomic DNA. The high magnitude difference in detection could be due to the soil PCR-inhibitors or the methodological bias involved in soil genomic DNA extraction methods. Soil enumeration of *A. flavus* populations indicated that using bahiagrass rotation with peanuts resulted in significant reduction of *A. flavus* population levels compared to other cropping sequences. This was also evident from the aflatoxin levels in peanut pods determined by HPLC. Bahiagrass inclusion in to the peanut cropping sequence might be improving the soil organic matter content as well as reducing the deleterious microflora and thereby improving the soil health. Overall our studies suggest that the higher diversity of microbial communities and complex interactions among them

could play an important role in reducing the population levels of *A. flavus* group of fungi.

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APPENDIX

Chapters 2 to 4 of this dissertation have either been submitted or in preparation to submit to the following journals.

Chapter II: Exploring soil bacterial communities in different peanut cropping sequences. (This manuscript is in preparation and to be submitted to Microbial Ecology).

Chapter III: Comparison of soil fungal community structure in different peanut rotation sequences using ARISA in relation to aflatoxin producing fungi. (This manuscript is in preparation and to be submitted to Soil Biology & Biochemistry).

Chapter IV: Influence of soil microbial communities, crop rotations and other field factors on aflatoxin contamination of peanuts. (This manuscript is in preparation and to be submitted to Plant Disease).

The three articles have the followings as co-authors: Sudini, H. (first author), Bowen, K. L., Liles, M. R., Arias, C. R., Hagan, A. K. and Huettel, R. N. It should be noted that Sudini, H. contributed more than half of the total effort for each of the articles. Other authors, who collaborated on the work (and allowed me to use materials in their lab) and my major advisor, Dr. Robin N. Huettel, whose program funded the work and who also guided me, jointly contributed less than 50% of the efforts towards the studies. I am grateful to all of them.