MANAGEMENT OF AFLATOXIN THROUGH DROUGHT STRESS PHENOTYPING, *ASPERGILLUS* SECTION *FLAVI* CHARACTERIZATION, AND ACCURATE QUANTIFICATION OF *ASPERGILLUS* AND AFLATOXIN CONTAMINATION

by

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(Under the Directions of Robert C. Kemerait, Jr. and Anthony E. Glenn)

ABSTRACT

Aflatoxin is a major concern in peanut production especially in areas experiencing drought and high temperatures. The best way to manage aflatoxin contamination would be to integrate host resistance, identification of causal organism, and develop improved management strategies. Phenotyping of seven peanut genotypes identified Tifguard and Tifrunner as having better drought-coping ability than the other genotypes. *Aspergillus* section *Flavi* isolates collected from different geographical locations were identified through morphological and genetic variation. Evaluation of the effect of sample size in aflatoxin extraction demonstrated that the standard subsampling of 300 g into 100 g can be reduced to subsampling 100 g into 25 g. Immunochromatographic test strips were confirmed to have comparable aflatoxin detection results with fluorometry method and can be used under continuous high or fluctuating temperatures. Also, a real-time PCR (qPCR) assay using species-specific primers targeting the *aflS* gene effectively detected *A. flavus* and/or *A. parasiticus* in peanut seeds.

INDEX WORDS: Aflatoxin, peanut, drought-tolerance, *Aspergillus flavus*, *A. parasiticus*, sample size, immunochromatographic test strips, real-time PCR

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Aflatoxins are naturally occurring toxic substances produced by a number of different *Aspergillus* species. These toxins have carcinogenic, hepatotoxic, and immunosuppressive properties that have been reported to cause high mortality and reduced productivity in livestock as well as reduced immunity and hepatocellular carcinoma (liver cancer) in humans (8; 59; 76). It contaminates a wide variety of agronomic crops including peanut, corn, cottonseeds, pepper seeds, tree nuts, cereals, soybean, and cassava (7; 10; 53; 63; 73).

The concern for aflatoxin started when approximately 100,000 turkey poults in 500 farms in England died in 1960 after ingesting Brazilian peanut meal that was highly contaminated with aflatoxins (7; 9; 13). It was soon discovered that these toxins also occur in the human diet and can pass from feed to milk with only slight modification (9; 18). After several years, outbreaks of acute aflatoxicosis (poisoning due to severe intoxication by aflatoxin) were reported in Kenya, India, Malaysia, and Thailand (7; 19). The widespread incidence of aflatoxin in staple foods in less developed countries of the world contributed to high numbers of diseases, ill health, and death of both humans and livestock. These threats to human and animal health led to the establishment of regulatory limits of aflatoxin content in more than 100 countries. Crops intended for human consumption have an aflatoxin regulatory limit of 20 parts per billion (ppb) in the United States (US) and 2 ppb in the European Union (9; 10; 15).

Aspergillus flavus and *A. parasiticus* are the two major aflatoxin producers that largely contaminate agricultural crops and commodities. *Aspergillus flavus* (from which the term aflatoxin is derived, "*A. flavus* toxin") is predominant on many commodities. *Aspergillus parasiticus* is more common in peanut than any other crop but is typically outcompeted by *A. flavus* when both fungi are present (1; 7; 9; 30; 50; 52). Other *Aspergillus* species were also identified to produce aflatoxins but were rarely identified to contaminate agricultural crops. These include *A. nomius, A. pseudonomius, A. pseudotamarii, A. bombycis, A. ochraceorosus, A. arachidicola, A. minisclerotigenes,* and *A. pseudocaelatus* (54; 55; 71; 76).

Four major groups of aflatoxins have been identified. These are designated as B_1, B_2, G_1, and G_2 as based on their blue (B) or green (G) fluorescence under long-wave ultraviolet light (ƛ = 365 nm) and relative chromatographic mobility (1 or 2). *Aspergillus flavus* produces B_1 and B_2 whereas *A. parasiticus* produces all four toxins. Aflatoxin B_1 has proven to be the most potent toxin and has caused death in most experimental and domesticated animals. Several reports have shown that the occurrence of the 'B' toxins are usually higher than the 'G's and the '1's higher than the '2's. Two additional toxins, M_1 and M_2, were identified as derivative forms of B_1 (1; 59; 61; 76). M_1 is modified in the digestive tract of dairy cattle and may still be found active in milk, cheese, and other dairy products. This poses a relevant threat to the European economy especially in regions where dairy production is the main industry in social and economic businesses (11).

Cultivated peanut (*Arachis hypogaea* L.), also known as groundnut, is an important commodity worldwide yet is one of the most susceptible crops to *Aspergillus* infection and aflatoxin contamination (19). Peanut ranks as the second most economically important legume next to soybean and the fourth most important oilseed crop next to soybean, rapeseed (canola),

and cottonseed. It is an important staple crop in many areas of the world and is often consumed as an important dietary component. Peanut is considered as a nutritious snack and is used as feed or feed additives because of its high protein, unsaturated fat, carbohydrate, vitamin, and mineral contents (4; 26; 33).

Peanut is native to South America but is now widely grown in tropical and subtropical countries throughout the world, being cultivated on about 26.5 M hectares globally with an annual production of 35.7 M tons (4; 20). China currently leads in the world production of peanut (17.00 M tons) followed by India (5.50 M tons) and the US (1.89 M tons). However, the average yield for peanut in the US (4.49 tons/ha) is higher than China (3.61 tons/ha) and India (1.02 tons/ha) (70). In the US, the state of Georgia ranks as the largest producer accounting for approximately 45% of the country's production while the rest is accounted from Texas, Alabama, North Carolina, South Carolina, Florida, Virginia, Oklahoma, Mississippi, and New Mexico (44).

Peanut is vulnerable to pre-harvest *Aspergillus* infection and aflatoxin contamination because of the manner in which the pod develops. Being an underground crop, the peanut pods are continually at risk of being in direct contact with populations of aflatoxigenic aspergilli in the soil (29). The low soluble solids (sugars) when dry and the high oil content makes peanut susceptible to aflatoxin formation (55). The fungi generally penetrate developing nuts through cracks and wounds in the shell. On some occasions, infection may also occur through the pegs and flowers (12; 55). Peanut can also be infected and contaminated during harvest, transportation, processing, and storage (5; 12; 49; 64; 66) making aflatoxin contamination recognized worldwide as the most important problem affecting the quality and production of peanut (5; 66).

The strict quality regulations and aflatoxin regulatory limits imposed by different, mostly developed, countries caused a great decline in the international peanut trade. Although it was intended for the safety of livestock and consumers, this greatly affected the exporting capacity of many developing countries (32). Losses due to aflatoxins in three Asian countries (Indonesia, Philippines, and Thailand) were estimated to be $900 M annually. The domestic US peanut industry is also affected. Losses in the US were estimated to be over $25.8 M annually from 1993 to 1996 (60). Most of the costs were shouldered by the shelling segments of the industry while grower losses were estimated to be approximately $2.6 M each year. In 2001, aflatoxin contamination caused an estimated loss of over $20 M to the peanut industry in the southeastern US (38). In international trade, an estimated $450 M is lost annually due to the implementation of aflatoxin regulatory limits (60).

Aspergillus spp. are distributed worldwide but appear most abundantly in warm and humid climates, thus, being more common in subtropical and warm temperate areas (7; 36). Unfortunately, more than 70% of the peanut-growing areas are also located in the arid and semi-arid regions of the world where the peanuts are frequently subjected to drought stress at different durations and intensities (34). *Aspergillus flavus* and *A. parasiticus* grow in environments with moisture level below field capacity, atmospheric relative humidity range of 90-98%, and a temperature range of 12-42^{o}C. Aflatoxin production becomes optimum at temperatures between 20-35^{o}C with relative humidity greater than 83% and seed moisture content of 10.5-11% (7; 31; 53).

The main sources of inocula are the conidia in the soil, mycelia in plant debris, and fungal sclerotia. When the conidia and/or sclerotia germinate into mycelia, they produce numerous conidiophores that release conidia into the air or surrounding soil. The airborne

conidia are blown by the wind or vectored by insects into peanut flowers. The soil-borne conidia are mainly transmitted by ground insects into peanut pods as they feed. These insects create wound sites that can serve as direct entry for fungal colonization. Natural cracks and plant parts that are damaged by nematodes or harvest equipment can also serve as direct entry points. In addition, plant tissues that are weakened by environmental stress can be easily invaded (1; 6; 29; 53; 56; 58). The conidia can rapidly grow to invade and colonize the pod. The fungi produce a hyphal colony between the cotyledons that produces aflatoxin when environmental conditions become favorable. The aflatoxin-contaminated seeds normally weigh less than healthy seeds and develop yellow to brown discoloration due to the external sporulation of the fungus. Considerable invasion and aflatoxin contamination, however, can occur without visible fungal growth. On the other hand, the presence of *Aspergillus* does not necessarily indicate the presence of aflatoxin. Approximately 40-80% of *A. flavus* isolates, and nearly all *A. parasiticus*, produce aflatoxin (31).

Prolonged drought and high temperature (with a mean soil temperature of 27-30^{o}C) are the two most important conditions that favor pre-harvest fungal invasion and aflatoxin contamination especially when it occurs during the last three to six weeks of the growing season. These two factors are interrelated and neither will lead to enhanced aflatoxin contamination alone (1; 16; 31; 50; 74). From the perspective of agriculture, Tuberosa (69) defines drought as the condition where the amount of water through rainfall and/or irrigation is not sufficient to meet the transpiration needs of the crop. Drought causes severe yield losses depending on its timing, intensity, and duration in addition to other location-specific environmental factors like irradiance and temperature (45). On a global scale, it causes an annual loss of $520 million in peanut production (34). Drought and heat stress cause the plants to lose moisture from pods and

seeds while greatly reducing the plant's physiological activities (32). The stressed plants experience permanent foliage wilting, leaf shedding, and receding of canopy between rows. Such conditions lead to further increase in soil temperature and evaporation of soil moisture (31) while favoring rapid fungal growth (12). At the same time, dry conditions reduce the production of plant biocompetitive (phytoalexins) and/or protective (phenols) compounds which normally function to inhibit fungal infections (31; 53). Efforts in the past have been directed towards adapting the environment to the needs of the crop such as the provision of irrigation to improve crop health and reduce aflatoxin contamination (51; 69). Irrigation, however, is not readily available for most peanut-growing areas (46). The modern emerging concept is to genetically tailor crop cultivars so as to improve their ability to withstand drought and other environmental constraints while optimizing their water and nutrient use (69).

Plant breeders have long aimed to produce aflatoxin-resistant peanut genotypes through genetic manipulation. Several approaches were suggested for the genetic control of pre-harvest aflatoxin contamination in peanut through the production of aflatoxin-resistant peanut lines. However, the screening procedures for aflatoxin resistance can be expensive, laborious, and require destructive techniques to directly measure seed infection and aflatoxin content. These constraints slowed the development of resistant genotypes (5). As an alternative, drought resistance or tolerance traits have been identified as indirect selection tools for resistance to pre-harvest aflatoxin contamination. Several studies using drought-tolerant peanut genotypes show that these genotypes generally display lower levels of pre-harvest aflatoxin contamination (5; 27; 28). According to Levitt (39), drought-resistant/tolerant plants can mitigate the negative effects of water deficit either through dehydration avoidance or dehydration tolerance. Dehydration avoidance uses the morpho-physiological features of the plant such as deep roots and osmotic

adjustment to enable the plant or its parts to maintain hydration. On the other hand, dehydration tolerance uses features like remobilization of stem water-soluble characteristics to allow the plant to maintain or partially maintain its proper function.

The use of molecular genetics was explored to aid in understanding stress response and developing peanut genotypes with drought stress tolerance (34). Deoxyribonucleic acid (DNA) molecular markers including random amplified polymorphic DNA (RAPD) (57), amplified fragment length polymorphism (AFLP) (25), and simple sequence repeat (SSR) markers (22; 41; 48) were recently developed to assess genetic variability and evolutionary patterns in different crops including peanut. Among these molecular markers, SSRs show higher level of DNA polymorphism in cultivated peanut. In addition, SSR markers are co-dominant and multi-allelic in inheritance and are easier to amplify with less DNA quantity (41; 67).

The aflatoxigenic species of *Aspergillus* resemble atoxigenic species used in food fermentation such as *A. oryzae* and *A. sojae*. Correct species identification is, therefore, very important. Texture of conidial wall, growth rate, conidial diameter and colony colors were reported as important criteria for differentiating species (21; 35). The species identification based on these criteria, however, is time-consuming, laborious, and complex as it requires significant training and expertise in laboratory mycology (1; 24; 47). Genetic sequence variation based on species-specific gene targets was developed as an alternative. Still, problems can persist due to potential similarity to a large variety of fungi (17; 23; 49). Improvements in genetic variation approaches were explored. One of these approaches includes the repetitive-sequence-based polymerase chain reaction (PCR) which is based on the amplification of intervening sequences located within short repetitive DNA sequences that are dispersed throughout an organism’s

genome (47; 72). It has been reported to provide accurate discriminations among bacterial and fungal isolates from both clinical and field sources (21; 23; 47; 72).

Aside from identification, quantification of fungal infection is also important. The early detection of aflatoxigenic species before toxin production has begun would be necessary to prevent toxins from entering the food chain (3; 49). To meet this, DNA-based detection methods such as conventional real time polymerase chain reaction (qPCR) were developed. This method is highly sensitive and allows specific detection of fungal species in a mixed population (2; 62). The qPCR can be performed using different chemistries including DNA-associating dyes (SYBR® Green) or fluorescently labelled sequence-specific oligoprobes (Taqman® oligoprobes) (42). It has been used to quantify the amounts of *Aspergillus* in several commodities including peanuts (49), wine grapes (62), wheat flour (59), and maize (64) where results show that qPCR can be used to predict probable toxigenic risk even when there is a very low level of infection.

Aflatoxin contamination of peanut is monitored regularly in commerce. This is done through the sampling of lots or stocks. An accurate and convenient estimation of the aflatoxin content is essential for the effective monitoring and management of aflatoxin contamination (75). In the US, peanut samples are first examined visually for the presence of characteristic green or yellow-green *Aspergillus* colonies. The detection of fungal colonies on any peanut pod or kernel causes the entire lot to be designated as Segregation III (Seg III) which cannot be used for direct human or animal consumption (53). Since aflatoxins can still be present without visible fungal growth, several chemical detection methods were developed to fill the limitations of visual inspection. These include the Fourier transform near-infrared spectroscopy (68), thin layer chromatography (65), fluorometry method (28), high performance liquid chromatography (HPLC) (43), liquid chromatography-tandem mass spectrometry (LC-MS) (14), and enzyme-

linked immunosorbent assay (ELISA) (40). Among these, the ELISA and HPLC are most commonly used (78). These methods are proven to be accurate, selective, sensitive, and effective. However, these methods are usually costly, largely intended for laboratory scientific research, and require special equipment and training (63; 78). This offers a disadvantage to developing countries where these methods are not very accessible but where the aflatoxin problem is greater. The immunochromatographic test strips, also known as lateral flow test strips, are low-cost, easy to handle, and usable on-site qualitative tests which were developed and integrated into routine quality monitoring procedures. These strips can be operated following simple procedures, produce immediate results, and do not require expensive instrumentation. In addition, the immunochromatographic test strips do not necessarily need to be refrigerated, making their use very promising in developing countries. However, very few studies regarding the use of these strips in the field have been documented (37; 77; 78).

This study was conducted to consider the interaction between the peanut crop and pre-harvest aflatoxin contamination. The overall aim of the study was to employ different methods and techniques to generate information that could be useful in battling the problem of aflatoxin and aflatoxigenic *Aspergillus* species in peanut. Like other disease management programs, the most effective strategy to manage aflatoxin contamination would be to integrate different methods and/or techniques related to aflatoxin production: the host, the causal organism, the environment, and the current management strategies being employed. These four factors were considered in the overall scope of this study.

The specific objectives of the study were to: (1) Evaluate the performance of potential drought-tolerant peanut genotypes under drought stress conditions and their response to aflatoxin contamination; (2) Identify and differentiate *Aspergillus* section *Flavi* isolates that were collected

from peanuts grown in different geographical locations through morphology and genetic variation; and (3) Accurately detect *Aspergillus* infection and aflatoxin contamination in peanut. Objective 3 was achieved through the following sub-objectives: (a) Evaluate the effect of sample size in the accuracy of determining aflatoxin contamination; (b) Assess the sensitivity and accuracy of immunochromatographic test strips over time and temperature regimes in the qualitative detection of aflatoxin contamination in peanut and as compared to the Vicam fluorometry method; and, (c) Quantify the amount of *A. flavus* and *A. parasiticus* in peanut seeds through quantitative real-time PCR. Each objective is presented in the succeeding chapters of this thesis.

Chapter 2 (Objective 1) is focused on aflatoxin management through the improvement of the host crop. Seven peanut genotypes were assessed for drought tolerance and their response to aflatoxin contamination. Drought tolerance was evaluated through visual drought stress ratings, chlorophyll fluorescence (PI_{ABS}, Fv/Fm, and PHI_{EO}), soil plant analysis development (SPAD) chlorophyll meter reading (SCMR), canopy temperature (CT), canopy temperature depression (CTD), canopy reflectance through normalized difference vegetation index (NDVI), stomatal conductance, and pod yield. The aflatoxin contamination of each genotype was also quantified through the standard Vicam fluorometry method and then correlated with the drought stress responses. The genotypes that were identified to show better drought-tolerant traits and less aflatoxin contamination could be considered as candidates to be integrated into peanut breeding programs with the hope of developing aflatoxin-resistant lines. Also, the evaluation methods that showed significant correlations with the detection of amount of aflatoxin contamination are recommended for future studies regarding selection of drought-tolerance traits in peanuts.

Chapter 3 (Objective 2) is focused on the aflatoxin-producing organisms. Isolates were obtained from peanut samples collected in Texas, Alabama, and Georgia of the United States; Haiti; and Philippines. Isolates belonging to the genus *Aspergillus* section *Flavi* were characterized morphologically based on colony surface color, colony reverse color, growth diameter, presence or absence of sclerotia, color of sclerotia if present, and texture of conidial wall. These isolates were identified genetically through conventional DNA sequencing using primers that target the internal transcribed spacer (ITS) region, beta tubulin, and translation elongation factor 1-α (TEF1-α). These isolates were also identified using repetitive-sequence-based PCR (rep-PCR) DNA fingerprinting via the DiversiLab system. An awareness of the presence of the aflatoxigenic species can guide producers in implementing appropriate aflatoxin management strategies in the production area. On the other hand, the presence of non-aflatoxigenic strains can be considered in developing potential biological control agents against pre-harvest aflatoxin contamination as has been done with Aflaguard® in the US and Aflasafe® in Africa.

Chapters 4-6 (Objective 3) considered three management approaches involved in aflatoxin detection in peanut. In chapter 4, the effect of using different sample sizes in aflatoxin detection was assessed. Field samples subjected to conditions that favor aflatoxin production and Seg III peanuts obtained from a commercial buying point were used. The effect of subsampling of 300 g and 100 g peanuts into 100 g and 25 g was compared to direct sampling of 25 g. The possibility of using smaller sample sizes while not compromising the precision of aflatoxin detection would help reduce the cost of solutions/chemicals used during aflatoxin detection. In chapter 5, the efficiency of immunochromatographic test strips to qualitatively detect aflatoxin levels at a 20 ppb cut-off limit was compared the quantitative results obtained using the standard

Vicam fluorometry method. The ability of the test strips to perform well under tropical temperatures, which is the usual temperature in developing countries, was also evaluated. The test strips were incubated at continuous high (34^{o}C) and fluctuating (34^{o}C for 8 hours and 25^{o}C for 16 hours) temperatures. The maximum length of time that the test strips remained accurate and sensitive in detecting aflatoxin levels was determined. Establishing the efficiency of these strips under these tropical temperatures would benefit peanut production areas, especially in developing countries, where high-end aflatoxin detection technologies are not very accessible. In chapter 6, a quantitative real-time PCR (qPCR) assay that is useful for the detection of *A. flavus* and *A. parasiticus* in peanut seeds was tested for applicability. This assay can be used to detect minute fungal infection in seeds before aflatoxin production has been initiated. This assay was also used to determine the relationship between fungal infection and aflatoxin contamination.

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CHAPTER 2

PHENOTYPING PEANUT GENOTYPES FOR DROUGHT TOLERANCE[1]

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2.1 Abstract

Drought and heat stress enhance aflatoxin contamination of peanuts especially when such occur during the last three to six weeks of the growing season. Identifying drought-tolerant genotypes may aid in development of aflatoxin resistance in peanuts. This study was conducted to phenotype seven peanut genotypes (Tifguard, Tifrunner, Florida-07, 554CC, NC3033, C76-16, and A72) based on their response to drought stress. The phenotyping methods included visual ratings, chlorophyll fluorescence (PI_{ABS}, Fv/Fm, and PHI_{EO}), soil and plant analysis development (SPAD) chlorophyll meter reading (SCMR), canopy temperature (CT), canopy temperature depression (CTD), normalized difference vegetation index (NDVI), stomatal conductance, and pod yield. Based on these traits, Tifguard and Tifrunner showed better drought-coping mechanisms than the other genotypes. After the aflatoxin content of the different genotypes was measured, significant correlations were observed among aflatoxin contamination, visual ratings, SCMR, CT, CTD and NDVI.

Keywords: Drought tolerance, aflatoxin, visual ratings, SCMR, canopy temperature, canopy temperature depression, NDVI, stomatal conductance, pod yield

2.2 Introduction

Cultivated peanut (*Arachis hypogaea* L.) is an important crop but is also one of the most susceptible crops to aflatoxin contamination (27). It is cultivated on about 26.5 million hectares globally with an annual production of 35.7 million tons. It ranks as the 2nd most economically important legume next to soybean and the 4th most important oilseed crop next to soybean, rapeseed (canola), and cottonseed. Its high protein, unsaturated fat, carbohydrate, vitamin, and mineral contents make it an important dietary component in many countries, a nutritious snack, and healthy feed or feed additive (4; 36; 70).

Aflatoxin contamination in several crops has been repeatedly reported to cause reduced immunity, lesser productivity, hepatocellular carcinoma (liver cancer) and death in livestock and humans. This has produced significant economic problems for the international peanut trade and high losses to international and domestic producers (16; 18; 61; 77). Export losses due to implementation of aflatoxin regulatory limits in many countries amounts to $450 M annually. In the US, losses were estimated to be over $25.8 million annually from 1993 to 1996 where most of the costs have been shouldered by the shelling segments of the industry while grower losses were estimated to be approximately $2.6 million each year (62). In 2001, aflatoxin contamination caused an estimated loss of over $20 M to the peanut industry of southeast US (42).

Prolonged drought and high soil temperature, with mean of 27-30^{o}C, were identified as the two major factors contributing to enhanced pre-harvest fungal invasion and aflatoxin contamination. This is especially true when such occurred during the last three to six weeks of the growing season. These two factors are interrelated and neither will lead to increased aflatoxin concentration alone (2; 23; 35; 52; 76). Provision of irrigation was shown to improve drought stress in plants and reduce aflatoxin contamination (53). Irrigation, however, is not available for

most peanut-growing areas. Therefore, the development of drought-resistant genotypes had been viewed as a potential solution (50).

Early breeding efforts for the selection of drought-tolerant genotypes were based on pod yield alone. High-yielding cultivars that continued to produce well under drought conditions were selected as a priority to enable stable production (6; 38). However, the selection of genotypes using pod yield has been slow and has produced highly variable results as it is affected by large genotype by environment (G x E) interactions. In response, additional selection criteria, such as water-use efficiency (WUE) and transpiration efficiency (TE), were developed to select genotypes with drought tolerance traits (6; 48; 50). Water use efficiency is an important drought avoidance trait that uses soil water more efficiently for biomass production (11). Transpiration efficiency is an important component of WUE and is defined as biomass produced per unit of water transpired (6). Although these traits provide good results, the measurement of these traits can be very tedious. Hence, more easily measurable traits, such as those used in this study, have been developed and were successfully used as surrogate traits for WUE and TE (6; 48; 50). Furthermore, significant correlations were reported between aflatoxin contamination and visual ratings and leaf temperature (33), with SPAD chlorophyll meter reading (SCMR) and pod yield (6), and with ground-based reflectance (68). In this study, visual drought stress ratings, chlorophyll fluorescence, SCMR, canopy temperature (CT), canopy temperature depression (CTD), canopy reflectance through normalized difference vegetation index (NDVI), stomatal conductance, and pod yield were used to evaluate drought tolerance traits in seven peanut genotypes. Using a combination of these traits instead of relying on a single trait should lead to identification of genotypes with better drought-coping mechanisms.

Molecular genetics also has aided in understanding stress response and developing new peanut genotypes with stress tolerance (38). Deoxyribonucleic acid (DNA) molecular markers including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) markers were recently developed to assess genetic variability and evolutionary patterns in different crops (30; 45). Among these molecular markers, SSRs offers the advantage of showing higher level of DNA polymorphism in cultivated peanuts. These are sequence repeats that are generally less than five base pairs in lengths (14). They are co-dominant, multi-allelic in inheritance, relatively abundant, easily detectable and amplified, and provide extensive genome coverage (29; 45; 71)). Due to these reasons, SSR markers were used in this study to detect the genetic relationships of selected samples within and across seven peanut genotypes.

2.3 Materials and Methods

2.3.1 Plant materials and trial setup

Rainout shelter trials were set up at the National Environmentally Sound Production Agriculture Laboratory (NESPAL) and at the Gibbs Farm, Tift County, Georgia during the summers of 2012 and 2013. Seven peanut genotypes, namely: Tifguard (32), Tifrunner (31), C76-16, Florida-07 (24), 554CC, NC3033 (8) and A72 were provided by the peanut programs of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) and University of Georgia (UGA) Tifton Campus. The first six genotypes were tested for drought tolerance and aflatoxin resistance while A72, formerly identified as aflatoxin susceptible, served as the susceptible check. The plants were grown with sufficient water for 100 days before drought conditions were imposed.

At the NESPAL rainout shelter, three water treatments were imposed on six peanut genotypes (Tifguard, Tifrunner, C76-16, Florida-07, 554CC, and A72) following a split-plot design where water treatment served as the main plot and genotype as the subplot. There were two replicates per genotype for T_0 and three replicates per genotype for T_1 and T_2. The treatments were: T_0 (irrigated) = no heat stress imposed on plants with sufficient irrigation in both pod and root zones; T_1 (pod-zone stress, PZS) = drought and heat stress imposed in the pod zone of the plants but with irrigation in the root zone; and, T_2 (whole plant stress, WPS) = heat stress imposed in the root and pod zones of the plants without irrigation in both zones. These water treatments were applied from 100 days after planting (DAP) until harvest. After assessing the performance of the different genotypes during the 2012 trial, 554CC was replaced with NC3033 for the 2013 trial.

At the Gibbs Farm rainout shelter, six peanut genotypes (Tifguard, Tifrunner, C76-16, Florida-07, 554CC, and A72) were planted in a randomized complete block design with eight replications. Whole plant stress (sheltered trial) was implemented by covering the entire test plots with a mobile greenhouse from 100 DAP until harvest. Only a sheltered trial was conducted in 2012 while both sheltered and unsheltered (irrigated) trials were conducted in 2013.

2.3.2 Fungal inoculum preparation and application

The fungal inocula were prepared similarly to the organic matrix method described by Will *et al.* (75). Briefly, heat-sterilized cracked corn (25% moisture content) was inoculated with spore suspensions of seven-day old cultures of *Aspergillus flavus* (NRRL 3357) and *A. parasiticus* (NRRL 2999) containing approximately 1 x 10^6 conidia/ml of water. The inocula were incubated at 25^{o}C for three days then stored at 4^{o}C until used for field inoculation. These

were sprinkled by hand directly on the plant foliage then gently dislodged to the soil surface under the canopy.

2.3.3 Drought stress evaluation and data gathering

Different evaluation methods were used to compare the drought stress response of the different peanut genotypes. These were as follows:

Visual drought stress ratings. Plant drought stress was rated on a scale of 1-5 based on the criteria described in Figure 1. The chart was constructed based on the descriptions by previous studies (60; 68) and actual observations from the rainout shelter. Ratings were done twice daily at 8:00 AM and 1:00 PM twice a week. Morning ratings were done to assess permanent wilting of the plants while the afternoon ratings were done to assess drought stress as affected by solar heat.

Chlorophyll fluorescence. This was measured using a handheld fluorometer (FluorPEN FP 100, Photon System Instrument) by taking fluorescence readings from the second fully-expanded penultimate leaf of three randomly selected plants per plot. Readings were taken twice a week prior to dawn to allow dark adaptation of the plants for at least eight hours. Three parameters were measured, namely: PI_{ABS}, Fv/Fm, and PHI_{EO}.

Soil plant analysis development (SPAD) chlorophyll meter reading (SCMR). This was measured using a SPAD meter sensor (Minolta SPAD-502) following the procedure described by Nageswara *et al*. (48). Readings were taken from the second fully-expanded penultimate leaf of five randomly selected plants per plot. Extra care was taken to ensure that the meter sensor fully covered the lamina while avoiding the interference of the veins and midrib of the leaves. Readings were taken at 10:00 AM twice a week.

Canopy temperature (CT) and canopy temperature depression (CTD). CT was measured using an infrared thermometer (Extech IR400) following the procedure described by Fischer *et al*. (22). Four readings were taken from the same side of each plot at an angle of approximately 45^{o} from the horizontal plane, making sure that different regions of the plot were sampled and the laser was hitting the plant leaves.

CTD was calculated as: CTD = Ambient temperature – Canopy temperature

At the NESPAL rainout shelter, ambient temperatures were determined by air temperature sensors placed at the corners of the shelter. At Gibbs Farm, ambient temperatures were instantly measured after four readings from each plot following the procedure described by Fischer *et al.* (22). Measurements were taken twice a week during solar noon at around 12:00 NN.

Thermal imaging taken through the use of a FLIR Thermal Imager/Camera was used to measure the CT and compute the CTD of plants in the NESPAL rainout shelter during the 2013 trial. Data were downloaded and analyzed using the FLIR QuickReport software.

Canopy reflectance via normalized difference vegetation index (NDVI). This was measured using a handheld CropScan Multispectral Radiometer (CropScan, Inc.). Similar to the procedure described by Sullivan and Holbrook (68), readings were taken over the middle of each row from a height of 60-90 cm above the canopy at nadir position (0^{o} angle). Measurements were taken once a week at midday at around 12 NN.

Stomatal conductance. This was measured using a leaf porometer (Decagon SC-1 Leaf Porometer, Decagon Devices) following the instructions in the manufacturer's manual. The sensor head was attached to the second fully-expanded penultimate leaf of the plant with the

sensor measuring the adaxial (top) side of the leaf as based on the recommendation by Pallas (51).

Pod yield. Peanut pods from the Gibbs Farm were harvested from each 1.5 sq m plot at the end of the growing season. These were dried and then cleaned from rocks, soil, and other materials prior to weighing (g).

2.3.4 Data analysis for drought stress measurements

The collected data were analyzed using the two-way PROC ANOVA procedure in SAS ver. 9.2 (SAS Institute, Cary, NC) to test genotypic differences under the three water treatments in the NESPAL rainout shelter. The data collected from the Gibbs Farm rainout shelter were analyzed using the one-way PROC ANOVA procedure for the 2012 trial and two-way PROC ANOVA for the 2013 trial.

2.3.5 Aflatoxin extraction and quantification

The aflatoxin content of the peanut kernels harvested from the Gibbs Farm was measured through the standard Vicam fluorometry method. Briefly, representative samples (100 g) of shelled peanuts were added with 10 g NaCl and 200 ml of methanol/water (80:20 v/v), homogenized using a Waring blender at high speed for 1 min, and filtered through Whatman paper. Five ml of the filtrate was diluted with 20 ml HPLC water then re-filtered. A 10-ml filtrate was purified with Vicam immunoaffinity columns (Vicam Aflatest, MA) containing aflatoxin-specific (B_1, B_2, G_1 and G_2) monoclonal antibody and washed with 10 ml HPLC water before the aflatoxin was eluted with 1 ml methanol. The eluted fraction was diluted twice with HPLC water then measured with the fluorometer (Vicam Series 4 Fluorometer).

2.3.6 Evaluation of genetic uniformity within and across genotypes using SSR markers

Sixteen young terminal leaves were collected from each peanut genotype planted at the NESPAL shelter. Total genomic DNA was extracted following the cetyl trimethyl ammonium bromide (CTAB) method described by Tang *et al.* (71). After extraction, DNA quality was checked on 0.8% agarose gel and diluted to approximately 25 ng/µl. Samples were stored at 4^{o}C until further use.

Thirty-six previously reported SSR markers (28) (Table 20), identified to be polymorphic between Tifrunner and Florida-07, were used to test polymorphism within and among the seven peanut genotypes used in the study. The functional SSR markers were screened on selected samples through conventional PCR (GeneAmp® PCR System 9700). A volume of 0.4 µl extracted DNA was added to a 9.6 µl volume reaction containing 1 µl housekeeping actin depolymerizing factor (ADF) primers 400/401, 5 µl HPLC water, 1 µl 10x PCR buffer, 0.8 µl 2.5 mM dNTPs, 0.6 µl 25 mM $MgCl_2$, 1µl 10% PVP, 0.1 µl BSA, and 0.1 µl Taq polymerase. The thermocycling conditions used were: 94^{o}C for 5 min, 35 cycles of 94^{o}C for 30 s, 53^{o}C for 30 s, 72^{o}C for 30 s, and 72^{o}C for 1 min. Marker lengths were estimated by checking the amplification on 1.5% agarose gel.

Among the 36 markers, only 30 yielded amplifications. These were composed of 11 markers labelled with 6-FAM, 9 labelled with HEX, and 10 labelled with TAMRA (Table 20). These functional markers were subjected to 64^{o}C-58^{o}C 'touchdown' PCR in 10 µl volume reactions containing 1 µl forward and reverse primers, 0.5 µl diluted DNA, 5.6 µl water, 1 µl 10x PCR buffer, 0.08 µl 100 mM dNTPs, 0.6 µl 25 mM $MgCl_2$, 1µl 10% PVP, 0.1 µl BSA, and 0.1 µl Taq polymerase. The 'touchdown' PCR conditions consisted of 94^{o}C for 5 min, 36 cycles of 94^{o}C for 30 s, 62^{o}C for 30 s, and 72^{o}C for 30 s where the annealing temperature was decreased

by 1^{o}C per cycle until the temperature reached 58^{o}C and 36 cycles of 94^{o}C for 30 s, 58^{o}C for 30 s, 72^{o}C for 30 s, and 72^{o}C for 1 min. Successful amplifications were re-checked on 1.5% agarose gel. The amplicons were diluted 40x and multiplexed by color and/or amplification length (FAM + HEX + TAMRA fluorophores). One µl of the diluted multiplexed amplicons were mixed into 9 µl Hi-Di formamide (Applied Biosystems, Foster City, CA) with 20 µl GeneScan 500 internal lane standard labeled with ROX dye (GGF500R). The products were sent to the Georgia Genomics Facility (GGF), Athens, Georgia for genotyping.

2.3.7 Data analysis for SSR markers

The results obtained from GGF were analyzed using the software Gene Mapper version 4.0 (Applied Biosystems, Foster City, MA) for the detection of amplification bands and allele scoring. Comparison of the different peanut samples was based on the presence (1) or absence (0) of bands generated by each marker from which a binary matrix was generated and used for further analysis. The genetic distances between individual samples were calculated using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm using the software DendroUPGMA (http://genomes.urv.es/UPGMA/). The same program was used to construct a dendrogram showing the relationship of the different peanut samples by performing a bootstrap analysis of 100 random sets from the original data to assess the support for groupings within the original dendrogram.

2.4 Results

2.4.1 Rainout shelter trials

2.4.1.1 NESPAL rainout shelter

Three water treatments (pod zone stress = PZS, whole plant stress = WPS, and irrigated) were imposed at the NESPAL shelter starting from 100 DAP until harvest for a total of between five to six weeks. Six evaluation methods for drought tolerance traits were used: visual drought stress ratings; chlorophyll fluorescence measured as PI_{ABS}, Fv/Fm, and PHI_{EO}; SCMR; CT and CTD measured using infrared thermometer and thermal imaging; NDVI; and stomatal conductance.

Visual drought stress ratings. The irrigated plants generally showed lower stress ratings than those exposed to PZS and WPS (Tables 1-2). Moreover, morning ratings were lower than in the afternoon. Significant genotype-by-water treatment (G x T) effects were observed in both 2012 and 2013. Genotypes Tifguard and Tifrunner generally showed the lowest or one of the lowest morning and afternoon ratings across all water treatments in 2012 and 2013. C76-16 showed moderately low ratings under irrigated and PZS treatments but had high stress ratings under WPS, especially in 2013. Florida-07 had showed variable results of having high stress ratings in 2012 while having moderately low ratings under PZS and low ratings under WPS in 2013. Genotype 554CC showed moderately high ratings under PZS and the highest rating under WPS in 2012. Thus, 554CC was replaced with NC3033 in 2013. NC3033 was generally rated as moderately high to high stress, however, plants within a treatment showed dimorphic responses where some plants looked healthy and vigorous while some wilted and dried. The aflatoxin-susceptible check, A72, had the highest or one of the highest ratings across all treatments in both years except for WPS in 2012.

Chlorophyll fluorescence. The plants exposed to PZS and WPS generally showed reductions in their PI_{ABS}, Fv/Fm, and PHI_{EO} as compared to the irrigated plants (Tables 3-5). Conversely, there were a few differences in the results obtained from these three parameters. Data analysis for 2012 showed a significant G x T effect for PI_{ABS} but not for Fv/Fm and PHI_{EO}. In this year, exposure of plants to PZS and WPS showed that Tifguard, Tifrunner, C76-16, and 554CC had PI_{ABS} values that were significantly higher than Florida-07 and A72. The same trend for the genotypes was obtained for PHI_{EO} except that no significant G x T effect was observed. For Fv/Fm, Tifrunner had the highest value but was not significantly different from Tifguard. In 2013, significant G x T effects were observed for all three parameters. Tifguard and Tifrunner had the highest PI_{ABS}, Fv/Fm and and PHI_{EO} when exposed to PZS. When exposed to WPS, Tifguard, Tifrunner, Florida-07, and NC3033 had PI_{ABS}, Fv/Fm and PHI_{EO} that were significantly different from A72.

SCMR [soil plant analysis development (SPAD) chlorophyll meter reading]. A general decrease in SCMR was observed when the plants were exposed to PZS and WPS as compared to the irrigated treatment, except for Tifguard which had almost similar SCMR under irrigated and WPS conditions (Table 6). No significant G x T effect was observed in 2012 where Tifguard exhibited the highest SCMR. On the other hand, significant G x T effect was observed in 2013. Tifguard and Tifrunner had the highest SCMR under PZS, while only Tifguard under irrigated and WPS treatments.

CT (canopy temperature) and CTD (canopy temperature depression). In general, the irrigated plants had lower CT than the plants exposed to PZS and WPS. Evaluation of CT using an infrared thermometer in 2012 did not show significant differences among genotypes in the irrigated and PZS treatments (Table 7). Under WPS, Tifrunner had the lowest CT but was not

significantly different from C76-16 and A72. In 2013, the G x T effect was not significant. Tifguard and Tifrunner were observed to have the lowest CT. When evaluated using thermal imaging in the same year, Tifguard had the lowest CT but was not significantly different from Tifrunner and C76-16 (Table 9).

In terms of CTD, evaluation using an infrared thermometer in 2012 did not show significant genotypic differences under PZS (Table 8).Under WPS, only Tifrunner had a positive CTD. In 2013, evaluation using an infrared thermometer showed that Tifguard and Tifrunner had CTD values that were significantly higher than the rest of the genotypes. When evaluated using thermal imaging, Tifguard had the highest CTD but was not significantly different from Tifrunner and C76-16. It should, however, be noted that all genotypes yielded a negative CTD using thermal imaging (Table 9).

NDVI (normalized difference vegetation index). Higher NDVI values were obtained from scanning the canopy of the irrigated plants as compared to those of the plants exposed to PZS and WPS (Table 10). In 2012, the G x T effect was not significant. Tifrunner had the highest NDVI. On the other hand, significant G x T effect was observed in 2013. Tifguard and Tifrunner had significantly higher NDVI than the rest of the genotypes when exposed to PZS, while higher for Tifguard, Tifrunner, and Florida-07 under WPS.

Stomatal conductance. Stomatal conductance was measured only in 2013. The plants exposed to PZS and WPS generally showed a great reduction in stomatal conductance as compared to the irrigated plants (Table 11). When exposed to PZS, Tifguard, Tifrunner and C76-16 maintained a stomatal conductance that was significantly higher than A72. When exposed to WPS, Tifguard had significantly higher stomatal conductance than A72 but was not significantly

different from Tifrunner and Florida-07. The highest reduction in stomatal conductance from irrigated to stressed conditions was observed from Tifrunner.

2.4.1.2 Gibbs farm rainout shelter

At the Gibbs Farm, a sheltered trial (whole plant stress) was conducted in 2012 while both sheltered and unsheltered (irrigated) trials were conducted in 2013. Plant stress was imposed from 100 DAP until harvest for a total of six weeks. Six evaluation methods for drought tolerance traits were used: visual drought stress rating; chlorophyll fluorescence measured as PI_{ABS}, Fv/Fm, and PHI_{EO}; SCMR; CT and CTD using thermal imaging; NDVI; and pod yield.

Visual drought stress ratings. Visual stress ratings in the morning were generally lower than in the afternoon (Table 12). In 2012, Tifguard, Tifrunner, C76-16 and Florida-07 had morning ratings that were significantly lower than A72. On the other hand, only Tifguard, Tifrunner and C76-16 had afternoon ratings that were significantly lower than A72. In 2013, Tifguard and Tifrunner had morning and afternoon ratings that were significantly lower than A72. Both morning and afternoon ratings produced significant positive correlations with CT and aflatoxin contamination as well as significant negative correlations with Fv/Fm, SCMR, CTD, and NDVI (Table 19).

Chlorophyll fluorescence. In 2012, no significant differences were observed in the PI_{ABS}, Fv/Fm and PHI_{EO} of the different genotypes. In 2013, Tifrunner had the highest PI_{ABS}, Fv/Fm and PHI_{EO} (Table 13). In addition to Tifrunner, genotypes Tifguard, C76-16 and Florida-07 had PI_{ABS}, Fv/Fm and PHI_{EO} that were significantly higher than A72. PI_{ABS} showed significant positive correlations with Fv/Fm and PHI_{EO} but not with the other evaluation methods (Table 19). Fv/Fm yielded significant correlations with PI_{ABS}, PHI_{EO}, visual ratings, SCMR, CT, CTD,

NDVI, and aflatoxin contamination but showed no significant correlation with pod yield. PHI_{EO} did not show significant correlation with any of the other evaluation methods.

SCMR [soil plant analysis development (SPAD) chlorophyll meter reading]. Highest SCMR was observed from Tifguard in both 2012 and 2013 (Table 14). Data analysis showed significant positive correlation between SCMR and Fv/Fm as well as significant negative correlations between SCMR and visual ratings and aflatoxin contamination (Table 19).

CT (canopy temperature) and CTD (canopy temperature depression). In 2012, Tifrunner had the lowest CT but was not significantly different from most of the other genotypes including A72 (Table 15). In 2013, no significant genotypic differences were observed. Nevertheless, significant correlations were obtained between CT and visual ratings, Fv/Fm, SCMR, CTD, and aflatoxin contamination. In terms of CTD, Tifrunner was significantly higher than A72 in 2012 but was not significantly different from Tifguard, C76-16 and Florida-07. The same with CT, no significant differences were observed among genotypes in 2013. Significant correlations were observed between CTD and Fv/Fm, visual ratings, CT, and aflatoxin contamination (Table 19).

NDVI (normalized difference vegetation index). In 2012, Tifguard, Tifrunner and Florida-07 had NDVI that was significantly higher than A72 (Table 16). In 2013, significant G x T effect was observed. Tifguard and Tifrunner had significantly higher NDVI than A72 when grown under the sheltered (drough-stressed) trial. Under the unsheltered (rainfed) trial, Tifguard, Florida-07 and 554CC had significantly higher NDVI as compared to A72. NDVI showed significant positive correlations with Fv/Fm, SCMR, and CTD as well as significant negative correlations with visual drought stress ratings, CT, and aflatoxin contamination (Table 19).

Pod yield. The harvested average pod yields ranged from 720-1405 g per plot (Table 17). In 2012, highest yield was obtained from C76-16 but was not significantly different from

Tifrunner and Florida-07. In 2013, Tifguard gave the highest yield but was not significantly different from Florida-07. Data analysis showed moderate negative correlation between pod yield and aflatoxin contamination. However, pod yield did not show significant correlation with the other evaluation methods (Table 19).

Aflatoxin contamination. A wide range of aflatoxin values were obtained from the plots. Therefore, the aflatoxin data were log-transformed to normalize the dataset. Results are shown in Table 18. Aflatoxin contamination was generally higher in the sheltered trial of 2012 than 2013. In 2012, Tifguard, Tifrunner, and Florida-07 had the numerically lowest aflatoxin contaminations, although it was not significantly different from C76-16 and A72. In the sheltered trial of 2013, only Tifguard was significantly different from the aflatoxin-susceptible check, A72. No significant differences were observed in the aflatoxin content among the genotypes under the unsheltered trial. Aflatoxin contamination showed significant positive correlations with visual ratings (r = 0.85 for both morning and afternoon ratings) and CT (r = 0.73) as well as significant negative correlations with Fv/Fm (r = -0.62), SCMR (r = -0.57), CTD (r = -0.81), NDVI (r = -0.79), and pod yield (r = -0.44) (Table 19).

2.4.2 Genetic relationship analysis of the peanut genotypes using SSR Markers

Plant samples (n = 16) within each genotype formed two to five branches, indicating that some samples had genetic differences ≥10% as compared to the rest of the samples. Nonetheless, each genotype formed its own individual clade. Tifguard was more closely related to Florida-07 than the rest of the genotypes while A72 was more closely related to C76-16 and 554CC. Tifrunner shared only ~40% similarity with Tifguard, Florida-07, A72, C76-16 and 554CC. All these six genotypes share a small amount of genetic similarity (<10%) with NC3033.

2.5 Discussion

Development of drought tolerance in peanut has been explored as an alternative to the more expensive, laborious, and destructive screening procedures for aflatoxin resistance. This came about as several studies reported that certain crop physiological traits which confer drought tolerance may be used as indirect selection criteria for the pre-harvest aflatoxin resistance in peanut. The peanut genotypes with drought tolerance traits generally showed lower levels of pre-harvest aflatoxin contamination indicating that they may possess some degrees of resistance to aflatoxin contamination (6; 33; 54). Considering that drought tolerance is a complex phenomenon that involves many mechanisms (72), it is useful to use combinations of several traits as selection criteria for drought tolerance rather than a single trait (55).

All the evaluation methods used in this study (visual ratings, chlorophyll fluorescence, SCMR, CT, CTD, NDVI, stomatal conductance, and pod yield) showed significant variation among genotypes in both rainout shelter locations suggesting their sensitivity to detect differences in genotypic response to drought tolerance. Significant G x T effects were frequently observed from the analysis of data from the NESPAL rainout shelter indicating that the genotypes may behave differently depending on water condition. This large G x T interaction has been reported to be very common in aflatoxin research and is acknowledged as the main reason for the inconsistent performance of peanut genotypes in response to aflatoxin contamination (6). It was, however, observed that the responses of Tifguard, Tifrunner and A72 across water treatments were usually more uniform. On the other hand, the performance of C76-16, Florida-07, 554CC and NC3033 showed variation in responses across water treatments and this may have been an important contribution to the significant G x T effects. The peanuts performed best under irrigated conditions while performing poorly under WPS due to the compounded effect of

drought and heat stress in the pod and root zones of the plants. The plants subjected to PZS showed intermediate performance between irrigated and WPS demonstrating that drought tolerance in peanut is affected by available water in the pod zone despite the amount of available water in the root zone.

Better visual appearance was observed in the irrigated than the drought-stressed plants. As with previous reports, the stressed plants experienced permanent foliage wilting, leaf shedding, and receding of canopy between rows (35). Leaf color change was also obvious. Such responses occurred as drought stress adversely affected photosynthesis, mineral nutrition, metabolism, and growth of the stressed plants (9; 38; 69). The general increase in the afternoon ratings as compared to the morning ratings may be attributed to the relative water content, osmotic potential, and leaf water potential of the plants. Peanut leaves have high relative water contents in the morning when solar radiation and vapor pressure deficits are low, followed by low water content around midday, and gradual increase in water content again after midday (21). These same patterns occur for the osmotic and water potentials (38). The afternoon ratings (measured around 1:00 PM) had a high probability of being affected by the high solar radiation and vapor pressure deficit of midday.

Plants exposed to drought conditions suffer from scarce water availability coinciding with high temperature, which leads to increased vulnerability to light stress and photoinhibition. Most plants adapt to these conditions by dissipating excess excitation energy thermally with the down regulation of their photosystem II (PSII) activity in order to protect their photosynthetic apparatus (63). The state of the PSII of the plants can be assessed through analysis of chlorophyll *a* fluorescence, which was measured in the present study using PI_{ABS}, Fv/Fm, and PHI_{EO}. The maximum quantum yield of PSII (Fv/Fm) measures the amount of light absorbed by chlorophyll

in PSII that is used in photochemical processes. Performance index (PI_{ABS}) is a multiparametric expression that takes into account all the main photochemical processes such as absorption and trapping of excitation energy, electron transport, and dissipation of excess excitation energy (43). Both parameters, together with the quantum yield of electron transport (PHI_{EO}), can be used to quantify the damage caused by environmental stress including high temperature, drought and excess light to the PSII and its effect on photosynthesis (17; 44; 47). As observed in the present study, the plants showed a reduction in chlorophyll fluorescence when exposed to drought stress which is similar to previous reports observed in cotton, peanut and barley (17; 46; 63). The ability of Tifguard, Tifrunner, C76-16 and Florida-07 to maintain higher chlorophyll fluorescence values under drought stress suggests that there was less damage to their PSII and that larger proportion of their photosynthetic structure remained more functionally intact (63).

Drought affects the chlorophyll content of crops, thereby, inhibiting leaf photosynthesis and photosynthetic capacity (37). The chlorophyll content per unit area of a leaf can be measured using SCMR through the light absorbance and/or transmittance characteristic of a leaf. SCMR has been reported to be positively correlated with chlorophyll content and chlorophyll density, thus, can be used to screen genotypic variation in photosynthetic capacity (7; 12; 49; 65). Maintenance of chlorophyll density under water-limited conditions had been suggested as a mechanism for drought resistance in peanut (37). In the present study, Tifguard consistently showed the highest SCMR in both locations suggesting that it contains higher chlorophyll content and has greater photosynthetic capacity than the other genotypes. Visual observation also showed that Tifguard has deep green leaf color which was retained by the plants despite exposure to drought stress.

The photosynthetic capacity of the plants under drought stress can also be assessed through NDVI (normalized difference vegetation index). This is a tool that uses the visible and near-infrared bands of the electromagnetic spectrum to analyze remote sensing measurements and assess live green vegetation (34). Healthy vegetation, which correlates to higher photosynthetic capacity, is detected as it absorbs most of the visible light that hits it and reflects a large portion of the near-infrared light. On the other hand, unhealthy or sparse vegetation reflects more visible light and less near-infrared light (74). This tool can be a good estimator of plant stress wherein it has been found that stressed or diseased plants have lower NDVI than healthy plants (5; 13; 56). The high NDVI of Tifguard, Tifrunner and Florida-07 indicate that a higher amount of green vegetation was detected by scanning the canopy of these plants as compared to scanning the canopy of the other genotypes. The lower NDVI of the other genotypes were probably affected by wilting or drying of plant canopy due to drought.

Plants exposed to drought conditions are also often subjected to high temperature (15). CT (canopy temperature) examines drought tolerance based on the negative correlation between leaf temperature and transpirational cooling (67). Peanut genotypes with lower CT, generally exhibited by Tifrunner, have higher transpiration and carbon dioxide exchange rate than genotypes with high CT (37). In relation to CT, CTD (canopy temperature depression) measures the deviation of plant temperature from ambient temperature. It is used to indicate overall plant water status resulting from the effects of several biochemical and morpho-physiological features acting at the stomata, leaf, and canopy levels (3; 73). High CTD is selected for drought and heat tolerance (39) Again, Tifrunner exhibited higher CTD than the rest of the genotypes under drought conditions. However, it was noticed that most of the genotypes yielded a negative CTD when stressed. A negative CTD is acquired when the temperature of the canopy is higher than

the ambient temperature. Genduoz *et al.* (26) explained that genotypes with a negative CTD indicate sensitivity to stress. This suggests that the genotypes used in this study might be drought-sensitive, with Tifrunner being least sensitive. However, reasons for the negative CTD may not be totally attributed to genotype response to drought. The measurement of the plant canopy and/or the ambient temperature may be affected by environmental factors like wind, evapotranspiration, cloudiness, air temperature, relative humidity, and continuous radiation leading to temperatures that are higher or lower than they truly are (57).

Plants exposed to drought conditions have been found to reduce their stomatal conductance to control water loss, diminish transpiration rate, prevent the dehydration of leaf tissue, and maintain turgescence (10; 17). Drought tolerance can be conferred by having a decreased stomatal conductance during the early stage of soil drying or decreased stomatal conductance when atmospheric vapour pressure deficit (VPD) is high. Contrary to the usual model that transpiration rate increases linearly as vapour pressure deficit (VPD) increases, Devi *et. al.* (20) reported that peanut genotypes have variation in this response wherein some genotypes have a breakpoint in transpiration rate when VPD increased. Lack of sensitivity to increasing VPD will cause the plants to continually increase their transpiration rates with increasing VPD. Limiting transpiration to a maximum rate when VPD is high will conserve water which can be available for use later in the season if when water is limited (19; 59; 64). All genotypes used in this study showed a general reduction in their stomatal conductance when exposed to drought stress as compared to the irrigated plants. A study conducted by Shekoofa *et. al.* (64) showed that the soil water thresholds for a decline in transpiration rate for C76-16, NC3033, Florida-07 and Tifrunner were 0.38, 0.44, 0.38 and 0.36, respectively. In addition, C76-16 had a transpiration rate breakpoint at 1.6 kPa, NC3033 and Florida-07 at 1.9 kPa, and

Tifrunner at 2.9 kPa. Results also showed that Tifguard and C76-16 under PZS and Tifguard under WPS maintained the highest stomatal conductance. Tifguard and C76-16 was not significantly different from Tifrunner under PZS, nor was Tifguard significantly different from Tifrunner and Florida-07 under WPS. According to Koolachart *et. al*. (41), genotypes that maintained higher stomatal conductance under drought have higher transpiration and CO_2 exchange rate, thus, maintaining higher photosynthetic capacity (41).

Highest pod yield was obtained from C76-16 in 2012 but was not significantly different from Tifrunner and Florida-07. In 2013, Tifguard had the highest pod yield but was not significantly different from Florida-07. The correlation analysis in the present study did not show significant relationships between yield and the different evaluation methods. However, the observations were similar to the study of Koolachart *et. al.* (41) showing that the peanut genotypes with higher SCMR and stomatal conductance and lower canopy temperature under terminal drought had higher pod yield under drought.

The drought-stressed plants had higher aflatoxin contamination as compared to irrigated plants. This result is similar to the study conducted by Payne *et al.* (53) where aflatoxin contamination was higher during years of drought but was reduced when irrigation was supplied. In 2012, the numerically lowest aflatoxin contaminations were exhibited by Tifguard, Tifrunner, and Florida-07. However, these were not significantly different from C76-16 and A72. Under the sheltered (drought-stressed) trial in 2013, Tifguard exhibited the numerically lowest aflatoxin contamination but was not significantly different from Tifrunner, C76-16 and Florida-07. It could also be noted that Tifguard and Tifrunner had lower aflatoxin contamination than C76-16 and Florida-07 when the data in sheltered trials from both years were averaged. There was no

significant difference among the aflatoxin contamination of the different genotypes in the unsheltered (rainfed) trial.

All the evaluation methods used at the Gibbs Farm rainout shelter, except PI_{ABS} and PHI_{EO}, produced significant high or moderate correlations with aflatoxin contamination. The correlations were positive for visual ratings and CT while correlations were negative for Fv/Fm, SCMR, CTD, NDVI, and pod yield. These results suggest that low visual ratings and CT as well as high Fv/Fm, SCMR, CTD, NDVI, and pod yield should be selected in breeding programs that aim to reduce pre-harvest aflatoxin contamination. The significant correlations between aflatoxin contamination and visual ratings and leaf temperature (33), and SCMR and pod yield (6), and ground-based reflectance (68) have been reported in previous studies.

Results of the correlation analysis also showed significant correlations among the different evaluation methods. This suggests the interrelatedness of these traits in the plant's mechanism of coping to drought stress. The significant correlations between visual ratings and Fv/Fm, SCMR, CT, CTD, and NDVI indicate that the effect of drought on other plant physiological traits will likely affect the visual appearance of the plant. The low visual ratings generally shown by Tifguard and Tifrunner suggest that these two genotypes possess physiological traits that allow them to adapt well to drought stress. The positive correlations between Fv/Fm and all the other evaluation methods indicate that the photosynthetic efficiency of the PSII is affected by the chlorophyll content (SCMR), canopy temperature, difference between canopy temperature and ambient temperature (CTD), and amount of green vegetation (NDVI). Similar to the report of Shahen and Isoda (63), SCMR had significant positive correlation with Fv/Fm and significant negative correlation with leaf temperature. This suggests that the decrease of chlorophyll content due to drought stress caused damage to the PSII and was

affected by high leaf temperature. The correlation analysis also showed positive correlation between SCMR and NDVI indicating that greater chlorophyll content is related to higher amount of green vegetation.

Each evaluation method has its own advantages and disadvantages in the evaluation of drought stress. The use of visual ratings offers an advantage over the other methods considering that no equipment is needed during plant evaluation. However, certain genotypes behaved differently in response to drought stress. Hence, the results can depend on the rater's subjective assessment of the status of the crop. The equipment used to measure chlorophyll fluorescence, SCMR, CT, CTD, and NDVI are light-weight, easy to use, rapid in giving measurements, and relatively low-cost. On the other hand, certain challenges were also faced using these methods. The evaluation of chlorophyll fluorescence required ratings before dawn, and thus, can be very challenging when measuring a large amount of genotypes or plant populations. It was observed that there were few differences in the results provided by the three chlorophyll fluorescence parameters (PI_{ABS}, Fv/Fm, PHI_{EO}) regarding genotypic responses to drought stress. Such difference in results was also reported by Lepedus *et al.* (43) between Fv/Fm and PI_{ABS} in their study with maize. This led to their recommendation that these parameters be combined when evaluating genotypes for drought tolerance. On the other hand, data from the Gibbs Farm showed very similar results for the PI_{ABS}, Fv/Fm, and PHI_{EO}. In that trial, Tifguard, Tifrunner, C76-16 and Florida-07 had fluorescence values that were significantly higher than the aflatoxin-susceptible check, A72. Furthermore, the correlation analysis showed that only Fv/Fm was significantly correlated with aflatoxin contamination. This suggests that Fv/Fm may be sufficient to evaluate chlorophyll fluorescence in peanut. It could be more beneficial to use Fv/Fm in combination with the other evaluation methods such as visual ratings and SCMR. The use of CT

and CTD can be affected greatly by weather conditions such as wind and cloud coverage. A possible reason why no significant difference in CT and CTD were observed among genotypes in the Gibbs Farm trials in 2013 may be due to the frequent windy weather. Such weather, especially towards the end of the season, may have kept the plant canopy cool. This may have resulted in similar CT measurements among plants. In the NESPAL trial, CT measurements were taken using infrared thermometer and thermal imaging. Results showed that CT measurements were lowest in Tifguard and Tifrunner via infrared thermometer. The lowest CT was obtained from Tifguard via thermal imaging but was not significantly different from Tifrunner and C76-16. Nevertheless, the results of both devices were similar. The slight difference in these results might be attributed to the difference in atmospheric factors like solar radiation during the time that the measurements were taken. By necessity, both methods cannot be measured at exactly the same time. Both infrared thermometer and thermal imaging can be used to measure CT and CTD but the choice of device will be dependent on the amount of area to be measured and the availability of equipment. Using an infrared thermometer is easier. However, the measurements must be taken quickly as a change in atmospheric factors over time can cause a change in the CT of the plants. Thermal imaging offers the advantage of taking an image and recording the CT measurements of several plant canopies in one shot, therefore, reducing the differences in temperatures over time. However, additional equipment and creative ways of use are needed to take images at an angle (usually above the plots) that can encompass the plants to be measured. The use of NDVI was very useful but can reflect various plant growth factors instead of exclusively reflecting the effect of one parameter, i.e. water availability (25). Stomatal conductance took much longer time to measure as compared to the other evaluation methods and required clear sky conditions during measurement. This limited the number of samples that could

be measured within the suggested 12:00 NN to 2:00 PM rating time. Nevertheless, although each evaluation method has its own advantages and disadvantages, the different methods also assessed different drought-coping mechanisms of the plants. This reiterates the usefulness of combining several measurements as selection criteria for drought tolerance (55).

In addition to comparing differences based on morphological and physiological responses to drought, the genetic relationships of the genotypes were also evaluated. A comprehensive list of SSR markers used in peanut was provided by Guo *et. al.* (28), from which 30 functional SSR markers that are polymorphic between Tifrunner and Florida-07 were used in the present study. The resulting dendrogram showed genetic variations among some plant samples within genotypes. This genetic variations within genotypes probably occurred due to outcrossing among plants as they were planted close to each other in the field (40). The samples of NC3033 divided into two distinct groups which show high genetic differences from each other. This likely explains why dimorphic responses to drought stress were observed within NC3033 rows/plots. Nevertheless, the seven genotypes separated into individual clades. Tifguard had the highest genetic similarity with Florida-07 indicative that these two genotypes shared a common parental lineage. Genotypes C76-16, 554CC and A72 showed higher genetic similarities to each other than the other genotypes. These are breeding lines used in Tifton, GA and may also have shared similar parental lineage. These six genotypes, all runner types, showed limited genetic similarity with NC3033 which is a Virginia-type peanut (8). Results of these SSR markers can be used to characterize individuals and breeding lines and identify candidate parental genotypes for breeding of drought tolerant lines (58). The genetic relationships can be used to identify genotypic similarities and/or variations that are not clearly differentiated by morphological or physiological traits. These morphological and physiological traits may be influenced by many

factors such as G x E effects and polygenic inheritance of agronomic traits (66). In addition, the evaluation of genetic relationships can help eliminate duplicates in the peanut collection (1). Tifguard and Florida-07 were shown to have the closest genetic distance but were not the genotypes which behaved most similarly in response to drought stress in the field. Tifguard and Tifrunner behaved most similarly in response to drought stress in the field but had lower genetic similarities. Thus, in the future, it would be advantageous to identify which traits found in Tifguard and Tifrunner but not found in Florida-07 are able to confer tolerance to drought stress and/or resistance to pre-harvest aflatoxin contamination.

2.6 Summary and Conclusions

The differences in drought-stress response of seven peanut genotypes were assessed based on seven evaluation methods. Among the genotypes evaluated, Tifguard and Tifrunner generally showed better drought-coping mechanisms than the other genotypes. Tifguard and Tifrunner would, therefore, be good candidates to be incorporated into plant breeding programs for the development of pre-harvest aflatoxin resistance. Results of the study also showed that aflatoxin contamination had high correlations with visual ratings, CT, CTD, and NDVI and moderate correlations with the Fv/Fm and SCMR ($P \geq 0.05$). Thus, these easily measurable evaluation methods can be helpful in improving breeding programs. The use of these methods is also less costly than the measurement of aflatoxin contamination, hence, can reduce the cost of developing resistant line or cultivars. Florida-07 shared the highest genetic similarity with Tifguard but these were not the genotypes which behaved most similarly in response to drought stress in the field. On the other hand, Tifguard and Tifrunner behaved more similarly in response to drought stress in the field but had lower genetic similarities. In the future, it would be

advantageous to identify which traits similar to Tifguard and Tifrunner but different from Florida-07 can confer tolerance to drought stress and/or resistance to pre-harvest aflatoxin contamination.

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Figure 1. General criteria used for the visual rating of drought stress ranging from a scale of 1-5.

A1. NESPAL Rainout Shelter

Table 1. Mean[a] morning visual drought stress ratings of the different genotypes under the different water treatments.

GENOTYPE	2012[b]			2013[b]		
	Pod Zone Stress	Whole Plant Stress	Irrigated	Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	1.207 d	1.240 d	1.245	1.411 e	1.578 c	1.239 d
Tifrunner	1.510 bcd	1.272 cd	1.130	1.600 de	2.061 bc	1.494 cd
C76-16	1.433 cd	1.973 ab	1.395	1.941 cd	3.389 a	1.803 bc
Florida-07	1.940 a	2.113 ab	1.255	2.150 bc	1.686 c	2.497 a
554CC/NC3033[c]	1.567 bc	2.230 a	1.300	2.406 ab	2.329 bc	2.100 ab
A72	1.823 ab	1.687 bc	1.475	2.715 a	2.831 a	2.353 a

[a] Ratings were based on the general visual appearance of the plants within a replicate. Mean ratings were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 2. Mean[a] afternoon visual ratings of the different genotypes under the different water treatments.

GENOTYPE	2012[b]			2013[b]		
	Pod Zone Stress	Whole Plant Stress	Irrigated	Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	1.223 c	1.373 c	1.285	1.589 c	2.417 bc	1.400 d
Tifrunner	1.590 b	1.384 c	1.340	1.982 bc	2.733 bc	1.725 cd
C76-16	1.510 bc	2.080 ab	1.455	2.161 b	3.806 a	2.053 bc
Florida-07	2.050 a	2.353 a	1.375	2.430 ab	2.097 c	2.869 a
554CC/NC3033[c]	1.677 b	2.433 a	1.430	2.709 a	2.767 bc	2.397 b
A72	1.833 ab	1.860 bc	1.540	2.769 a	2.986 ab	2.878 a

[a] Ratings were based on the general visual appearance of the plants within a replicate. Mean ratings were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test.
[c] 554CC was used in 2012 then replaced with NC3033 in 2013.

Table 3. Mean[a] PI_{ABS} values of the different genotypes under the different water treatments.

GENOTYPE	2012[b]			2013[b]		
	Pod Zone Stress	Whole Plant Stress	Irrigated	Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	5.192 b	5.496 a	4.589 bcd	5.587 a	5.303 a	6.226 a
Tifrunner	6.040 a	5.514 a	6.354 a	5.080 a	3.694 ab	6.145 a
C76-16	4.816 b	5.230 a	5.237 b	4.146 b	1.767 cd	6.058 a
Florida-07	3.766 c	3.804 b	5.459 b	2.640 c	3.669 ab	5.076 b
554CC/NC3033[c]	4.900 b	4.814 a	5.594 abc	1.604 c	2.407 bc	4.659 ab
A72	3.399 c	3.258 b	4.127 d	1.492 c	0.722 d	3.278 b

[a] Measurements were taken from three plants per replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 4. Mean[a] Fv/Fm values of the different genotypes under the different water treatments.

GENOTYPE	2012[b]	2013[b]		
		Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	0.829 ab	0.837 a	0.840 a	0.849 a
Tifrunner	0.842 a	0.812 a	0.685 ab	0.840 ab
C76-16	0.813 bc	0.612 b	0.428 c	0.823 ab
Florida-07	0.795 c	0.554 bc	0.754 ab	0.836 ab
554CC/NC3033[c]	0.820 b	0.354 d	0.634 b	0.789 bc
A72	0.815 b	0.524 b	0.345 c	0.766 c

[a] Measurements were taken from three plants per replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test. The single column in 2012 indicates that G x T effect was not significant, thus, only genotypic differences are shown. In 2013, G x T effect was significant, thus, all values are shown.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 5. Mean[a] PHI_{Eo} values of the different genotypes under the different water treatments.

GENOTYPE	2012[b]	2013[b]		
		Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	0.492 a	0.521 a	0.527 a	0.523 a
Tifrunner	0.504 a	0.475 a	0.391 b	0.514 ab
C76-16	0.463 b	0.360 b	0.218 c	0.495 ab
Florida-07	0.444 c	0.309 bc	0.432 ab	0.496 ab
554CC/NC3033[c]	0.470 b	0.182 d	0.358 b	0.453 bc
A72	0.429 c	0.257 cd	0.187 c	0.391 c

[a] Measurements were taken from three plants per replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test. The single column in 2012 indicates that G x T effect was not significant, thus, only genotypic differences are shown. In 2013, G x T effect was significant, thus, all values are shown.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 6. Mean[a] soil plant analysis development (SPAD) chlorophyll meter reading (SCMR) of the different genotypes under the different water treatments.

GENOTYPE	2012[b]	2013[b]		
		Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	43.637 a	39.979 a	43.566 a	43.586 a
Tifrunner	36.256 bc	36.200 a	33.334 b	38.143 b
C76-16	34.369 cd	35.630 b	24.580 c	39.182 b
Florida-07	35.704 bc	29.549 b	33.553 b	31.681 c
554CC/NC3033[c]	36.637 b	20.431 c	23.586 c	28.087 d
A72	33.055 d	23.104 c	15.832 d	30.075 cd

[a] Measurements were taken from five plants per replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test. The single column in 2012 indicates that G x T effect was not significant, thus, only genotypic differences are shown. In 2013, G x T effect was significant, thus, all values are shown.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 7. Mean[a] canopy temperature (^{o}C) of the different genotypes under the different water treatments measured using infrared thermometer.

GENOTYPE	2012[b]			2013[b]
	Pod Zone Stress	Whole Plant Stress	Irrigated	
Tifguard	29.110	32.418 a	27.562	30.606 c
Tifrunner	30.566	28.915 c	29.000	30.875 c
C76-16	29.640	30.442 abc	29.066	32.788 b
Florida-07	29.974	32.218 ab	26.868	32.468 b
554CC/NC3033[c]	29.496	31.980 ab	30.355	33.373 b
A72	28.755	29.959 bc	28.957	35.475 a

[a] Measurements were taken from five plants per replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test. In 2012, G x T effect was significant, thus, all values are shown. In 2013, the single column indicates that G x T effect was not significant, thus, only genotypic differences are shown.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 8. Mean[a] canopy temperature depression (^{o}C) of the different genotypes under the different water treatments measured using infrared thermometer.

GENOTYPE	2012[b]			2013[b]
	Pod Zone Stress	Whole Plant Stress	Irrigated	
Tifguard	-0.069	-3.149 c	1.574 ab	2.114 a
Tifrunner	-1.302	0.337 a	0.257 bc	1.845 a
C76-16	-0.361	-1.131 ab	0.202 bc	-0.068 b
Florida-07	-0.464	-2.975 c	2.357 a	-0.252 b
554CC/NC3033[c]	-0.378	-2.700 bc	-1.116 c	-0.653 b
A72	-0.462	-0.758 a	0.349 bc	-2.755 b

[a] Measurements were taken from five plants per replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test. In 2012, G x T effect was significant, thus, all values are shown. In 2013, the single column indicates that G x T effect was not significant, thus, only genotypic differences are shown.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 9. Mean[a] canopy temperature (CT, ^{o}C) and canopy temperature depression (CTD, ^{o}C) of the different genotypes under the different water treatments measured using thermal imaging.

GENOTYPE	2013[b]	
	CT	CTD
Tifguard	33.716 c	-0.525 a
Tifrunner	34.457 bc	-1.267 ab
C76-16	34.447 bc	-1.256 ab
Florida-07	35.413 ab	-2.223 bc
554CC/NC3033[c]	35.276 ab	-2.085 b
A72	36.710 a	-3.786 c

[a] Measurements were taken from five plants per replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 10. Mean[a] normalized difference vegetation index (NDVI) of the different genotypes under the different water treatments.

GENOTYPE	2012[b]	2013[b]		
		Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	0.674 b	0.785 a	0.824 a	0.817 a
Tifrunner	0.713 a	0.737 a	0.680 ab	0.734 b
C76-16	0.637 bc	0.599 b	0.388 d	0.628 c
Florida-07	0.665 bc	0.601 b	0.717 ab	0.553 cd
554CC/NC3033[c]	0.629 cd	0.573 bc	0.565 c	0.600 c
A72	0.591 d	0.456 c	0.528 cd	0.505 d

[a] Readings were taken throughout the canopy of each replicate. Mean values were calculated from three replicates for each genotype exposed to pod-zone stress and whole plant stress and two replicates for each genotype exposed to irrigated treatment.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test. The single column in 2012 indicates that G x T effect was not significant, thus, only genotypic differences are shown. In 2013, G x T effect was significant, thus, all values are shown.
[c] 554CC was used in 2012, then replaced with NC3033 in 2013.

Table 11. Mean[a] stomatal conductance (mmol/m^2s) of the different genotypes under the different water treatments.

GENOTYPE	2013[b]		
	Pod Zone Stress	Whole Plant Stress	Irrigated
Tifguard	34.020 a	33.321 a	45.720 ab
Tifrunner	27.405 ab	24.489 ab	70.130 a
C76-16	30.573 a	10.038 c	38.280 bc
Florida-07	9.333 c	18.699 abc	16.160 c
NC3033	15.554 bc	17.064 bc	44.300 abc
A72	6.276 c	11.220 bc	22.500 bc

[a] Measurements were taken from three plants per replicate. Mean values were calculated from two replicates for each genotype under each treatment.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test.

A2. Gibbs Farm Rainout Shelter

Table 12. Mean[a] morning (AM) and afternoon (PM) visual ratings of the different genotypes under sheltered and unsheltered conditions.

GENOTYPE	2012[b]		2013[b]	
	AM	PM	AM	PM
Tifguard	1.53 cd	1.65 cd	1.411 c	1.489 c
Tifrunner	1.34 d	1.61 d	1.435 c	1.515 c
C76-16	1.70 cd	1.88 bcd	1.517 bc	1.569 bc
Florida-07	1.95 bc	2.13 abc	1.508 bc	1.604 bc
554CC	2.25 ab	2.28 ab	1.756 a	1.839 a
A72	2.44 a	2.55 a	1.609 b	1.665 b

[a] Ratings were based on the general visual appearance of the plants within a replicate. Mean ratings were calculated from eight replicates per genotype.
[b] Means with different letters within a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test.

Table 13. Mean[a] PI_{ABS}, Fv/Fm ratio and PHI_{EO} values of the different genotypes under sheltered and unsheltered conditions measured using infrared thermometer.

GENOTYPE	2012[b]			2013[b]		
	PI_{ABS}	Fv/Fm	PHI_{EO}	PI_{ABS}	Fv/Fm	PHI_{EO}
Tifguard	5.171	0.816	0.477	5.224 b	0.839 b	0.484 b
Tifrunner	5.667	0.832	0.499	6.119 a	0.851 a	0.511 a
C76-16	5.372	0.828	0.485	5.275 b	0.839 b	0.481 bc
Florida-07	5.289	0.821	0.486	5.116 b	0.839 b	0.478 bc
554CC	5.326	0.830	0.493	4.801 bc	0.830 cd	0.465 cd
A72	5.103	0.825	0.483	4.262 c	0.827 d	0.448 d

[a] Measurements were taken from three plants per replicate. Mean values were calculated from eight replicates per genotype.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test.

Table 14. Mean[a] soil plant analysis development (SPAD) chlorophyll meter reading (SCMR) of the different genotypes under sheltered and unsheltered conditions.

GENOTYPE	2012[b]	2013[b]
Tifguard	42.45 a	46.433 a
Tifrunner	39.88 b	41.945 b
C76-16	39.12 bc	40.248 c
Florida-07	38.54 bcd	41.928 b
554CC	36.58 d	42.362 b
A72	37.32 cd	38.855 d

[a] Measurements were taken from five plants per replicate. Mean values were calculated from eight replicates per genotype.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test.

Table 15. Mean[a] canopy temperature (CT, ^{o}C) and canopy temperature depression (CTD, ^{o}C) of the different genotypes under sheltered and unsheltered conditions.

GENOTYPE	2012[b]		2013[b]	
	CT	CTD	CT	CTD
Tifguard	29.962 ab	-1.259 abc	24.324	0.871
Tifrunner	29.418 b	-0.530 a	23.740	1.455
C76-16	30.518 ab	-1.236 abc	23.943	1.251
Florida-07	30.064 ab	-1.175 ab	24.853	0.342
554CC	31.293 a	-2.196 c	24.328	0.867
A72	30.833 ab	-2.046 bc	23.962	1.233

[a] Measurements were taken from five plants per replicate. Mean values were calculated from eight replicates per genotype.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test.

Table 16. Mean[a] normalized difference vegetation index (NDVI) of the different genotypes under sheltered and unsheltered conditions.

GENOTYPE	2012[b]	2013[b]	
	Sheltered	Sheltered	Unsheltered
Tifguard	0.718 a	0.761 ab	0.773 ab
Tifrunner	0.736 a	0.784 a	0.756 abc
C76-16	0.662 bc	0.739 bc	0.747 bc
Florida-07	0.697 ab	0.745 bc	0.780 a
554CC	0.620 d	0.731 bc	0.765 ab
A72	0.633 cd	0.719 c	0.734 c

[a] Readings were taken throughout the canopy of each replicate. Mean values were calculated from eight replicates per genotype.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test. Only sheltered trial was conducted in 2012 while both sheltered and unsheltered trials were conducted in 2013.

Table 17. Mean[a] pod yield (g) per 1.5 m plot of the different genotypes harvested from sheltered and unsheltered trials.

GENOTYPE	2012[b]	2013[b]
	Sheltered	
Tifguard	1,090.75 bc	1,346.00 a
Tifrunner	1,308.25 ab	1,035.10 b
C76-16	1,405.25 a	1,030.90 b
Florida-07	1,336.50 ab	1,260.50 ab
554CC	720.00 d	790.00 c
A72	1,038.50 c	766.30 c

[a] Mean values were calculated from eight replicates per genotype.
[b] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test. Only sheltered trial was conducted in 2012 while both sheltered and unsheltered trials were conducted in 2013. No significant G x T effect was observed in 2013, thus, only genotypic differences are shown.

Table 18. Mean[a] aflatoxin content (log-transformed, ppb)[b] of the different genotypes measured from sheltered and unsheltered trials.

GENOTYPE	2012[c]	2013[c]	
	Sheltered	Sheltered	Unsheltered
Tifguard	1.173 b	0.7431 bc	0.7500
Tifrunner	1.093 b	1.0227 ab	0.7159
C76-16	1.457 ab	1.0780 ab	0.7443
Florida-07	1.091 b	1.2775 ab	0.5797
554CC	1.878 a	1.6305 a	0.8295
A72	1.707 ab	1.4135 a	0.6021

[a] Mean values were calculated from eight replicates per genotype.
[b] A wide range of aflatoxin values were obtained from the experiment. The data were log-transformed to normalize the dataset.
[c] Means with different letters within a column are significantly different at P≤0.05 as determined by Fisher LSD test. Only sheltered trial was conducted in 2012 while both sheltered and unsheltered trials were conducted in 2013.

Table 19. Correlation between aflatoxin contamination, yield (g/1.5 m) and the drought stress evaluation methods used in the study.

	AFLA TOXIN	YIELD	CHLOROPHYLL FLUORESCENCE			VISUAL RATINGS		SCMR	CT (^{o}C)	CTD (^{o}C)
			PI_{ABS}	Fv/Fm	PHI_{EO}	AM	PM			
Pod Yield	-0.442**									
PI_{ABS}	-0.274	0.255								
Fv/Fm	-0.620**	-0.121	0.555**							
PHI_{EO}	-0.257	0.251	0.982**	0.588**						
Visual Rating (AM)	0.847**	-0.399	-0.286	-0.414*	-0.249					
Visual Rating (PM)	0.848**	-0.263	-0.235	-0.504**	-0.208	0.977**				
SCMR	-0.572**	0.318	0.217	0.426*	0.210	-0.599**	-0.622**			
CT (^{o}C)	0.733**	0.001	-0.087	-0.636**	-0.099	0.728**	0.837**	-0.502**		
CTD (^{o}C)	-0.808**	0.188	0.148	0.418*	0.151	-0.793**	-0.847**	0.372	-0.917**	
NDVI	-0.788**	0.169	0.249	0.624**	0.240	-0.842**	-0.872**	0.750**	-0.809**	0.704**

Significant correlation at P≤0.05 and P≤0.10 are indicated by ** and *, respectively as determined by Fisher LSD test.

B. SSR Markers

Table 20. Amplification results of the initial screening of SSR markers. SSR marker information was adapted from Guo et al (28).

#	MARKER	FORWARD PRIMER SEQUENCE (5'-3')	REVERSE PRIMER SEQUENCE (5'-3')	LABEL[a]	SCREENING RESULT
1	GM0002	TCAACGCGACACAAGAAGTC	GTCGGTAAATCCGACGAAAA	FAM	No amplification
2	GM0004	AAGGGGTTAAGGGCATGACT	CCACAAATGGGTCGTCGAT	FAM	Amplified
3	GM0011	ACCGTTACGAACGCTTTGTC	TCCCTCTCATACGACACCCT	FAM	Amplified
4	GM0023	ATGTGGGGAGGTCGGTAAC	TCACAGGTTTTGTGTGCTCG	FAM	Amplified
5	GM0038	CTCTCCGCCATCCATGTAAT	ATGGTGAGCTCGACGCTAGT	FAM	Amplified
6	GM0070	TCACAATCAGAGCTCCAACAA	CAGGTTCACCAGGAACGAGT	FAM	Amplified
7	GM0071	CGAAAACGACACTATGAAACTGC	CCTTGGCTTACACGACTTCCT	FAM	Amplified
8	GM0074	GAAGGACCCCATCTATTCAAA	TCCGATTTCTCTCTCTCTCTCTC	FAM	Amplified
9	GM0076	AATGGGGTTCACAAGAGAGAGA	CCAGCCATGCACTCATAGAATA	FAM	Amplified
10	GM0090	TTAGCGACAAAGGATGGTGAG	TAGGGACGAAAATAGGGACTGA	FAM	Amplified
11	GM0099	CCATGTGAGGTATCAGTAAAGAAAGG	CCACCAACAACATTGGATGAAT	FAM	Amplified
12	GM0175	CCGAGGTAGAATCGCAAGC	GTAATGCCAGGGAGAATCAGC	FAM	Amplified
13	GM0009	CAGCAAAGAGTCGTCAGTCG	GAAAGTTCACTTGAGCAAATTCA	HEX	Amplified
14	GM0024	GAATTTATAAGGCGTGGCGA	CCATCCCTTCTTCCTTCACA	HEX	Amplified
15	GM0048	CTTTCTTCCCCCTTGAACCT	GATCAAGTGAAAATGTTAGTATAAG	HEX	No amplification
16	GM0346	CTGATGCATGTTTAGCACACTT	TGAGTTGTGACGGCTTGTGT	HEX	Amplified
17	GM0405	TGGGCCTAAACCCAACCTAT	CCACAAACAGTGCAGCAATC	HEX	Amplified
18	GM0424	AATGCATGAGCTTCCATCAA	AACCCCATCTTAAAATCTTACCAA	HEX	No amplification
19	GM0429	TACAGCATTGCCTTCTGGTG	CCTGGGCTGGGGTATTATTT	HEX	Amplified
20	GM0443	CCTCCCTGCTTGATCCAATA	AACTGTAGCGAATGTGTTACATGG	HEX	Amplified
21	GM0028	GCCCATATCAAGCTCCAAAA	TAGCCAGCGAAGGACTCAAT	HEX	Amplified
22	GM0032	TGAAAGATAGGTTTCGGTGGA	CAAACCGAAGGAGGAACTTG	HEX	Amplified
23	GM0072	GGCAGGGGAATAAAACTACTAACT	TTTTCCTTCCTTCTCCTTTGTC	HEX	No amplification
24	GM0089	GCCAAAGGGGACCATAAAC	TCCATCTTCCATCTCATCCAC	HEX	Amplified

25	GM0056	GATCCAACTGTGAATTGGGC	CACACCAGCAACAAGGAATC	TAMRA	Amplified
26	GM0066	GAAATTTTAGTTTTCAGCACAGCA	TTTTCCCCTCTTAAATTTTCTCG	TAMRA	Amplified
27	GM0079	AGGTTGGAACTATGGCTGATTG	CCAGTTTAGCATGTGTGGTTCA	TAMRA	Amplified
28	GM0098	TGAGTCTGTGGAAGAATAAGAGAAG	TGAGTCTATCGCCGCCTAC	TAMRA	Amplified
29	GM0108	CTCGCTATACTAGGTTTTGGGTGT	TGGTTTGCCTTTCTAGCCATTA	TAMRA	No amplification
30	GM0126	TGTCTCTCTTCCTTTCCTTGCT	CCTTTTGCTTCTTTGCTTCC	TAMRA	Amplified
31	GM0382	TGAGTTGTGACGGCTTGTGT	GATGCATGTTTAGCACACTTGA	TAMRA	Amplified
32	GM0422	GGGAATAGCGAGATACATGTCAG	CAGGAGAGAAGGATTGTGCC	TAMRA	No amplification
33	GM0477	AAAAGAAAGACCTTCCCCGA	GCAGGTAATCTGCCGTGATT	TAMRA	Amplified
34	GM0496	TCTGTTGAGAACCACCAGCA	GTGCTAGTTGCTTGACGCAC	TAMRA	Amplified
35	GM0508	CATGTCTCCATGAGCATTTCA	TGGATGTGGACAGCATATCG	TAMRA	Amplified
36	GM0540	ATTCCCATGTCGTCAAGACC	GCGACGGTATTGGCTTTTAG	TAMRA	Amplified

[a]Forward primers were fluorescent dye-labeled with FAM, HEX or TAMRA

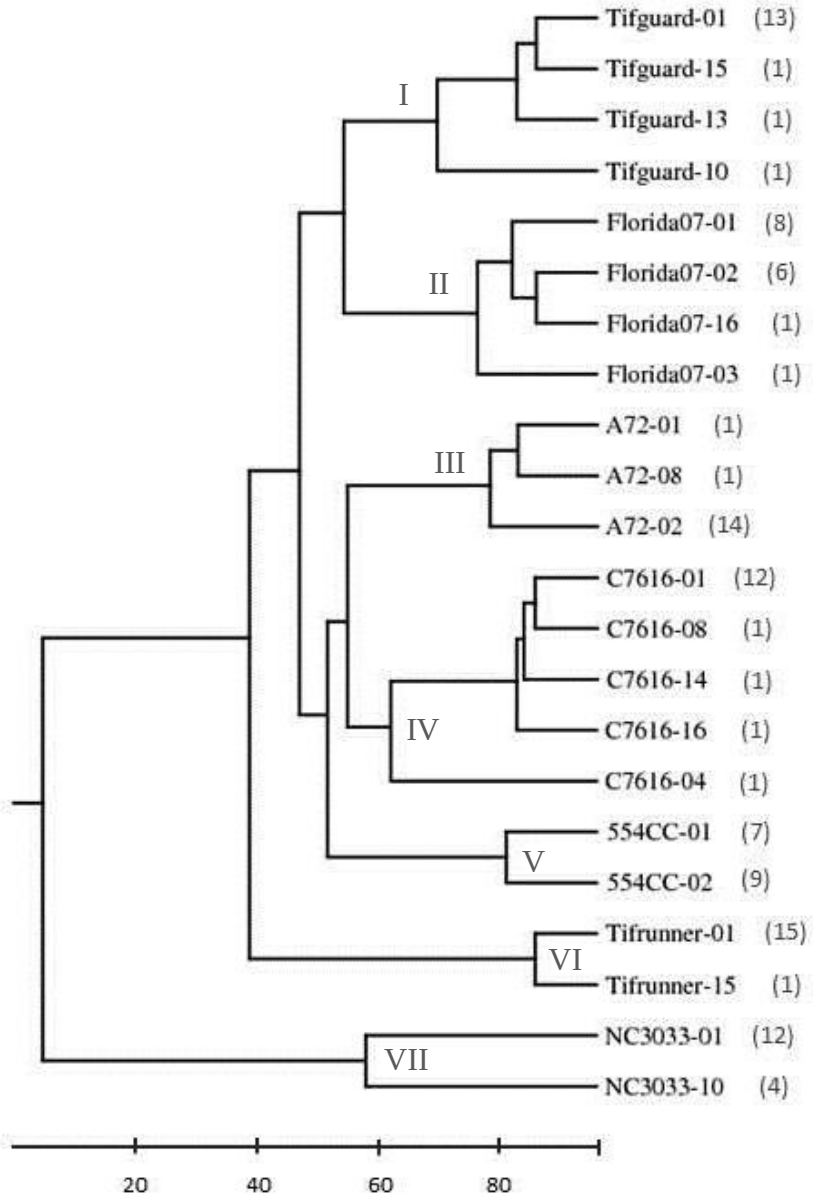

Figure 2. Collapsed dendogram showing the genetic relationship of the seven peanut genotypes analysed using 30 SSR markers. Sample names are composed of the genotype followed by sample number (16 samples per genotype; n=112). Due to ample amount of samples, genetic similarity was assessed where samples with ≥90% similarity were collapsed so that only one representative sample is shown. The number of samples showing ≥90% similarity is enclosed in parenthesis.

CHAPTER 3

CHARACTERIZATION OF *ASPERGILLUS* SECTION *FLAVI* ISOLATES COLLECTED FROM DIFFERENT GEOGRAPHICAL LOCATIONS[1]

[1] Luis, J.M., Glenn, A.E. and Kemerait Jr., R.C. To be submitted to *Mycologia.*

3.1 Abstract

The genus *Aspergillus* section *Flavi* includes species and strains that are aflatoxigenic and those that are atoxigenic which are used in food fermentation or as biological control agents. Correct identification of species and strains is, therefore, very crucial. This study aimed to characterize isolates (n = 99) from Georgia, Texas, and Alabama of the United States; Haiti; and Philippines through morphological characterization, conventional DNA sequencing, and repetitive-sequence-based PCR (rep-PCR) DNA fingerprinting. The rep-PCR approach is based on the amplification of intervening sequences located within the short repetitive DNA sequences dispersed throughout an organism's genome. Morphological characterization showed observable differences in colony surface color and conidial texture among isolates but no obvious differences in colony reverse color, growth diameter, and production and color of sclerotia. Genetic sequence variation based on beta-tubulin did not discriminate among *A. flavus*, *A. parasiticus* and *A. oryzae*. However, using both internal transcribed spacer (ITS) region and translation elongation factor (TEF1-α) produced more specific results. Rep-PCR of a subset of isolates identified 93.34% (42) of the isolates as *A. flavus*, 4.44% (2) as *A. parasiticus* and 2.22% (1) as *A. oryzae*.

Keywords: *Aspergillus flavus*, *A. parasiticus*, *A. oryzae*, ITS, sclerotia, conidia, beta-tubulin, TEF1-α, rep-PCR DNA fingerprinting

3.2 Introduction

Aspergillus is large genus of asexual fungi (Fungi Imperfecti or Deuteromycetes) with over 180 recognized species divided into ten sections (28; 30). Many of these species have great impact as human, animal, and plant pathogens, as producers of toxic secondary metabolites, as food spoilage agents, and as important microorganisms used in food fermentation and industrial bioprocesses. Section *Flavi* is one of the most economically important sections because it includes *A. flavus* and *A. parasiticus* which are the major aflatoxin producers affecting agronomic crops (29; 32). Aflatoxins are of great concern because of their hepatotoxic and immunosuppressive properties that can cause mortality, reduced productivity, weaker immunity and hepatocellular carcinoma (liver cancer) in livestock and humans (4; 36). *Aspergillus flavus* is the predominant species on many susceptible commodities including peanut, corn, cottonseeds, pepper seeds, tree nuts, cereals, soybean, and cassava. *Aspergillus parasiticus* is more common on peanut than on any other crop, but is typically outcompeted by *A. flavus* when both fungi are present (1; 2; 5; 15; 23; 24; 34). *Aspergillus nomius* has also been reported as a strong aflatoxin producer but is rarely identified in soils and food commodities (33). In contrast to these aflatoxigenic species and strains, some atoxigenic strains of *A. flavus* are atoxigenic and serve as useful biological control agents against toxigenic strains (7). Section *Flavi* also includes *A. oryzae* and *A. sojae* which also atoxigenic and widely used in food fermentation and industrial bioprocesses in Asian countries (32). The close morphological and phylogenetical relatedness of these aflatoxigenic and atoxigenic species and strains (3; 32) warrants the need for correct species identification in order to properly identify species that are safe for food processing as well as identify species that pose risks to human and livestock health. The identification of

atoxigenic *A. flavus* strains can also be used in further development of biological control agents against aflatoxigenic strains.

The classic systematics of *Aspergillus* section *Flavi* has been based primarily on cultural and microscopic characteristics (17; 20; 32). The texture of the conidial wall, growth rate, conidial diameter and colony colors were reported as important criteria for differentiating species (12; 20). The identification based on these criteria, however, can be time-consuming, laborious, and complex since it requires significant training and expertise in laboratory mycology (1; 14; 22). In addition, the extensive divergence in morphological characters caused by the high level of genetic variability is adding difficulty to the identification of fungal species. Therefore, recent studies regarding species identification has shifted to polyphasic approaches which combine morphological and genetic approaches (37).

Molecular methods have been widely used in the identification of *Aspergillus* species. Several results obtained from these molecular methods generally correlate with morphological and physiological traits that are observed through cultural and microscopic methods (27; 32). Variation in the genetic sequences of the isolates on species-specific gene targets such as the internal transcribed spacer (ITS) region (13; 22), partial calmodulin (37), β-tubulin (10), translation elongation factor 1-α (TEF1-α) (26), and RNA polymerase II (RPB2) (25) have been explored. Still, some problems can persist due to potential similarity of the sequenced fungus to other fungal genera and species (13).

A recently introduced approach for species and strain identification is the use of the semi-automated repetitive-sequence-based polymerase chain reaction (rep-PCR) DNA fingerprinting. This approach is based on the amplification of intervening sequences that are located within the short repetitive DNA sequences dispersed throughout an organism’s genome (22; 38). It has been

reported to provide accurate discriminations among bacterial and fungal isolates from clinical and field sources (12; 13; 22; 38) as well as to predict and distinguish the black-spored morphotypic *Aspergillus* species belonging to section *Nigri* (22).

This study aimed to identify *Aspergillus* section *Flavi* isolates that were collected from peanut samples from different geographic locations through the combined use of cultural and microscopic morphological characterization, conventional DNA sequencing, and rep-PCR DNA fingerprinting. It also aimed to evaluate the discriminatory ability of these various approaches in species identification.

3.3 Materials and Methods

3.3.1 Collection, preparation, and storage of isolates

Peanut samples collected from 10 peanut-growing states of the United States (US) were provided by Dr. Charles Bacon of the Richard B. Russell Research Center, USDA-ARS, Athens, Georgia. Isolates NRRL 3357 (*A. flavus*) and NRRL 2999 (*A. parasiticus*) were obtained from USDA-ARS, Tifton, Georgia. These two strains plus the commercial atoxigenic *A. flavus* biological control strain (NRRL 218822) used in Aflaguard® were used as standards. Peanut samples from Haiti and Philippines were also collected. The kernels were surface-sterilized with 10% bleach for five min, rinsed thrice with distilled water, plated in Czapek Yeast Agar (CYA) and incubated at 34^{o}C until fungal growths appeared. Streptomycin (1 µl/ml) was added into the culture media to prevent bacterial contamination. The *Aspergillus* colonies belonging to section *Flavi* were identified based on their green to olive brown colony colors in CYA (17; 20). Pure cultures were obtained through single spore isolation and maintained in CYA at 34^{o}C or stored in 15% glycerol at -80^{o}C for future use.

3.3.2 Morphological characterization

Observations were made on the macroscopic characteristics of the isolates that were grown on CYA incubated at 34^{o}C for seven days. These characteristics included colony surface and reverse colors, growth diameter, presence or absence of sclerotia, and color of sclerotia if present. Colors of the colony and sclerotia were assessed visually and described based on Ridgway's Color Standards and Color Nomenclature (28). Wet mounts were prepared to observe the texture of the conidial wall under the compound microscope.

3.3.3 Conventional sequencing

Fungal genomic DNA was recovered from 7-day old cultures using the cetyl trimethyl ammonium bromide (CTAB) method modified from Graham *et al*. (11). The internal transcribed spacer (ITS) region, beta-tubulin, and translation elongation factor 1-α (TEF1-α) were sequenced using primer pairs ITS5/ITS4 (ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3'/ ITS4: 5'TCC TCCGCTTATTGATATGC-3'), Tub2F/Tub2R (Tub2F: 5'-TCGAGAACTCCGATGAGACCTT -3'/ Tub2R: 5'-GTCAGAGGAGCAAATCCAACCA-3'), and TEF983F/TEF2218R (TEF983F: 5'-ATGGGTAAGGAGGACAAGAC-3'/ TEF2218R: 5'-GGAAGTAACAGTGATCATGTT-3') as described by White *et al*. (39), Glass and Donaldson (10), and Peterson *et al*. (26), respectively. The resulting amplicons were purified using the QIAquick PCR Purification Kit (QIAgen Sciences, MD) and sent to the USDA-ARS Eastern Regional Research Center Facility, Wyndmoor, PA for sequencing. Sequencing errors were detected and corrected using the software Sequencher v. 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). The isolates were identified based on their % similarity to the referenced strains in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.4 DNA extraction and repetitive-sequence-based-PCR DNA fingerprinting

Single-spored cultures were grown in CYA at 34^{o}C for seven days. From these, a 10 µl loop of fungal culture was used for genomic DNA extraction using the UltraClean™ Microbial DNA isolation kit (Mo Bio Laboratories, Solano Beach, CA) following the manufacturer's instructions. DNA yield was increased by heating the fungal material at 65^{o}C for 10 min before extraction and extending bead beating from 10 to 30 min as recommended by Bacterial Barcodes when testing fungi. The DNA concentration was determined using a Nanodrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE) and standardized to approximately 25 ng/µl. The DNA was subjected to rep-PCR for DNA fingerprinting using the DiversiLab *Aspergillus* kit (Bacterial Barcodes, Inc., Houston, TX) following the manufacturer's instructions. The detection and analysis of the rep-PCR results were done through the DiversiLab system. Fragments of various sizes and intensities of the samples loaded into a micro-chip were detected by the Agilent 2100 Expert Bioanalyzer (Agilent Tenchnologies, Palo Alto, CA). Results generated include electropherograms and virtual gel images of the samples. Further analysis was performed through the web-based DiversiLab software version 3.3 using Pearson's correlation coefficient to generate the distance matrices used for the unweighted pair group method analysis (UPGMA) dendrogram. The rep-PCR DNA fingerprints of the unknown isolates were compared to previously identified fingerprints in the DiversiLab *Aspergillus* library.

3.4 Results

3.4.1 Collected isolates

Some *Aspergillus* species outside the section *Flavi* have been recognized as aflatoxin producers but only the species belonging to this section remain associated with the

contamination of agronomic food and food commodities. Thus, only the species belonging to section *Flavi* were included in this study.

The initial screening of peanut samples collected from the 10 states of US showed highest percentage of seed infection incidences in Georgia (33.33%), Texas (21.43%), and Alabama (22.86%) (Table 21). These states were chosen for further collection of isolates, leading to collection of 29, 24 and 24 isolates from Georgia, Texas and Alabama, respectively (Table 22). Isolates from Philippines (16) and Haiti (6) were also collected. All these amount to a total of 99 isolates. The standards for *A. flavus* (NRRL 3357), *A. parasiticus* (NRRL 2999) and the atoxigenic strain of *A. flavus* (NRRL 21882, Aflaguard®) were used as controls.

3.4.2 Morphological characterization

Morphological characterization showed several similarities among the 99 isolates collected across the geographical location sites composed of Georgia, Texas and Alabama of US; Haiti; and Philippines. The morphological characterization of these isolates was compared with NRRL 3357, NRRL 2999, and Aflaguard®. Color descriptions used for colony surface color, colony reverse color and sclerotia were based on Ridgway's Color Standards and Color Nomenclature (31).

Colony surface color. A variety of colors were observed ranging from five shades of green (dark green as ivy green; and shades of olive green as cress green, olive green, parrot green, and rainette green), two shades of yellow (aniline yellow and primuline yellow), and shades of green with shades of yellow areas (Tables 23-24). Isolated with dark green color (ivy green) was initially identified as *A. parasiticus* (5 isolates, 5.05%), shades of olive green as *A.*

flavus (91 isolates, 91.92%), and shades of yellow as *A. oryzae* (3 isolates, 3.03%) based on species description provided by Klich (17).

Colony reverse color. This refers to the color of the colony when observed from the back of the CYA plate. In the study, eight colors were observed ranging from cream/uncolored to cinnamon buff (Tables 23 and 25). When compared to the standard isolates, Aflaguard has cream/uncolored reverse color but both NRRL 3357 and NRRL 2999 had light drab colors. This suggests that the color of the reverse side of the colony may be used to characterize *Aspergillus* section *Flavi* isolates but would not be helpful in identifying them into species.

Production and color of sclerotia. A total of 24 isolates (24.24%) did not produce sclerotia (Tables 23 and 26). The remaining 75 isolates (75.75%) produced either gray, walnut brown or black sclerotia. When compared to the standard isolates, both NRRL 3357 and NRRL 2999 produced brown sclerotia. This indicates that the color of sclerotia is not a helpful criterion to differentiate between species.

Colony growth diameter. The mean growth diameter of the 99 isolates ranged from 70-85 mm (Tables 23 and 27). The standard NRRL 3357 had a mean growth diameter of 85 mm while both NRRL 2999 and Aflaguard® had 75 mm. This indicates that the growth of *A. flavus* and *A. parasiticus* can overlap and, hence, is not helpful in the identification of species.

Texture of conidial wall. The standard isolate NRRL 2999 is characterized by having a rough conidial wall while both NRRL 3357 and Aflaguard® had smooth to finely roughened conidial walls. Such observations were similar to the species description provided by Klich (17). Rough conidial walls were identified by their pronounced irregular wall surfaces and obvious ornamentations; smooth conidial walls by even and regular surface appearance; while finely roughened conidial walls by uneven wall surfaces with mildly noticeable ornamentations.

Among the 99 isolates, four (4.04%) had rough conidial walls and identified as *A. parasiticus*. There were 91 isolates (93.93%) with smooth conidial walls and four isolates (4.04%) with finely roughened conidial walls. All these isolates (95 isolates, 97.98%) were considered as *A. flavus* (Tables 23 and 28).

In summary, morphological characterization using the different parameters gave varying results. Combining the characterizations based on colony surface color and conidial wall texture gave more reliable identification.

3.4.3 Conventional sequencing

Seven isolates were used for conventional sequencing by targeting the internal transcribed spacer (ITS) region, beta-tubulin, and translation elongation factor 1-α (TEF1-α) using the primer pairs ITS5/4, Tub2F/Tub2R, and TEF983/TEF2218R, respectively (Table 29). The DNA sequences of these isolates were compared to referenced sequences in the GenBank using BLAST. Results showed that the discrimination of the isolates using these three primer pairs varied. Out of six isolates tested, the ITS primers discriminated five isolates as *A. flavus*, and identified one isolate as 99% similar to *A. flavus, A. parasiticus,* and *A. oryzae*. The beta-tubulin primers identified all six isolates tested as 99% or 98% similar to *A. flavus, A. oryzae* and *A. parasiticus*. Of the four isolates tested using TEF1-α primers, two isolates were discriminated as *A. flavus* and two as 99% similar to *A. flavus* and *A. oryzae*. Although the results are limited to a small number of sequenced samples, results of the experiment showed the lack of capability of the primers Tub2F/Tub2R to discriminate among *A. flavus, A. parasiticus,* and *A. oryzae*. Both the ITS and TEF1-α primers were able to discriminate some isolates as *A. flavus*. Certain isolates

were not discriminated between *A. flavus* and *A. oryzae* most probably due to the high similarity between the genomes of these two species (9).

3.4.4 rep-PCR DNA fingerprinting

Of the 99 isolates, 45 representative isolates composed of 10 isolates from Georgia, nine from Alabama, nine from Texas, five from Haiti, and 12 from Philippines were used for rep-PCR DNA fingerprinting. Isolates NRRL 3357, NRRL 2999, and Aflaguard® were also included.

Results of the rep-PCR identified two isolates (4.44%) from Alabama (AL1E and AL2A) as *A. parasiticus* based on their high genetic similarity to NRRL 2999 and AG-50 from the DiversiLab Library (Table 30 and Figure 3). On the other hand, AL1E and AL2A showed only about 60% genetic similarity to the rest of the isolates. Forty-two isolates (93.34%) were identified as *A. flavus* based on their high genetic similarities to NRRL 3357 and several referenced samples in the DiversiLab Library. Analysis based on the genetic distance matrices revealed that these *A. flavus* isolates shared high genetic similarities of above 95%. The dendrogram did not show marked clustering of these isolates according to geographical location. However, it was observed that the isolates from Georgia, Texas and Alabama mostly clustered together while a majority of the isolates from Philippines (except PHD1, PHL2A2, and PHA1A) clustered with the isolates from Haiti. Three isolates from Georgia (GA1C, GA2D and GA1A) formed a group with Aflaguard®. Results also identified one isolate (2.22%) from Texas (TX3A) as *A. oryzae* as it showed closest genetic similarity to AG-26 from the DiversiLab Library. The ability of rep-PCR to identify TX3A despite high genetic similarity to the *A. flavus* isolates indicates its high sensitivity and specificity to discriminate among *Aspergillus* section *Flavi* species. High discriminatory power to identify strains was also observed.

3.5 Discussion

Morphological characterization based on cultural and microscopic methods has been widely used to discriminate species of the genus *Aspergillus* section *Flavi* (17; 37). In agreement to the results of Rodrigues *et al.* (32), a better chance of species identification was achieved by combining colony surface color and texture of conidial wall. *Aspergillus flavus* was identified by different shades of green and yellow green colonies, *A. parasiticus* by dark green colonies, and *A. oryzae* by yellow colonies as grown on Czapek Yeast Extract Agar (CYA) based on the descriptions given by Klich (17). However, a few identifications based on surface color did not match the identification by rep-PCR. This may have occurred due to the observer's subjective assessment of the colony color because visual color assessment can also be influenced by the quality of lighting, texture of the colonies, and other factors (15; 19). Horn *et al.* (16) suggest the use of color image processing with digital color images of cultures and filtered conidia using the RGB (red, green, blue) model as an alternative to visual color assessment so as to avoid this problem in the future. For the texture of conidial wall, *A. parasiticus* was identified by a rough conidial wall while *A. flavus* by a smooth to finely roughened wall (18). Identification of the *A. parasiticus* isolates based on their rough conidial wall conformed to the results of rep-PCR. However, it was difficult to morphologically distinguish between *A. flavus* and *A. oryzae* because both species exhibit smooth to finely roughened conidial walls (17). This overlap in morphological characteristics suggests the need for more thorough identification methods. In addition, morphological variability at the intra-specific level and inter-specific similarity among species generally occur (32).

The collected isolates were subjected to conventional DNA sequencing and compared to referenced samples in GenBank for species identification. As stated by Rodrigues *et al.* (32),

molecular identification using a single DNA sequence can be accurate but cannot clearly discriminate between very closely related species in *Aspergillus* section *Flavi*, especially those that are related to *A. flavus*. Therefore, three target genes were used. Sequence variation using primers that target beta-tubulin (Tub2F/Tub2R) did not discriminate among *A. flavus*, *A. parasiticus*, and *A. oryzae* suggesting that there is limited sequence variation among the species detected by the primers in this gene. The primers targeting the ITS region (ITS5/ITS4) and TEF1-α (TEF983F/TEF2218R) were able to identify selected isolates as *A. flavus*. However, a few isolates remained indistinguishable between *A. flavus* and *A. oryzae*. As shown by Rokas *et al* (35), *A. flavus*, *A. parasiticus*, and *A. oryzae* have high degrees of DNA relatedness wherein *A. flavus* and *A. oryzae* are almost identical. Difficulty of discriminating between these two species can be associated to their highly similar genomes (3). Both species has a genome size of about 37 Mb (3), which when evaluated using an array based genome comparison only show 709 genes that are uniquely polymorphic between the two species (9). Better identification of isolates into species was obtained by combining the results of ITS and TEF1-α. This led to the identification of all isolates as *A. flavus* which conforms to the results obtained from using rep-PCR.

The use of rep-PCR via the DiversiLab system produced the most clear-cut results. The majority of the isolates (42 isolates, 93.34%) were identified as *A. flavus* with only two (4.44%) as *A. parasiticus* and one (2.22%) as *A. oryzae*. As previously known, *A. flavus* is the predominant species on many agronomic commodities. Only two *A. parasiticus* isolates were obtained because this species is normally outcompeted by *A. flavus* when both fungi are present (1). No *A. parasiticus* was isolated from the Philippines supporting the report of Frisvad *et al.* (8) that *A. parasiticus* is geographically restricted to USA, South America, and Australia. One isolate (2.22%) from Texas (TX3A) was identified as *A. oryzae*. When assessed, TX3A shares

94% genetic similarity to the *A. flavus* isolates. This shows the high ability of rep-PCR to discriminate among *Aspergillus* section *Flavi* isolates. Clustering within the *A. flavus* isolates also indicates the high sensitivity of the method to discriminate among strains.

A defined clustering based on geogprahical location was not observed among distribution of *A. flavus* in the obtained dendrogram. Some clustering was observed across different region and/or subregion but there were also genetic variations. The isolates from Georgia, Texas and Alabama mostly clustered together while the isolates from Haiti clustered with a majority of the isolates from Philippines (with the exemption of PHD1, PHL2A2, and PHA1A). Moreover, all four isolates from Mt. Province, Philippines clustered together while those from La Union and Tuguegarao showed high genetic similarities with those from Cap-Haitian, Haiti. According to Orum *et al.* (21), environmental factors like temperature, soil condition, day length, crop sequence history, rainfall, and management practice may influence *A. flavus* communities. The US has a temperate climate while both Haiti and Philippines are both tropical. Therefore, all the above mentioned environmental factors are more likely to be similar for Georgia, Texas and Alabama as well as for Haiti and Philippines. A cooler temperature is also exhibited by Mt. Province due to its higher land elevation as compared to the hotter temperatures of La Union and Tuguegarao which is similar to Cap-Haitian, Haiti. Having similar environmental factors most likely led to planting of similar peanut types and implementation of similar management strategies in the field. Thus, it may be possible that similar environmental factors and field practices favored the selection of common strains. Three isolates from Georgia (GA1C, GA2D, and GA1A) clustered with Aflaguard®. Since NRRL 21882 (Aflaguard®) was isolated from Georgia (6), these isolates may be possible variants of Aflaguard® and can be explored as possible biological control agents.

3.6 Summary and Conclusions

Ninety-nine isolates belonging to *Aspergillus* section *Flavi* were collected from Georgia, Texas, Alabama of US; Haiti; and, Philippines. These were characterized based on morphology and genetic variation. The identification of unknown isolates based on morphological characteristics can be beneficial when used for screening purposes. The color of colony surface and texture of conidial wall were most helpful in identifying species belonging to *Aspergillus* section *Flavi*. Using this method can reduce the cost of reagents used in molecular methods. However, this method can be time-consuming and requires sufficient mycology training to be successful. An alternative to morphological characterization is the identification of unknown samples based on genetic sequence variation. The primers that target beta-tubulin (Tub2F/Tub2R) did not discriminate among *A. flavus, A. parasiticus,* and *A. oryzae*, while using the primers that target ITS (ITS5/ITS4) and TEF1-α (TEF983F/TEF2218R) together produced more specific results. Rep-PCR was observed to be the most sensitive method. It identified 93.34% of the isolates as *A. flavus*, 4.44% as *A. parasiticus*, and 2.22% as *A. oryzae*. The isolates did not show a marked clustering based on geographic location. However, isolates from Georgia, Texas and Alabama mostly clustered together whereas isolated from Haiti clustered with most of the isolates from Philippines. Three isolates from Georgia formed a group with Aflaguard® suggesting their being possible variants of Aflaguard® which can be explored as biological control against aflatoxigenic strains.

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Table 21. Initial screening for infection by *Aspergillus* section *Flavi* of peanut samples collected from 10 states of the US.[a]

STATE	NO. OF FARMS/ SOURCES	SEED INFECTION		
		No. of Plated Seeds	No. of Infected Seeds	% Incidence[b]
Alabama	7	35	8	22.86
Florida	6	30	5	16.67
Georgia	3	15	5	33.33
Mississippi	1	5	0	0.00
New Mexico	2	10	0	0.00
North Carolina	9	45	2	4.44
Oklahoma	3	15	2	13.33
South Carolina	4	20	3	15.00
Texas	14	70	15	21.43
Virginia	4	20	2	10.00
TOTAL	53	265	42	15.85

[a] Five surface-sterilized seeds from each location/source were plated in Czapek Yeast Extract Agar (CYA) and incubated at 34^{o}C until fungal growths appeared. Isolates belonging to *Aspergillus* section *Flavi* were identified based on their characteristic colony colours described by Klich (17).
[b] % incidence was calculated by dividing the number of infected seeds by the number of plated seeds then multiplied by 100.

Table 22. Final number and frequency of *Aspergillus* section *Flavi* isolates collected from Georgia, Texas, and Alabama of US; Haiti; and Philippines that were used in the study.

COUNTRY/ STATE	SEED INFECTION			OCCURRENCE (%)[c]
	No. of Plated Seeds	No. of Infected Seeds[a]	% Incidence[b]	
Georgia	40	29	72.50	29.30
Texas	100	24	24.00	24.24
Alabama	55	24	43.64	24.24
Philippines	50	16	32.00	16.16
Haiti	35	6	17.14	6.06
TOTAL	280	99	35.36	100.00

[a] Each infected seed corresponds to one isolate
[b] % incidence was calculated by dividing the number of infected seeds by the number of plated seeds then multiplied by 100.
[c] % occurence was calculated by dividing the number of infected seeds by the total number infected seeds then multiplied by 100.

Table 23. List and morphological characterization of *Aspergillus* isolates collected from US, Haiti, and Philippines.

#	SAMPLE	LOCATION/ SOURCE	COLONY CHARACTERISTICS			SCLEROTIA[a]	CONIDIAL WALL TEXTURE[b]
			Surface Color[a]	Reverse Color[a]	Growth Diameter (mm)		
1.	NRRL 3357	USDA-ARS Tifton, GA	cress green	light drab	85	walnut brown	smooth
2.	NRRL 2999	USDA-ARS Tifton, GA	ivy green	light drab	75	walnut brown	rough
3.	Aflaguard®	Commercial Product	parrot green	cream/uncolored	75	none	smooth
4.	GA1A	Tifton, GA (Seg III peanuts)	cress green	light drab	76	none	smooth
5.	GA1B	Tifton, GA (Seg III peanuts)	parrot green	pale orange-yellow	81	walnut brown	smooth
6.	GA1C	Tifton, GA (Seg III peanuts)	rainette green	light drab	81	none	smooth
7.	GA1D	Tifton, GA (Seg III peanuts)	rainette green	salmon	83	none	finely rough
8.	GA1E	Tifton, GA (Seg III peanuts)	rainette green	salmon	85	none	smooth
9.	GA1F	Tifton, GA (Seg III peanuts)	rainette green	light drab	78	none	smooth
10.	GA1G	Tifton, GA (Seg III peanuts)	rainette green	light drab	82	none	smooth
11.	GA1H	Tifton, GA (Seg III peanuts)	rainette green	salmon buff	77	walnut brown	smooth
12.	GA1I	Tifton, GA (Seg III peanuts)	cress green with PYA	light drab	77	walnut brown	smooth
13.	GA1J	Tifton, GA (Seg III peanuts)	ivy green	light drab	80	none	smooth
14.	GA1K	Tifton, GA (Seg III peanuts)	cress green	light drab	75	walnut brown	smooth
15.	GA1L	Tifton, GA (Seg III peanuts)	rainette green with PYA	light drab	76	black	smooth
16.	GA1M	Tifton, GA (Seg III peanuts)	cress green with PYA	light drab	85	none	smooth
17.	GA2A	Tifton, GA (Seg III peanuts)	rainette green	light drab	82	none	smooth
18.	GA2B	Tifton, GA (Seg III peanuts)	rainette green	salmon	82	walnut brown	smooth
19.	GA2C	Tifton, GA (Seg III peanuts)	cress green with PYA	drab gray	81	black	smooth
20.	GA2D	Tifton, GA (Seg III peanuts)	parrot green	drab gray	82	black	smooth
21.	GA2E	Tifton, GA (Seg III peanuts)	rainette green	drab gray	84	black	smooth
22.	GA2F	Tifton, GA (Seg III peanuts)	cress green with PYA	salmon buff	83	none	smooth
23.	GA2G	Tifton, GA (Seg III peanuts)	rainette green	salmon buff	84	walnut brown	smooth
24.	GA2H	Tifton, GA (Seg III peanuts)	rainette green	salmon	83	walnut brown	smooth
25.	GA2I	Tifton, GA (Seg III peanuts)	ivy green	light drab	83	none	smooth
26.	GA2J	Tifton, GA (Seg III peanuts)	cress green	salmon buff	78	none	smooth
27.	GA2K	Tifton, GA (Seg III peanuts)	olive green	light drab	80	walnut brown	smooth

28.	GA2L	Tifton, GA (Seg III peanuts)	olive green	drab gray	82	black	smooth
29.	GaA	UGA-Tifton, GA (Field trial)	olive green	cinnamon buff	82	none	smooth
30.	GaA2	UGA-Tifton, GA (Field trial)	ivy green	cinnamon buff	85	none	smooth
31.	Yma	UGA-Tifton, GA (UGA seed lab)	cress green	salmon	85	black	smooth
32.	DrB1a	UGA-Tifton, GA (UGA seed lab)	cress green with PYA	light drab	83	none	smooth
33.	TX1A	Seagraves, TX (Peanut farm)	rainette green	salmon buff	85	walnut brown	smooth
34.	TX1B	Seagraves, TX (Peanut farm)	rainette green with PYA	salmon buff	85	puritan gray	smooth
35.	TX3A	Seminole, TX (Peanut farm)	primuline yellow	light drab	85	walnut brown	smooth
36.	TX3B	Seminole, TX (Peanut farm)	rainette green	pale orange-yellow	85	puritan gray	smooth
37.	TX3C	Seminole, TX (Peanut farm)	cress green with PYA	light drab	84	walnut brown	smooth
38.	TX3D	Seminole, TX (Peanut farm)	rainette green with PYA	pale orange-yellow	81	gray	smooth
39.	TX3E	Seminole, TX (Peanut farm)	rainette green	cream/uncolored	82	gray	smooth
40.	TX4A	Brownfield, TX (Peanut farm)	cress green	salmon buff	83	black	smooth
41.	TX4B	Brownfield, TX (Peanut farm)	rainette green	light drab	81	gray	smooth
42.	TX4C	Brownfield, TX (Peanut farm)	parrot green	light drab	84	gray	smooth
43.	TX7B	Brownfield, TX (Peanut farm)	cress green with PYA	salmon buff	83	black	smooth
44.	TX7C	Brownfield, TX (Peanut farm)	cress green	light drab	82	black	smooth
45.	TX12A	Brownfield, TX (Peanut farm)	rainette green	salmon	83	black	smooth
46.	TX12B	Brownfield, TX (Peanut farm)	citrine green	light drab	79	gray	smooth
47.	TX12C	Brownfield, TX (Peanut farm)	primuline yellow	salmon buff	78	gray	smooth
48.	TX13A	Seagraves, TX (Peanut farm)	rainette green with PYA	pale orange-yellow	81	black	smooth
49.	TX14A	Seagraves, TX (Peanut farm)	cress green with PYA	salmon buff	82	gray	smooth
50.	TX14B	Seagraves, TX (Peanut farm)	olive green	salmon buff	80	gray	smooth
51.	TX14C	Seagraves, TX (Peanut farm)	olive green	light drab	85	gray	smooth
52.	TX14D	Seagraves, TX (Peanut farm)	rainette green with PYA	light drab	84	gray	smooth
53.	TX15A	Lubbock, TX (Peanut farm)	parrot green with PYA	light drab	85	black	smooth
54.	TX15B	Lubbock, TX (Peanut farm)	rainette green	pale orange-yellow	83	walnut brown	smooth
55.	TX15C	Lubbock, TX (Peanut farm)	cress green	salmon buff	83	walnut brown	smooth
56.	TX15D	Lubbock, TX (Peanut farm)	cress green	pale orange-yellow	85	black	smooth
57.	AL1A	Eufaula, AL (Peanut farm)	rainette green with PYA	salmon buff	85	walnut brown	smooth
58.	AL1B	Eufaula, AL (Peanut farm)	rainette green	salmon buff	83	black	smooth
59.	AL1C	Eufaula, AL (Peanut farm)	rainette green with PYA	pale orange-yellow	82	black	smooth
60.	AL1D	Eufaula, AL (Peanut farm)	ivy green	light drab	82	black	rough

61.	AL1E	Eufaula, AL (Peanut farm)	cress green with AYA	light drab	83	gray	rough
62.	AL2A	Slocomb, AL (Peanut farm)	aniline yellow	pale orange-yellow	85	none	rough
63.	AL2B	Slocomb, AL (Peanut farm)	rainette green with PYA	salmon buff	84	walnut brown	smooth
64.	AL2C	Slocomb, AL (Peanut farm)	cress green	pale orange-yellow	82	walnut brown	smooth
65.	AL2D	Slocomb, AL (Peanut farm)	cress green	light drab	80	gray	smooth
66.	AL3A	Ashford, AL (Peanut farm)	rainette green	salmon buff	82	walnut brown	smooth
67.	AL3B	Ashford, AL (Peanut farm)	rainette green with PYA	cream/uncolored	85	gray	smooth
68.	AL3C	Ashford, AL (Peanut farm)	rainette green	salmon buff	82	black	smooth
69.	AL3D	Ashford, AL (Peanut farm)	olive green	salmon buff	80	gray	smooth
70.	AL3E	Ashford, AL (Peanut farm)	cress green with PYA	salmon buff	84	black	smooth
71.	AL3F	Ashford, AL (Peanut farm)	rainette green with PYA	salmon buff	82	walnut brown	smooth
72.	AL3G	Ashford, AL (Peanut farm)	olive green	light drab	84	walnut brown	smooth
73.	AL4A	Headland, AL (Peanut farm)	olive green with AYA	salmon buff	85	walnut brown	smooth
74.	AL4B	Headland, AL (Peanut farm)	parrot green with PYA	cream/uncolored	81	gray	smooth
75.	AL4C	Headland, AL (Peanut farm)	parrot green with PYA	pale orange-yellow	85	walnut brown	smooth
76.	AL4D	Headland, AL (Peanut farm)	olive green	salmon	84	black	smooth
77.	AL4E	Headland, AL (Peanut farm)	ivy green	pale orange-yellow	85	walnut brown	smooth
78.	AL4F	Headland, AL (Peanut farm)	cress green	light drab	85	black	smooth
79.	AL4G	Headland, AL (Peanut farm)	ivy green	light drab	85	black	smooth
80.	AL4H	Headland, AL (Peanut farm)	parrot green	light drab	84	walnut brown	smooth
81.	PHA1A	Tuguegarao, PH (Buying point)	rainette green with PYA	salmon buff	78	walnut brown	smooth
82.	PHA1B	Tuguegarao, PH (Buying point)	parrot green with PYA	salmon buff	82	walnut brown	smooth
83.	PHB1A	Tuguegarao, PH (Buying point)	parrot green	cinnamon buff	80	black	smooth
84.	PHB1B	Tuguegarao, PH (Buying point)	parrot green	cream/uncolored	80	black	smooth
85.	PHC1A	Tuguegarao, PH (Buying point)	rainette green with PYA	cinnamon buff	77	black	smooth
86.	PHC2A	Tuguegarao, PH (Buying point)	cress green	salmon buff	77	black	smooth
87.	PHD1	Tuguegarao, PH (Buying point)	cress green	cinnamon buff	80	black	smooth
88.	PHD2	Tuguegarao, PH (Buying point)	cress green	cinnamon buff	84	black	smooth
89.	PHE1	Tuguegarao, PH (Buying point)	parrot green	drab gray	78	gray	smooth
90.	PHE2	Tuguegarao, PH (Buying point)	parrot green	drab gray	77	gray	smooth
91.	PHC2	Mt. Province, PH (Culture)	cress green	drab	81	walnut brown	finely rough
92.	PHC3	Mt. Province, PH (Culture)	cress green	cream/uncolored	78	walnut brown	smooth
93.	PHC4	Mt. Province, PH (Culture)	parrot green	light drab	81	walnut brown	smooth

94.	PHC5	Mt. Province, PH (Culture)	cress green	cream/uncolored	80	walnut brown	smooth
95.	PHL2A1	La Union, PH (Peanut farm)	cress green	cream/uncolored	84	gray	finely rough
96.	PHL2A2	La Union, PH (Peanut farm)	cress green	cinnamon buff	83	gray	finely rough
97.	HT2A	Cap Haitian, HT (Peanut farm)	parrot green	salmon buff	85	black	smooth
98.	HT2B	Cap Haitian, HT (Peanut farm)	parrot green	salmon buff	84	gray	smooth
99.	HT2C	Cap Haitian, HT (Peanut farm)	parrot green	cream/uncolored	82	walnut brown	smooth
100.	HT301	Cap Haitian, HT (Peanut farm)	parrot green	salmon buff	80	gray	smooth
101.	HT404	Cap Haitian, HT (Peanut farm)	parrot green	cinnamon buff	78	none	smooth
102.	HT504	Cap Haitian, HT (Peanut farm)	rainette green	salmon buff	81	walnut brown	smooth

[a] Observations were made from 7-day old cultures grown on CYA at 34^{o}C. Color descriptions were based on Ridgway's Color Standards and Color Nomenclature (31). AYA = aniline yellow areas; and, PYA = primuline yellow areas.

[b] Rough conidial walls were characterized by pronounced irregular wall surfaces and obvious ornamentations; smooth conidial walls by even and regular surface appearance; while finely roughened conidial walls by uneven wall surfaces with mildly noticeable ornamentations.

Table 24. Colony surface color at CYA incubated for seven days at 34°C for 99 isolates collected from Georgia, Texas, and Alabama of US; Haiti; and Philippines.

COLONY SURFACE COLOR[a]	NO. OF ISOLATES					TOTAL	OCCURRENCE (%)	INITIAL IDENTIFICATION
	Georgia	Texas	Alabama	Haiti	Philippines			
A. Shades of Green (6)								
1. Cress green	3	4	3	-	8	18	18.18	*A. flavus*
2. Ivy green	2	-	3	-	-	5	5.05	*A. parasiticus*
3. Olive green	3	3	3	-	-	9	9.09	*A. flavus*
4. Parrot green	4	1	1	5	5	16	16.16	*A. flavus*
5. Rainette green	11	6	3	1	-	21	21.21	*A. flavus*
Subtotal	23	14	13	6	13	69	69.70	
B. Shades of Yellow (2)								
1. Aniline yellow	-	-	1	-	-	1	1.01	*A. oryzae*
2. Primuline yellow	-	2	-	-	-	2	2.02	*A. oryzae*
Subtotal	0	2	1	0	0	3	3.03	
C. With Primuline Yellow Area (PYA) (3)								
1. Cress green with PYA	5	3	1	-	-	9	9.09	*A. flavus*
2. Parrot green with PYA	-	1	2	-	1	4	4.04	*A. flavus*
3. Rainette green with PYA	1	4	5	-	2	12	12.12	*A. flavus*
Subtotal	6	8	8	0	3	25	25.25	
D. With Aniline Yellow Area (AYA) (2)								
1. Olive green with AYA	-	-	1	-	-	1	1.010	*A. flavus*
2. Cress green with AYA	-	-	1	-	-	1	1.010	*A. flavus*
Subtotal	0	0	2	0	0	2	2.020	
TOTAL	29	24	24	6	16	99	100.00	

[a] Color description was based on Ridgway's Color Standards and Color Nomenclature (31). The standard isolates used include NRRL 3357 (*A. flavus*) as cress green, NRRL 2999 (*A. parasiticus*) as ivy green, and Aflaguard® (atoxigenic *A. flavus*) as cress green.

Table 25. Colony reverse color at CYA incubated after seven days at 34°C of 99 isolates collected from Georgia, Texas, and Alabama of US; Haiti; and Philippines.

COLONY REVERSE COLOR[a]	NO. OF ISOLATES					TOTAL	OCCURRENCE (%)
	Georgia	Texas	Alabama	Haiti	Philippines		
1. Cream/uncolored	0	1	2	1	5	9	9.09
2. Salmon buff	4	8	9	4	3	28	28.28
3. Pale yellow-orange	5	1	1	-	-	7	7.07
4. Drab gray	1	5	5	-	-	11	11.11
5. Light drab	4	-	-	-	2	6	6.06
6. Drab	-	-	-	-	1	1	1.01
7. Cinnamon buff	13	9	7	-	1	30	30.30
8. Salmon	2	-	-	1	4	7	7.07
TOTAL	29	24	24	6	16	99	100.00

[a] Color description was based on Ridgway's Color Standards and Color Nomenclature (31). The standard isolates used include NRRL 3357 (*A. flavus*) and NRRL 2999 (*A. parasiticus*) which both have light drab reverse colors, and Aflaguard® (atoxigenic *A. flavus*) as cream/uncolored.

Table 26. Production and color of sclerotia at CYA incubated for seven days at 34°C of 99 isolates collected from Georgia, Texas, and Alabama of US; Haiti; and Philippines.

PRODUCTION AND COLOR OF SCLEROTIA[a]	NO. OF ISOLATES					TOTAL	OCCURRENCE (%)
	Georgia	Texas	Alabama	Haiti	Philippines		
1. No sclerotia produced	15	7	1	1	-	24	24.24
2. Puritan gray	-	12	5	2	4	23	23.23
3. Walnut brown	8	5	10	2	6	31	31.31
4. Black	6	-	8	1	6	21	21.21
TOTAL	29	24	24	6	16	99	100.00

[a] Color description was based on Ridgway's Color Standards and Color Nomenclature (31). The standard isolates used include NRRL 3357 (*A. flavus*) and NRRL 2999 (*A. parasiticus*) which both produced walnut brown sclerotia, and Aflaguard® (atoxigenic *A. flavus*) that did not produce sclerotia.

Table 27. Growth diameter (mm) at CYA incubated for seven days at 34°C of 99 isolates collected from Georgia, Texas, and Alabama of US; Haiti; and Philippines.

MEAN GROWTH DIAMETER (mm)	
Georgia	76 – 85
Texas	78 – 85
Alabama	80 – 85
Haiti	78 – 85
Philippines	77 – 84
NRRL 3357 (*A. flavus*)	85
NRRL 2999 (*A. parasiticus*)	75
NRRL 21882 (Aflaguard®)	75

Table 28. Conidial wall texture at CYA incubated for seven days at 34°C of 99 isolates collected from Georgia, Texas, and Alabama of US; Haiti; and Philippines.

TEXTURE OF CONIDIAL WALL	NO. OF ISOLATES					TOTAL	OCCURRENCE (%)	INITIAL IDENTIFICATION[a]
	Georgia	Texas	Alabama	Haiti	Philippines			
1. Rough	-	-	4	-	-	4	4.04	*A. parasiticus*
2. Smooth	28	24	22	6	13	91	91.92	*A. flavus*
3. Finely roughened	1	-	-	-	3	4	4.04	*A. flavus*
TOTAL	29	24	24	6	16	99	100.00	

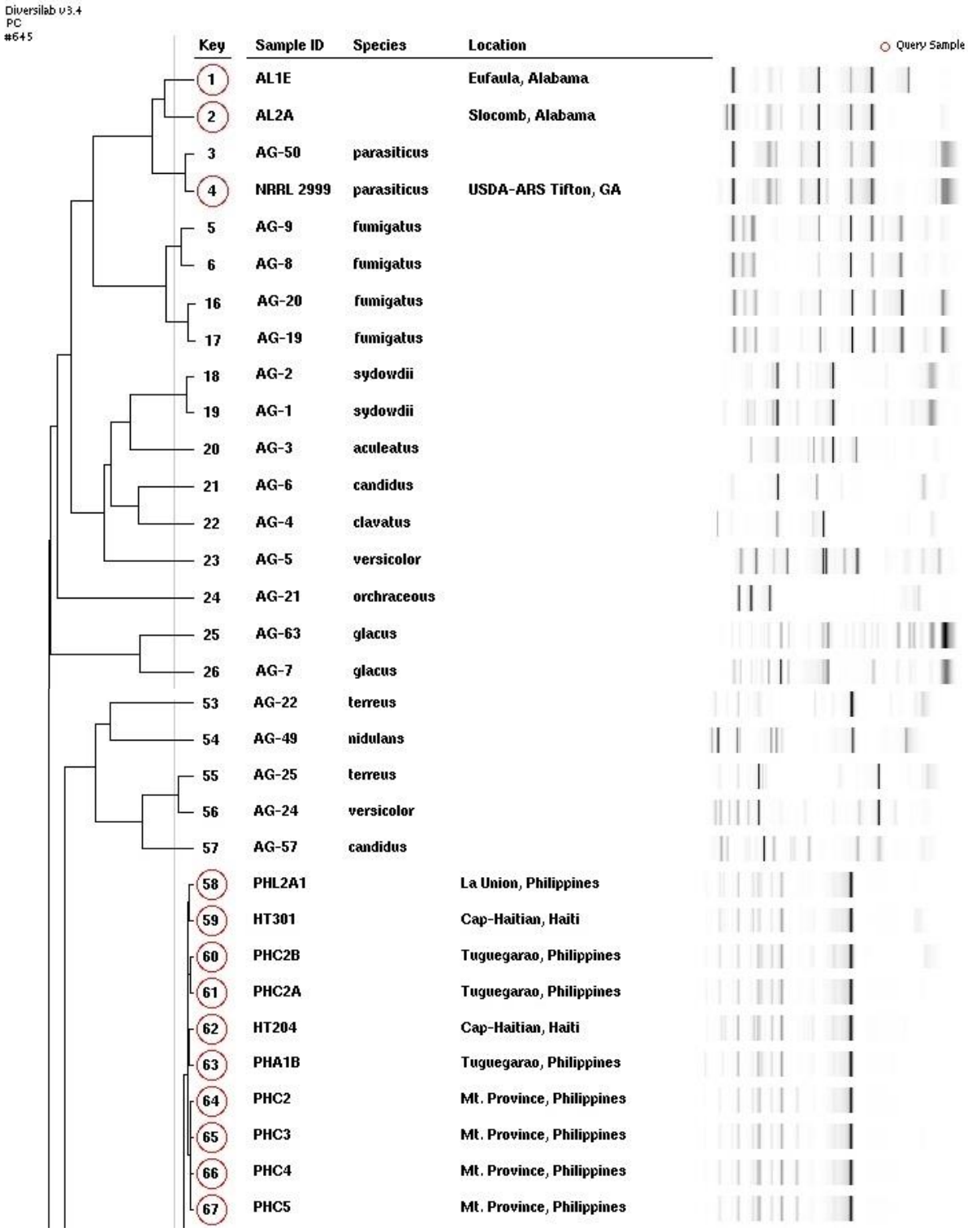
[a] The standard isolates used include NRRL 2999 (*A. parasiticus*) which has rough condial wall, NRRL 3357 (*A. flavus*) and Aflaguard® (atoxigenic *A. flavus*) that both have smooth conidial texture.

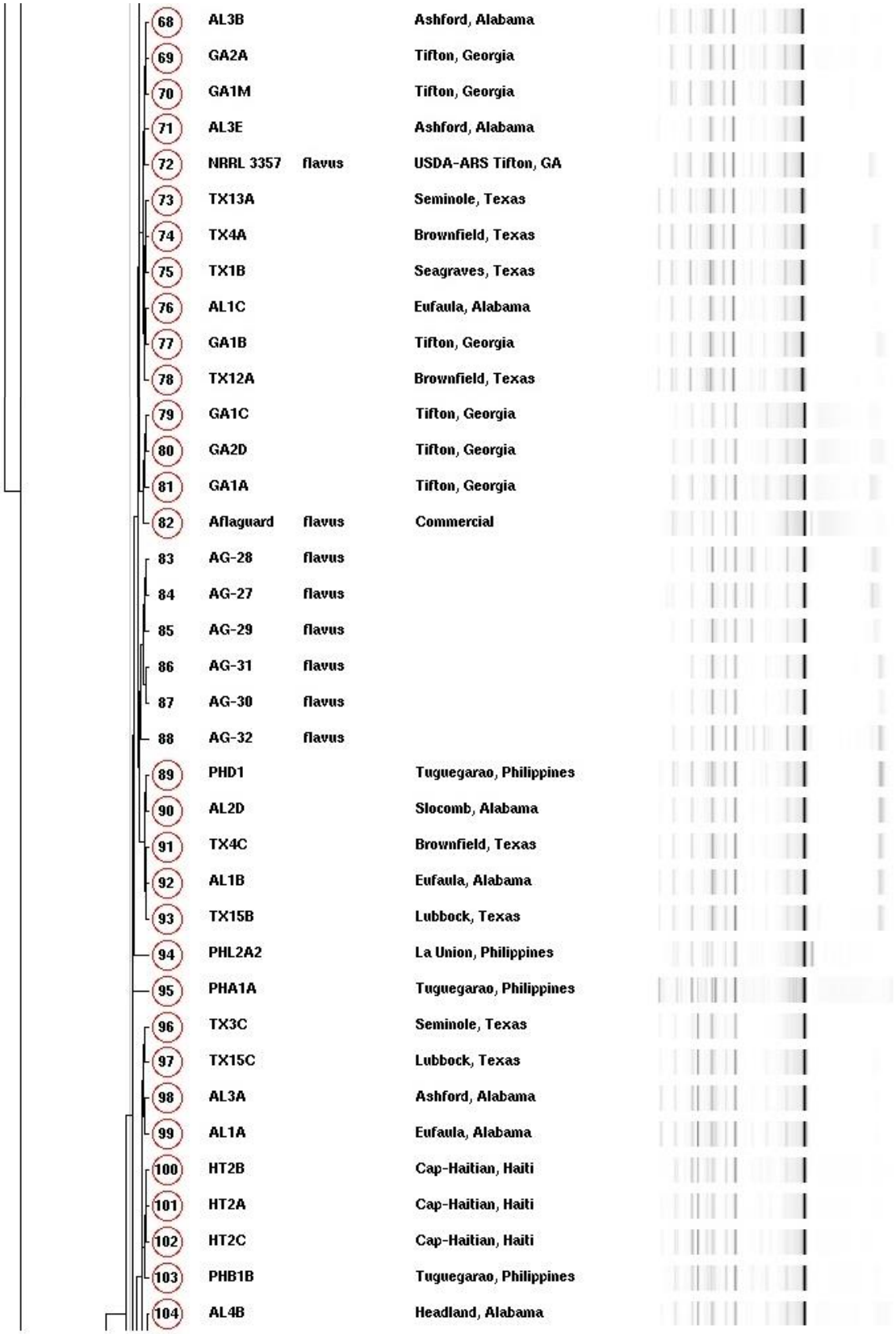
[b] Rough conidial walls were characterized by pronounced irregular wall surfaces and obvious ornamentations; smooth conidial walls by even and regular surface appearance; while finely roughened conidial walls by uneven wall surfaces with mildly noticeable ornamentations.

Table 29. Identification of seven *Aspergillus* section *Flavi* isolates based on the DNA sequences of their ITS region, beta-tubulin, and TEF1-α.

SAMPLE ID	TARGET	IDENTIFICATION[a]
GA1A	ITS	*A. flavus* (99%); *A. oryzae* (99%)
	Beta-tubulin	*A. flavus* (99%); *A. oryzae* (99%); *A. parasiticus* (99%)
	TEF1-α	*A. flavus* (100%)
GA1B	ITS	*A. flavus* (100%)
	Beta-tubulin	*A. flavus* (99%); *A. oryzae* (99%); *A. parasiticus* (99%)
GA1C	ITS	*A. flavus* (100%)
	Beta-tubulin	*A. flavus* (99%); *A. oryzae* (99%); *A. parasiticus* (99%)
	TEF1-α	*A. flavus* (99%); *A. oryzae* (99%)
GA2A	Beta-tubulin	*A. flavus* (99%); *A. oryzae* (99%); *A. parasiticus* (99%)
GA2B	ITS	*A. flavus* (99%)
	Beta-tubulin	*A. flavus* (98%); *A. oryzae* (98%); *A. parasiticus* (98%)
	TEF1-α	*A. flavus* (100%)
GA2C	ITS	*A. flavus* (100%)
	Beta-tubulin	*A. flavus* (99%); *A. oryzae* (99%); *A. parasiticus* (99%)
	TEF1-α	*A. flavus* (99%); *A. oryzae* (99%)
GaA	ITS	*A. flavus* (100%)

[a] Isolates were identified based on their % genetic similarity to referenced samples in GenBank.


Diversilab v3.4
PC
#645
Key
Sample ID
Species
Location
Query Sample
1 AL1E Eufaula, Alabama
2 AL2A Slocomb, Alabama
3 AG-50 parasiticus
4 NRRL 2999 parasiticus USDA-ARS Tifton, GA
5 AG-9 fumigatus
6 AG-8 fumigatus
16 AG-20 fumigatus
17 AG-19 fumigatus
18 AG-2 sydowdii
19 AG-1 sydowdii
20 AG-3 aculeatus
21 AG-6 candidus
22 AG-4 clavatus
23 AG-5 versicolor
24 AG-21 orchraceous
25 AG-63 glacus
26 AG-7 glacus
53 AG-22 terreus
54 AG-49 nidulans
55 AG-25 terreus
56 AG-24 versicolor
57 AG-57 candidus
58 PHL2A1 La Union, Philippines
59 HT301 Cap-Haitian, Haiti
60 PHC2B Tuguegarao, Philippines
61 PHC2A Tuguegarao, Philippines
62 HT204 Cap-Haitian, Haiti
63 PHA1B Tuguegarao, Philippines
64 PHC2 Mt. Province, Philippines
65 PHC3 Mt. Province, Philippines
66 PHC4 Mt. Province, Philippines
67 PHC5 Mt. Province, Philippines


68 AL3B Ashford, Alabama
69 GA2A Tifton, Georgia
70 GA1M Tifton, Georgia
71 AL3E Ashford, Alabama
72 NRRL 3357 flavus USDA-ARS Tifton, GA
73 TX13A Seminole, Texas
74 TX4A Brownfield, Texas
75 TX1B Seagraves, Texas
76 AL1C Eufaula, Alabama
77 GA1B Tifton, Georgia
78 TX12A Brownfield, Texas
79 GA1C Tifton, Georgia
80 GA2D Tifton, Georgia
81 GA1A Tifton, Georgia
82 Aflaguard flavus Commercial
83 AG-28 flavus
84 AG-27 flavus
85 AG-29 flavus
86 AG-31 flavus
87 AG-30 flavus
88 AG-32 flavus
89 PHD1 Tuguegarao, Philippines
90 AL2D Slocomb, Alabama
91 TX4C Brownfield, Texas
92 AL1B Eufaula, Alabama
93 TX15B Lubbock, Texas
94 PHL2A2 La Union, Philippines
95 PHA1A Tuguegarao, Philippines
96 TX3C Seminole, Texas
97 TX15C Lubbock, Texas
98 AL3A Ashford, Alabama
99 AL1A Eufaula, Alabama
100 HT2B Cap-Haitian, Haiti
101 HT2A Cap-Haitian, Haiti
102 HT2C Cap-Haitian, Haiti
103 PHB1B Tuguegarao, Philippines
104 AL4B Headland, Alabama

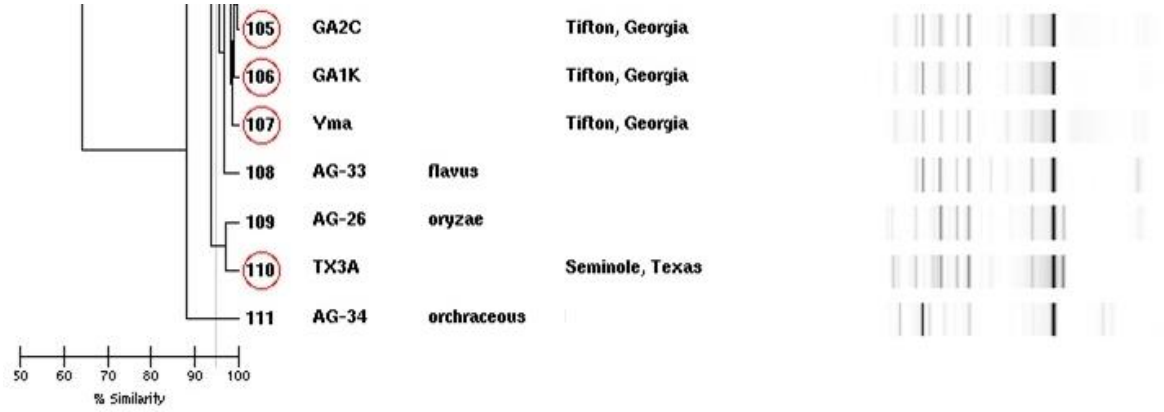


Figure 3. Dendogram and gel-like images generated through the DiversiLab system. Samples in red circles are representative isolates collected from Georgia, Texas, and Alabama of US; Haiti; and Philippines.

Table 30. Distribution of *Aspergillus* section *Flavi* isolates as discriminated by rep-PCR DNA fingerprinting. The standards used include NRRL 3357 (*A. flavus*), NRRL 2999 (*A. parasiticus*), and Aflaguard® (atoxigenic *A. flavus*).

SPECIES	STATE/COUNTRY	NO. OF ISOLATES	%
A. flavus	Georgia	9	20.00
	Texas	8	17.78
	Alabama	8	17.78
	Haiti	5	11.11
	Philippines	12	26.67
	Subtotal	42	93.34
A. parasiticus	Alabama	2	4.44
A. oryzae	Texas	1	2.22
TOTAL		45	100.00

CHAPTER 4

ACCURATE DETECTION OF *ASPERGILLUS* INFECTION AND AFLATOXIN CONTAMINATION: A. EFFECT OF SAMPLE SIZE IN THE ACCURACY OF DETERMINING AFLATOXIN CONTAMINATION IN PEANUT[1]

[1] Luis, J.M., and Kemerait Jr., R.C. To be submitted to *Food Additives and Contaminants.*

4.1 Abstract

Proper sampling and accurate estimation of aflatoxin content are required for effective aflatoxin management. At the same time, the use of a smaller sample size reduces the cost of chemicals used in aflatoxin extraction. Thus, the objective of the study was to evaluate the use of different sample and subsample sizes in aflatoxin detection. Peanut samples from a field trial and commercial buying point were collected. Subsampling of 100 g or 25 g from 300 g or 100 g ground peanuts gave remarkably narrower confidence intervals of aflatoxin contents than direct sampling of 25 g ($P \geq 0.05$). The narrowest confidence interval among samples was obtained from subsampling of 25 g from 100 g. Therefore, the standard method of subsampling 100 g from 300 g peanuts can be reduced into 25 g from 100 g. Reduction in the subsample size reduces cost and still gives reliable results. Direct sampling of 25 g runs the risk of either hitting or missing kernels with high aflatoxin content.

Keywords: Aflatoxin, peanut, sampling, sample size

4.2 Introduction

Cultivated peanut (*Arachis hypogaea* L.) is one of the most widely grown legumes in the world being cultivated on about 26.5 million hectares globally with an annual production of 35.7 million tons (3). It ranks as the second most economically important legume next to soybean and the fourth most important oilseed crop next to soybean, rapeseed (canola), and cottonseed. Peanut is consumed as an important dietary component, considered as a nutritious snack, and used as oil, feed or feed additive because of its high protein, unsaturated fat, carbohydrate, vitamin, and mineral contents (2; 15; 17; 20).

Peanut, however, is one of the host crops most susceptible to aflatoxin contamination. The hepatotoxic and immunosuppressive properties of aflatoxins has led to several reports of high mortality and reduction of productivity in livestock as well as reduced immunity and liver cancer in humans. The concern for the risks of aflatoxin to human and livestock health highly affected peanut trade. This led to significant economic problems both in international trade and domestic products (5; 10; 14; 18). At the same time, the concern for human and livestock health led to the implementation of strict aflatoxin control programs and regulatory limits in more than 100 countries. All food and food products intended for human consumption should not contain more than 20 parts per billion (ppb) of total aflatoxin in the United States (US) and 2 ppb in the European Union (7; 8; 12).

In the US, the cleanliness of a farmer's load (or lot) of peanuts is initially tested through visual inspection at buying points. Peanut kernels from a 1,800 g sample are graded and inspected for the presence of *Aspergillus flavus* through an aflatoxin inspection method often called as the visual *A. flavus* (VAF) method. The presence of this fungus on a single peanut kernel in a sample taken from a farmer's load would cause the whole load to be classified as

Segregation (Seg) III. Seg III peanuts cannot be used for direct human or animal consumption, thus, are usually crushed for oil. Peanut samples that do not show visible signs of *A. flavus* but contain 2% damaged kernels are classified as Seg II. These are also usually crushed for oil. Seg I peanuts, which do not contain any visible fungal growth nor contain 2% damaged kernels, are used in the edible market (1; 25). The classification of the lot as Seg I does not guarantee the safeness of the peanuts because aflatoxins can still be present without visible fungal growth (16). Therefore, the presence or absence of aflatoxin in the peanut lot is further verified at buying points, shelling plants, and/or processing sites through mechanical and chemical methods of aflatoxin extraction and quantification. The Codex Alimentarius Commission (4; 6) had set general standards in dealing with toxins in several food and feed including peanut. Codex established a sampling plan that requires a 20 kg sample of raw shelled peanuts to be taken from the lot and tested against an aflatoxin limit of 15 ppb total aflatoxin. Since the estimate of the peanut's average level of aflatoxin is based upon the analysis of samples taken from the lot (22), proper sampling and accurate estimation of aflatoxin content are required for the effective monitoring and management of aflatoxin contamination (26). On the contrary, the determination of the accurate concentration of aflatoxin in the peanut lot could be very challenging because the amount of aflatoxin contamination is not uniformly distributed throughout the population of the peanuts. In addition, only a very small percentage of seeds are usually contaminated but one peanut seed could be contaminated with several hundred thousands to a million ppb of aflatoxin. A study conducted by Cucullu *et al.* (9) showed that 5% or less of the population of peanuts were contaminated with aflatoxin. Nevertheless, the level of contamination on a single kernel reached up to 1,000,000 ppb or more. With this, improper sampling can lead to inaccurate or misleading

results that are either (too) high or low as compared to the actual aflatoxin contamination (11; 13; 19; 21).

Whitaker *et al.* (25) reported that sample size is one of the most important parameters in sampling for aflatoxin. Thus, this study aimed to compare the use of different sample sizes in the accuracy of quantifying aflatoxin contamination in peanuts. The practical application of the study would be to help buying points and factories in developing countries to identify a suitable sampling size for aflatoxin testing. Considering that the materials used in aflatoxin extraction can be costly, the use of larger or smaller size has to be considered.

The factory of Meds and Foods for Kids (MFK) in Haiti produces peanut-based Ready-to-Use Therapeutic Food (RUTF). As safety and cleanliness of the peanuts used for their production is of utmost importance, the peanuts that the factory purchases from local farmers are always checked for aflatoxin contamination. The factory currently uses a sample size of 300 g per roasted batch of 100-120 kg for aflatoxin extraction and quantification. The factory will soon be using a new smaller but much faster roaster that will use 70 kg per batch. It was the goal of this study to model this situation on a smaller scale to determine a reasonable sample size per batch. A second objective was to come up with a confidence interval for these test sample sizes so as to compare the positive and negative effects of using bigger or smaller sample size.

4.3 Materials and Methods

4.3.1 Sample preparation

Peanut samples in 50-pound sacks were obtained from a field trial and a commercial buying point. Two sacks were obtained from each source. The samples from the field plots were subjected to conditions favorable for aflatoxin contamination while Seg III peanuts were

obtained from the commercial buying point. From each sack, the following sampling sizes were obtained (n = 25 per sack): (a) 300 g shelled peanut samples ground by a blender and thoroughly mixed before subsampling into 100 g samples (300-100 g); (b) 300 g shelled samples ground by a blender and thoroughly mixed before subsampling into 25 g samples (300-25 g); (c) 100 g shelled samples ground by a blender and thoroughly mixed before subsampling into 25 g (100-25 g); and, (d) direct sampling of 25 g shelled peanuts.

The raw shelled peanuts were kept in paper bags until ready for grinding. Once ground, peanuts were kept in ziplock bags until ready for aflatoxin extraction.

4.3.2 Aflatoxin extraction and quantification

Aflatoxin was extracted from all sample sizes. For the 100 g subsamples, 10 g of NaCl and 100 ml of methanol/water (80:20 v/v) were mixed into the samples. These were homogenized using a Waring blender at high speed for 1 min before filtering through Whatman filter paper. Five ml of the filtrate was diluted with 20 ml HPLC water then re-filtered. Ten ml filtrate was purified with Vicam immunoaffinity columns (Vicam Aflatest, MA) containing the aflatoxin-specific (B_1, B_2, G_1 and G_2) monoclonal antibody and washed with 10 ml HPLC water before the aflatoxin was eluted with 1 ml methanol. The eluted fraction was diluted twice with HPLC water then measured/read by the fluorometer (Vicam Series 4 Fluorometer). For the 25 g samples or subsamples, 2.5 g of NaCl and 50 ml of methanol/water (80:20 v/v) were added into the samples. The rest of the procedures were followed the same as done with the 100 g subsamples.

4.3.3 Data analysis

The mean aflatoxin content (actual ppb and $\log_{10}$ ppb), standard deviation, range, and confidence interval of each sample size were computed. The differences among the mean aflatoxin content ($\log_{10}$) of the different sample sizes were analyzed using the one-way PROC ANOVA procedure in SAS ver. 9.2 (SAS Institute, Cary, NC). Boxplot distributions were generated to identify differences in the trend of aflatoxin quantification within and across sample sizes.

4.4 Results

The aflatoxin content of the peanut samples collected from the field plots and the commercial buying point ranged from 2.4-830.0 ppb and 1.2-280.0 ppb, respectively (Table 32). Due to the large variability among samples, the data was log-transformed in order to normalize the dataset. The aflatoxin content of the peanut samples obtained from the field plots was generally higher than in those obtained from the buying point (Table 31 and Figure 4). As established by the Food and Drug Administration (FDA), the acceptable regulatory limit for total aflatoxin in peanut is 20 ppb when intended for human consumption (7; 8; 12). The peanuts collected from the field plots had nine samples (36% occurrence) from Sack I and six samples (24% occurrence) from Sack II that contained aflatoxin levels above this acceptable limit. Samples obtained from the buying point had only three samples (12% occurrence) and one sample (4% occurrence) from Sack I and Sack II, respectively, that exceeded the limit.

For the field samples, the highest variation in aflatoxin values was obtained from S4 (25 g) as shown by wider dispersion of values in the boxplot analysis in Figure 4. This is supported by the mean comparison analysis showing that the mean aflatoxin content obtained using S4 (25

g) (1.050 and 1.166 ppb for Sacks I and II, respectively) is significantly higher than those of the larger sampling sizes (Table 31). No significant difference in the aflatoxin measurements were observed when using S1 (300-100 g), S2 (300-25 g), and S3 (100-25 g). For the Seg III peanut samples from the buying point, the lowest mean aflatoxin content was obtained from S3 (100-25 g). No significant differences among sample sizes were observed from Sack I. In Sack II, The aflatoxin content of the samples obtained using S4 (25 g) (0.419 ppb) did not show significant difference from using S1 (300-100 g) and S2 (300-25 g).

As suggested by Whitaker *et al*. (21; 23), it is advantageous to use confidence intervals (CI) and confidence levels instead of actual values in data analysis because the true aflatoxin contamination of a peanut lot cannot be determined with 100% certainty. Results of the study showed that using larger original sample sizes of 300 and 100 g before subsampling gave remarkably narrower CI for the mean aflatoxin contents of the peanuts as compared to directly using 25 g ($P \geq 0.05$) (Table 32). Furthermore, subsampling from 100 into 25 g (S4) showed the narrowest CI for both the field trial and Seg III peanuts. The CI obtained from subsampling 300 g samples into either 100 g (S1) or 25 g (S2) showed comparable results.

4.5 Discussion

Overall, this study evaluated the impact of using different sample sizes in the precision to predict the average level of aflatoxin contamination of peanuts from a given source. The difference between using a larger sample size of 300 g and smaller sample sizes of 100 g and 25g was assessed. In addition, the effect of subsampling the original 300 g samples into 100 g and 25 g was also compared. In the US, shelled peanuts are tested for aflatoxin prior to processing for food use. The average level of aflatoxin in a lot is estimated by analyzing a sample drawn from

the lot. Thus, it is important that the sample drawn from the lot provides an accurate estimation of the level of aflatoxin in the population of peanuts (24).

Samples in the study were taken from a field plot and a commercial peanut buying point. The field samples were subjected to conditions conducive for aflatoxin while Seg III peanuts were obtained from the buying point. Results of the study showed a great variation of aflatoxin measurements, ranging from 1.2 - 830.0 ppb, among the peanut samples within and across the sample sizes. In general, the field samples contained higher aflatoxin levels than the Seg III peanuts obtained from the buying point. Assumptions for higher aflatoxin content in the field samples include: (a) the presence of higher populations of aflatoxigenic fungi in the field location had led to greater exposure to pre-harvest aflatoxin contamination; and (b) the exposure of the samples to aflatoxin-inducing conditions had favored increased aflatoxin production. On the other hand, the aflatoxin content of the Seg III peanuts yielded relatively low aflatoxin measurements. Here, only four out of 50 samples (8% occurrence) had aflatoxin contents above 20 ppb. It is possible that the peanuts brought by the farmers to the buying point had already been cleaned from 'bad-looking' pods. Classifying the peanut lot as Seg III, despite the fact that only a small percentage of the lot was contaminated with aflatoxin levels above 20 ppb, clearly indicates the strict implementation of *Aspergillus* infection screening and aflatoxin control programs in the US.

Data analysis showed that using the original sample sizes of 300 g and 100 g before subsampling into 100 g or 25 g greatly reduced the range between the upper and lower limits of the CI for the aflatoxin measurements as compared to direct sampling of 25 g. This conforms to the report of Whitaker *et al.* (24) that larger sample size increased sampling accuracy. Among the three subsampled sizes (S1 = 300-100 g, S2 = 300-25 g, and S3 = 100-25 g), S3 (100-25 g)

showed the narrowest CI for aflatoxin at 95% confidence suggesting that it has better precision in estimating mean aflatoxin levels. As assessed, this sample size also exhibited the smallest standard deviation indicating that the aflatoxin measurements using S3 (100-25 g) showed close values to its mean.

The high aflatoxin contents obtained using direct sampling of 25 g as compared to the larger sample sizes may be attributed to the ratio of contaminated and non-contaminated kernels in the sample (24). If a contaminated kernel was included in a sample size of 25 g, the aflatoxin level of that kernel directly affected the total aflatoxin level of the whole sample. On the other hand, if a contaminated kernel was included in an original sample size of 300 g then further subsampled into 100 g, then the aflatoxin concentration of the contaminated kernel diluted the amount of non-contaminated kernels that led to a lower mean aflatoxin reading. Otherwise, the contaminated kernel was lost when not picked by the random subsampling, resulting in much lower aflatoxin reading.

4.6 Summary and Conclusions

This study evaluated the effect of using different sample sizes on the accuracy in predicting the level of aflatoxin of a given peanut lot. Based on the results of the study, the standard method of subsampling peanut samples from 300 g into 100 g (S1 = 300-100 g) for aflatoxin detection can be reduced into subsampling from 300 g into 25 g (S2 = 200-25 g) when assessing both field and commercial samples. This was shown by the insignificant difference in their mean aflatoxin content and CI of aflatoxin content at 95% confidence level. It should, however, be emphasized that the 300 g original sample should truly represent the whole lot as made possible by appropriate sample collection methods. As long as the original sample is

thoroughly mixed, taking a subsample of 25 g does not differ significantly from 100 g subsample. This reduction in the subsample (75 g) gave enormous savings in terms of the cost of chemicals used in the aflatoxin quantification.

The recommended S1 (300-100 g) can further be reduced into subsampling from 100 g into 25 g (S3 = 100-25 g). Results of the study showed better precision in determining aflatoxin level among samples using this sample size. Such reduction in original sample size (200 g) saved a significant amount of peanuts that needed to be taken out from the sack to be ground for aflatoxin analysis. This can be specifically helpful in a situation where the peanut yield is low or if more than one sampling is required. The reduction in the subsample also gave enormous savings for the cost of chemicals used in the aflatoxin extraction.

Direct sampling is not recommended due to the risk of either hitting or missing kernels with very high aflatoxin content, therefore, giving unreliable results. When contaminated kernels are missed, it can be good for the income of farmers but is a health hazard to the consumer. When the kernels with very high aflatoxin content are picked, it will be a loss to the farmer and which might not really be the real picture of the aflatoxin content of the whole peanut lot.

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Table 31. Aflatoxin content, standard deviation, and coefficient of variance of the peanut samples obtained from different sample sizes.

SAMPLE ID[a]	FIELD PLOTS						COMMERCIAL BUYING POINT					
	Sack I[b]			Sack II[b]			Sack I[b]			Sack II[b]		
	Mean (ppb)	Mean (log_{10} ppb)[c]	St Dev	Mean (ppb)	Mean (log_{10} ppb)[c]	St Dev	Mean (ppb)	Mean (log_{10} ppb)[c]	St Dev	Mean (ppb)	Mean (log_{10} ppb)[c]	St Dev
S1 (300-100 g)	8.184	0.760 b	0.303	8.215	0.756 b	0.292	4.276	0.585	0.192	3.629	0.483 a	0.262
S2 (300-25 g)	8.812	0.775 b	0.310	11.525	0.864 b	0.302	6.040	0.725	0.218	5.896	0.693 a	0.292
S3 (100-25 g)	6.212	0.745 b	0.195	6.895	0.774 b	0.195	3.932	0.552	0.192	2.908	0.419 b	0.188
S4 (25 g)	62.356	1.050 a	0.578	43.412	1.166 a	0.465	5.708	0.587	0.343	28.564	0.621 ab	0.535
Overall Mean	21.391	0.832		17.512	0.890		4.989	0.612		10.249	0.542	

[a] Sample ID indicates the following: S1 (300-100 g) = 300 g samples subsampled into 100 g; S2 (300-25 g) = 300 g samples subsampled into 25 g; S3 (100-25 g) = 100 g samples subsampled into 25 g; and, S4 (25 g) = direct sampling of 25 g.
[b] A total of 25 samples were taken from each sack. Means with different letters in a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test.
[c] The data was log-transformed to normalize the large variability of aflatoxin content among the samples in the dataset.

Table 32. Range and confidence interval of the aflatoxin contents obtained from different sample sizes estimated at 95% confidence level.

SAMPLE ID[a]	FIELD PLOTS				COMMERCIAL BUYING POINT			
	Sack I[b]		Sack II[b]		Sack I[b]		Sack II[b]	
	Range (ppb)	Confidence Interval	Range (ppb)	Confidence Interval	Range (ppb)	Confidence Interval	Range (ppb)	Confidence Interval
S1 (300-100 g)	2.50 - 48 (2)[c]	3.98 - 12.37	2.90 - 69 (1)[c]	3.13 - 13.31	1.80 - 14 (0)[c]	3.32 - 5.24	1.20 - 12 (0)[c]	2.59 - 4.67
S2 (300-25 g)	3.20 - 56 (2)[c]	3.91 - 13.72	3.10 - 120 (1)[c]	2.59 - 20.46	1.80 - 20 (1)[c]	4.63 - 7.45	1.60 - 16 (0)[c]	4.36 - 7.43
S3 (100-25 g)	2.70 - 20 (1)[c]	4.80 - 7.63	3.40 - 36 (1)[c]	4.46 - 9.34	2.00 – 8 (0)[c]	3.20 - 4.66	1.30 - 9.9 (0)[c]	2.25 - 3.57
S4 (25 g)	2.40 - 830 (4)[c]	0.00 - 136.76	6.40 - 630 (3)[c]	0.00 - 92.63	1.40 - 28 (2)[c]	3.02 - 8.39	1.30 - 280 (1)[c]	6.71 - 50.42

[a] Sample ID indicates the following: S1 (300-100 g) = 300 g samples subsampled into 100 g; S2 (300-25 g) = 300 g samples subsampled into 25 g; S3 (100-25 g) = 100 g samples subsampled into 25 g; and, S4 (25 g) = direct sampling of 25 g.
[b] A total of 25 samples were taken from each sack. Means with different letters in a column are significantly different at $P \leq 0.05$ as determined by Fisher LSD test.
[c] The numbers in parentheses indicate the number of samples with aflatoxin levels above 20 ppb.

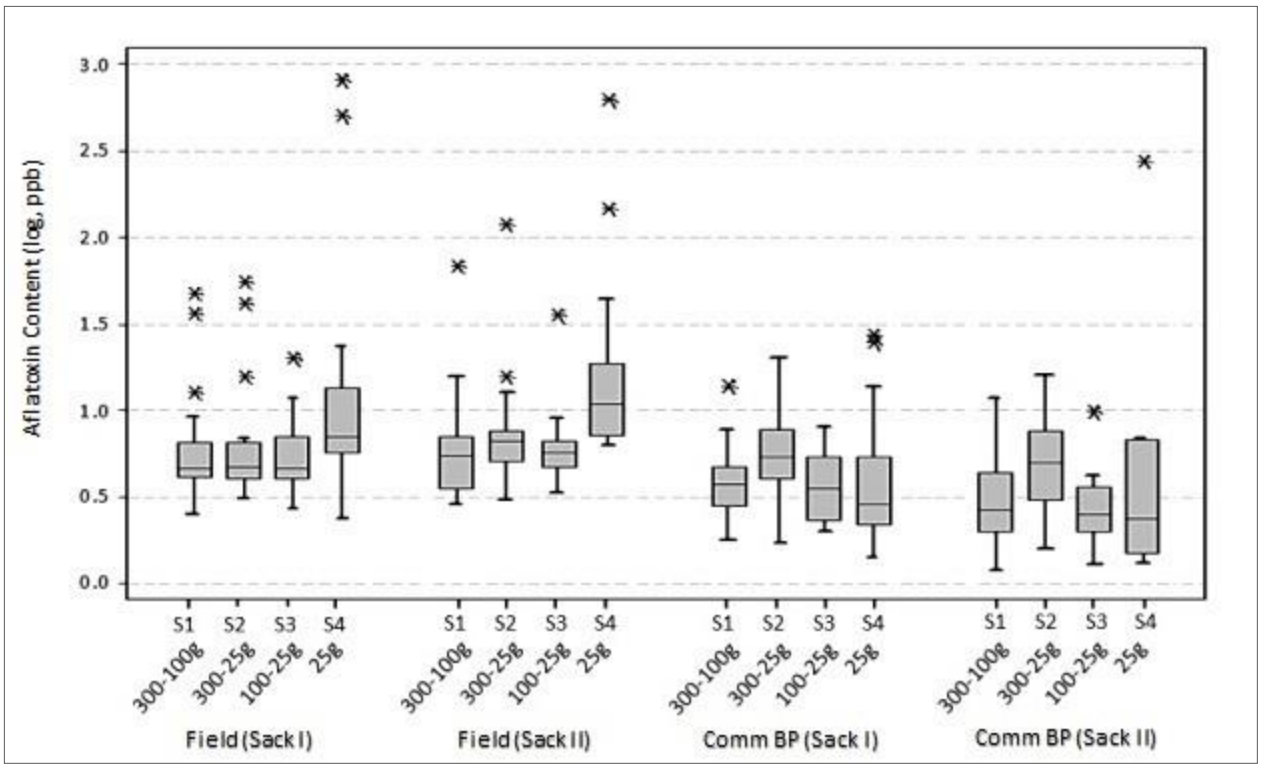

Figure 4. Boxplot distribution of the aflatoxin content of the peanut samples obtained from different sample sizes. Sample ID indicates the following: S1 (300-100 g) = 300 g samples subsampled into 100 g; S2 (300-25 g) = 300 g samples subsampled into 25 g; S3 (100-25 g) = 100 g samples subsampled into 25 g; and, S4 (25 g) = direct sampling of 25 g.

CHAPTER 5

ACCURATE DETECTION OF *ASPERGILLUS* INFECTION AND AFLATOXIN CONTAMINATION: B. ASSESSMENT OF THE SENSITIVITY AND ACCURACY OF IMMUNOCHROMATOGRAPHIC TEST STRIPS IN THE QUALITATIVE DETECTION OF AFLATOXIN CONTAMINATION[1]

[1] Luis, J.M., and Kemerait Jr., R.C. To be submitted to *Food Additives and Contaminants.*

5.1 Abstract

Most mechanical and chemical methods of aflatoxin detection are effective, accurate and sensitive but are not very accessible to many developing countries. In these areas, immunochromatographic test strips can be considered as an alternative. The first objective of this study was to assess the efficiency of immunochromatographic test strips in the qualitative detection of aflatoxin at a 20 ppb cut-off limit as compared to the standard fluorometry method. The second objective was to assess the effect of continuous high or fluctuating temperatures to the sensitivity and accuracy of the test strips. Results showed a high correspondence (95%) between the results obtained from the test strips (AflaCheck™) and the Vicam fluorometry method. When exposed to continuous high (34°C) and fluctuating (34°C for 8 hours and 25°C for 16 hours) temperatures, AflaCheck™ and AgraStrip® retained their sensitivity and accuracy in testing aflatoxin levels until 32 and 47 weeks (8 and 12 months), respectively. This documents the reliable use of the test strips in tropical peanut production areas where technologies like the fluorometer for aflatoxin quantification and refrigerators for storage are not very accessible.

Keywords: Immunochromatographic test strips, aflatoxin, fluorometry

5.2 Introduction

Aflatoxins have carcinogenic, hepatotoxic, and immunosuppressive properties that have caused high mortality and reduction of productivity in livestock as well as reduced immunity and liver cancer in humans (2; 10; 16). Due to these risks to human and livestock health, aflatoxin contamination is regularly monitored in peanut and many agricultural crops (15).

Several mechanical and chemical methods for the detection, extraction, and quantification of aflatoxin were developed. These include Fourier transform near-infrared spectroscopy (13), fluorometry method (5), high performance liquid chromatography (HPLC) (8), liquid chromatography-tandem mass spectrometry (LC-MS) (4), and enzyme-linked immunosorbent assay (ELISA) (7). Among these, ELISA and HPLC are the most commonly used (18). These methods are proven to be accurate, selective, sensitive, and effective. However, they are usually costly, largely intended for laboratory scientific research, and require special equipment and training (12; 18). In addition, most of these technologies are not accessible to developing countries where aflatoxin is of greater concern (9; 14). The improvement of standards of living in developing countries demanded increased attention to food safety and monitoring of aflatoxins (11). There was a demand in the food and feed sectors of both developed and developing countries to develop rapid aflatoxin testing methods that are low-cost, easy to handle, usable on-site, independent of other instruments, and could be easily integrated into the production process. Immunochromatographic test strips, also known as lateral flow test strips, were developed and are now firmly integrated into routine quality-monitoring procedures. These strips are easily operated following simple manufacturer's procedures, produce immediate results, and do not require expensive instrumentations. In addition, they are not required to be refrigerated, thus, facilitating use, especially in developing countries (6; 17; 18). Test strips,

however, have manufacturer-recommended storage temperatures that are lower than the actual temperatures in some peanut production areas. Considering these conditions, this study addressed two objectives: (1) To validate the sensitivity and accuracy of immunochromatographic test strips in the qualitative detection of peanut aflatoxin at a 20 ppb cut-off limit in comparison to the quantitative fluorometry method; and, (2) To evaluate the effect of continuous high and fluctuating temperatures to the sensitivity and accuracy of the strips.

5.3. Materials and Methods

5.3.1 Performance of the test strips in comparison to the Vicam fluorometry method

5.3.1.1 Experimental setup

A total of 108 immunochromatographic test strips from Vicam (AflaCheck™) were used in this study. Fifty four strips were used to test peanut samples with aflatoxin contents <20 ppb as measured by the fluorometer and another 54 strips to test samples with aflatoxin contents ≥20 ppb. The number of test strips used was based on the suggested number of not less than 50 positive and 50 negative samples for qualitative assay studies (1; 3).

Peanut samples were collected from different field trials in Tifton, Georgia. These samples were subjected to aflatoxin extraction and quantification using the Vicam fluorometry method. Briefly, representative samples (100 g) of shelled peanuts were added with 10 g of NaCl and 200 ml of methanol/water (80:20 v/v), homogenized using a Waring blender at high speed for 1 minute and filtered through Whatman paper. Five ml of the filtrate was diluted with 20 ml HPLC water then re-filtered. Ten ml filtrate was purified with Vicam immunoaffinity columns (Vicam Aflatest, MA) containing aflatoxin-specific (B_1, B_2, G_1 and G_2) monoclonal antibody and

washed with 10 ml HPLC water before the aflatoxin was eluted with 1 ml methanol. The eluted fraction was diluted twice with HPLC water and measured with the Vicam fluorometer (Vicam Series 4 Fluorometer).

Samples with fluorometer readings <20 ppb and ≥20 ppb were identified and tested in triplicates using AflaCheck™ test strips. All procedures were done according to the manufacturer's instructions.

5.3.1.2 Sensitivity and accuracy validation

The AflaCheck™ immunochromatographic test strips used in this study were designed to qualitatively detect aflatoxin contents either <20 or ≥20 ppb. A positive reaction indicating the detection of aflatoxin level ≥20 ppb is displayed by the production of one visible line (Figure 5). A negative reaction which indicates the detection of aflatoxin level <20 ppb is displayed by the production of two visible lines. The non-production of any line is an invalid result.

5.3.2 Performance of the immunochromatographic test strips when exposed to continuous high and fluctuating temperatures at increasing time duration

5.3.2.1 Experimental setup

Immunochromatographic test strips from two companies, AflaCheck™ (Vicam, MA) and AgraStrip® (Romer Labs, MO), were stored in three temperature regimes: T_0 = room temperature (25^{o}C); T_1 = high temperature (34^{o}C); and, T_2 = fluctuating at 34^{o}C (8 hours) and room temperature (16 hours). High temperature (34^{o}C) was imposed by warming the test strips inside an incubator. Fluctuating temperatures were imposed by incubating the test strips for 8 hours at 34^{o}C then bringing them out at room temperature for 16 hours overnight until the next day.

5.3.2.2 Sensitivity and accuracy validation

As the test trips were continually incubated under the three temperature regimes, 10 test strips were taken from each treatment at certain time durations. These strips were used to test the following solutions prepared from calibrated aflatoxin standards: (a) ≥20 ppb, to examine the test strips for positive results; (b) 10 ppb, to examine the test strips for negative results; and, (c) distilled water, as control.

The test strips were incubated and tested for a maximum duration of 53 weeks (1 year).

5.4 Results

5.4.1 Performance of the test strips in comparison to the Vicam fluorometry method

The aflatoxin content of the peanut samples as quantified through the Vicam fluorometry method ranged from 1.9 to 1,200.0 ppb. Samples with reactions indicative of aflatoxin contents <20 and ≥20 ppb were chosen and tested in triplicates. Results of the test strips yielded a high correspondence of 95% with the results of the fluorometry method (Figure 6). All samples with aflatoxin contents <20 ppb as read by the fluorometer yielded a negative reaction in the strips. Also, all samples with aflatoxin contents >20 ppb as read by the fluorometer yielded a positive reaction with the strips. However, three samples with exactly 20 ppb as read by the fluorometer yielded a negative reaction in the strips instead of the expected positive result.

5.4.2 Performance of the test strips when exposed to continuous high and fluctuating temperatures at increasing time durations

AflaCheck^{TM} (Vicam) retained its sensitivity and accuracy in yielding positive and negative results for 32 weeks when incubated under the three temperature regimes (Figure 7).

However, it was observed that starting at Week 29, some of the strips incubated at continuous high (34^{o}C) and fluctuating (34^{o}C for 8 hours, 25^{o}C for 16 hours) temperatures started to have a slower flow absorbing rate of the liquid towards the pad. Some strips under these temperature regimes also produced blurry pads with pink dye as the liquid was being absorbed. Starting at Weeks 35 and 38, increasing invalid results were observed from the test strips incubated at continuous high and fluctuating temperatures, respectively. Starting at Week 47, at least 30% of the strips under both temperature regimes yielded results that were contradictory to what were expected. It should however be noted that starting on the same week, the strips maintained at room temperature (25^{o}C) also started yielding invalid results.

In comparison, AgraStrip® (Romer Labs) yielded accurate positive and negative results until 47 weeks of incubation under the three temperature regimes (Figure 8). This showed the considerably longer shelf life of AgraStrip® as compared to AflaCheck™. The only observed problem was at Week 50 when three (30% occurrence) invalid results were obtained from test strips incubated at continuous high temperature.

5.5 Discussion

Aflatoxin contamination of peanut is a worldwide concern. Adequate knowledge and several methodologies are currently available to control aflatoxins in food and food products. However, most of these technologies are only readily available in developed countries which have the capability to establish analytical methods to screen for toxins and establish strong regulatory controls. The techniques used in developed countries require sophisticated infrastructure, stable electricity, readily available supplies, and experienced technicians. Most developing countries lack the resources, infrastructure, sustainable supplies, and personnel for

efficient regulatory system (9; 14). In these places, the use of relatively affordable simple-to-use materials would be a great advantage. The high correspondence (95%) between the results of the immunochromatographic test strips and the fluorometry method in detecting aflatoxin levels obtained in this study indicates that the strips can be a good option when a fluorometer is not available. Although the results are only qualitative, the negative reaction shown by the strips for aflatoxin detection <20 ppb would be useful in determining that the sampled peanut lot is safe for human consumption. On the other hand, the positive reaction of the strips for aflatoxin contamination ≥20 ppb should already guarantee the rejection of the peanut lot for human consumption.

In the second objective, room temperature (25^{o}C) was within the manufacturer-recommended storage temperatures for both AflaCheck™ and AgraStrip®. High temperature (34^{o}C) was purposely imposed to mimic the usual temperature in tropical environment. Exposing the test strips to fluctuating temperatures (34^{o}C for 8 hours and 25^{o}C for 16 hours) mimics two natural conditions in the field: fluctuating day and night storage temperatures, and the usage of the test strips in the field during the day then being stored at room temperature during the night when the test strips are not in use. Results of the study showed that AflaCheck™ and AgraStrip® retained their accuracy and sensitivity in detecting aflatoxin levels under continuous high or fluctuating temperatures for 32 and 47 weeks (around 8 and 12 months), respectively. Thus, the use of these strips in the absence of fluorometer or other technology for aflatoxin contamination in field locations that exhibit constant high or fluctuating temperatures is recommended. Given that the test strips were incubated at temperatures beyond the manufacturer's recommendation, the accurate performance of the test strips is economically significant, especially in developing countries without access to better equipment or technology. It should also be noted that the

expiration date for the strips is 1 – 1½ years when kept under the manufacturer-recommended storage temperatures of 15 - 30^{o}C and 2 -25^{o}C for AflaCheck™ and AgraStrip®, respectively.

5.6 Summary and Conclusions

This study assessed the sensitivity and accuracy of immunochromatographic test strips to detect aflatoxin levels at a 20 ppb cut-off limit. A high correspondence of 95% between the use of the test strips and the fluorometry method was obtained. In addition, incubating the test strips at continuous high (34^{o}C) and fluctuating (34^{o}C for 8 hours and 25^{o}C for 16 hours) temperatures did not alter its efficiency in yielding accurate results for 32 and 47 weeks (around 8 and 12 months) for AflaCheck™ and AgraStrip®, respectively. These test strips may, therefore, be used for the qualitative detection of aflatoxin at a 20 ppb cut-off limit in peanut production areas or clinical laboratories that lack specialized equipment like the fluorometer or in tropical locations where refrigeration is not a part of normal storage practice.

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Figure 5. Illustration of the expected reactions of the chromatographic test strips (left = AflaCheck™ from Vicam; right = AgraStrip® from Romer Labs). A positive reaction indicates the detection of aflatoxin level ≥20 ppb by the production of one visible line; a negative reaction indicates the detection of aflatoxin level <20 ppb by production of two visible lines; and an invalid result if no line developed.

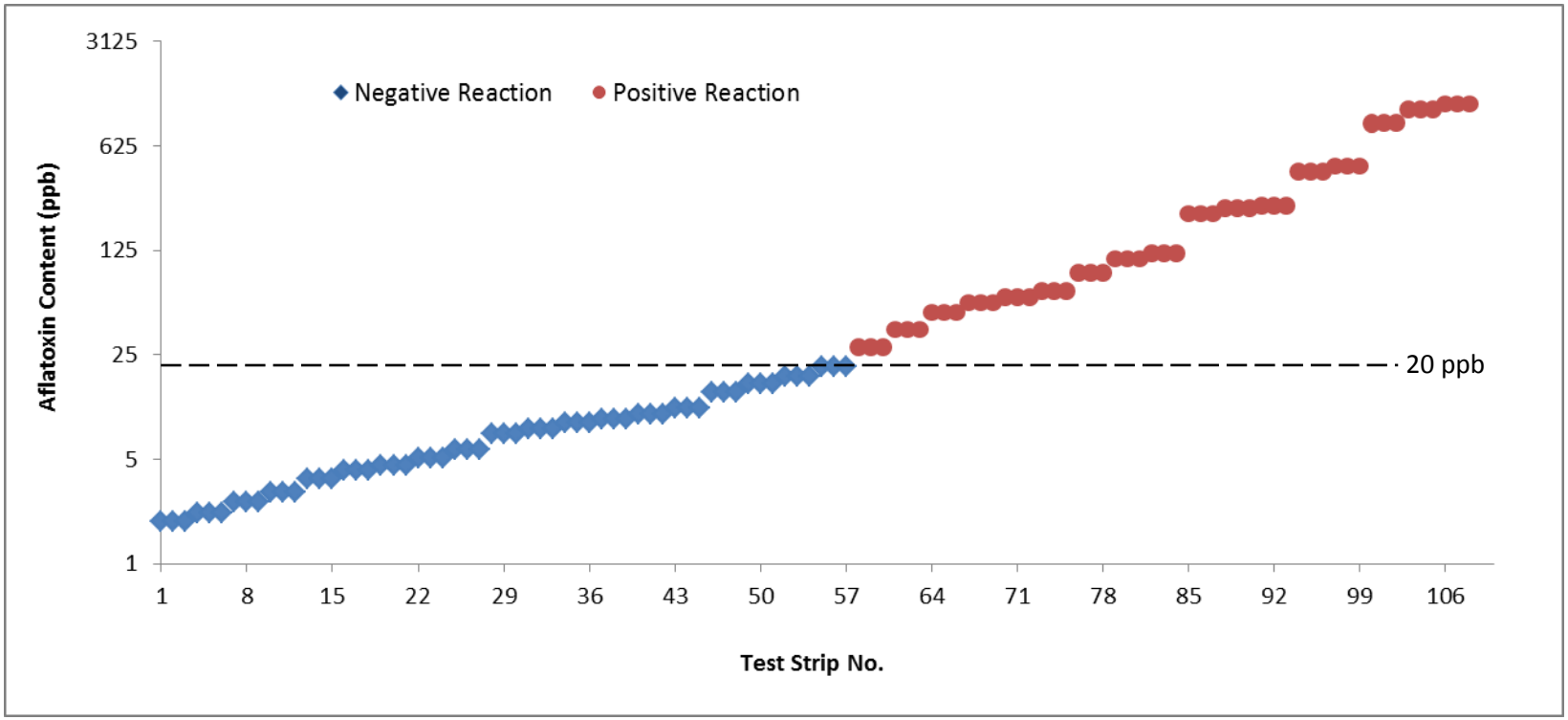
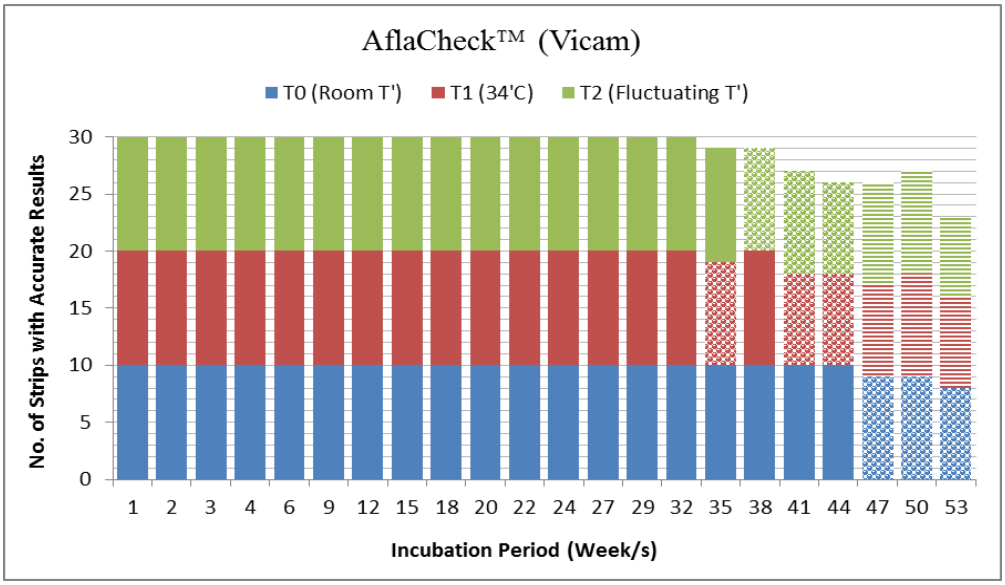
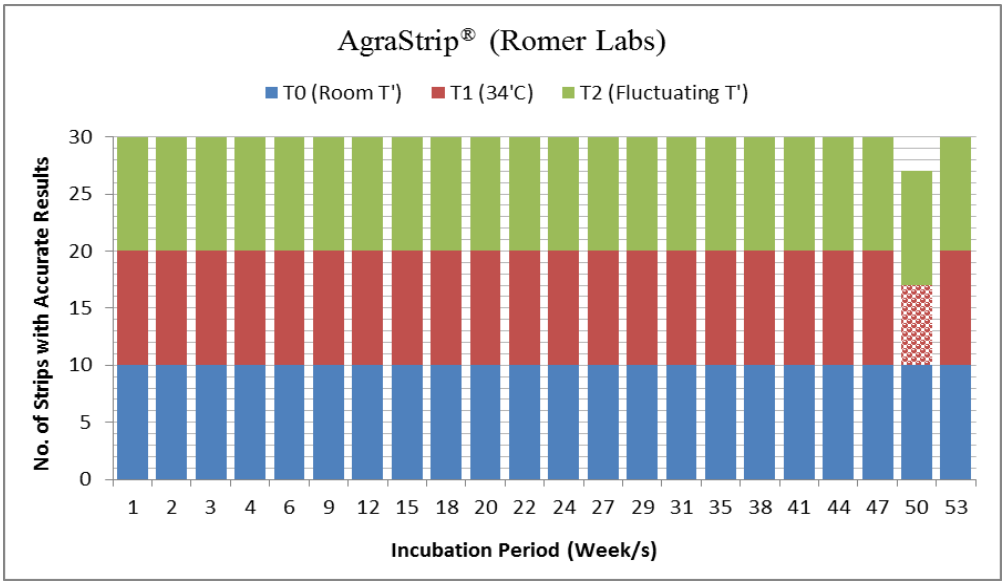


Figure 6. Results obtained from the immunochromatographic test strip assay. The test strips were used to qualitatively detect the aflatoxin content of peanut samples previously quantified through the Vicam fluorometry method. A total of 108 strips were used (54 test strips each for samples with aflatoxin contents <20 and ≥20 ppb). As expected, samples with aflatoxin content <20 ppb yielded positive reaction while samples with aflatoxin contents >20 ppb had negative reactions. However, samples with exactly 20 ppb of aflatoxin content yielded negative reactions instead of the manufacturer-claimed positive reaction if used with the test strips. This produced a 95% correspondence between the results obtained by the test strip and those by fluorometry method.





Figures 7-8. Bar graphs showing the number of immunochromatographic strips from Vicam and Romer Labs that yielded accurate detection of aflatoxin levels in calibrated solutions. A total of 30 test strips were tested per week (10 for each temperature regime); each treatment was tested on 5 solutions calibrated to contain aflatoxin levels <20 ppb, 3 solutions containing 10 ppb, and 2 samples on distilled water as control. Specific problems observed are indicated by dotted (due to invalid result as no line was produced) and horizontal (positive result instead of a negative result and vice versa) lines.

CHAPTER 6

ACCURATE DETECTION OF *ASPERGILLUS* INFECTION AND AFLATOXIN CONTAMINATION: C. QUANTIFICATION OF *ASPERGILLUS FLAVUS* AND *A. PARASITICUS* IN PEANUT SEEDS THROUGH QUANTITATIVE REAL-TIME PCR (qPCR)[1]

[1] Luis, J.M., Chu, Y., Ozias-Akins, P., Glenn, A.E., Holbrook, C.C. and Kemerait Jr., R.C. To be submitted to *Food Additives and Contaminants.*

6.1 Abstract

Aspergillus flavus and *A. parasiticus* are the major aflatoxigenic species contaminating peanut. Exposure of peanuts to drought towards the end of the growing season enhances fungal invasion and aflatoxin contamination. In this study, a real-time PCR (qPCR) assay was used to quantify the amount of *A. flavus* and/or *A. parasiticus* in peanut seeds using species-specific primers that target the *aflS* gene. Peanut samples were collected from four peanut genotypes that were field-inoculated with *A. flavus* and *A. parasiticus* and exposed to drought conditions for five weeks. The amount of aflatoxin contamination was quantified through Vicam flourometry method while the quantification of fungal genomic DNA was based on a comparison of the qPCR cycle threshold value to a standard curve of known DNA amounts. The test demonstrated that the assay can detect as low as 10 pg of fungal DNA. Statistical analysis showed significant moderate correlation between fungal infection and aflatoxin contamination.

Keywords: *Aspergillus flavus*, *A. parasiticus*, aflatoxin, peanut, real-time PCR, *aflS* gene

6.2 Introduction

Cultivated peanut (*Arachis hypogaea* L.) is an important crop but is one of the most susceptible host to *Aspergillus* infection and aflatoxin contamination (10). Infection and contamination can occur during pre-harvest, harvest, transportation, processing, and storage (8; 10; 22; 26). This is of great concern because aflatoxins have hepatotoxic, carcinogenic, and immunosuppressive properties that can cause high mortality and reduction of productivity in livestock as well as reduced immunity and liver cancer in humans (7; 22; 23).

In order to avoid these risks to human and livestock health, the Food and Drug Administration (FDA) in the US established that all food and feed products intended for human, dairy cattle, and immature livestock should not contain more than 20 parts per billion (ppb) of aflatoxin (27). The standard methods of testing the aflatoxin content of peanuts involve mechanical and chemical methods of aflatoxin extraction and quantification. Peanuts with aflatoxin contents lower than 20 ppb are accepted for direct human and livestock consumption while those with aflatoxin contents higher than 20 ppb are rejected (27; 29). The direct quantification of aflatoxin content, however, fails to consider the possibility that the peanuts might be infected with aflatoxigenic fungi but has not yet produced aflatoxin during the time that the peanuts were tested. Such situation can be risky as these fungi have the potential to produce aflatoxin once the environmental conditions become favorable. The visual inspection of peanuts for the presence of *A. flavus* is included in peanut control programs (29) but minute fungal infections can still be invisible to the eye. This led to the objectives of this study which were: 1) To determine the lowest amount of *A. flavus* and *A. parasiticus* that can be detected in peanut seeds; and, 2) To determine if the amount of aflatoxin contamination is associated with the amount of fungal infection. The second objective would be beneficial in determining the

acceptance or rejection of tested samples for direct human and animal consumption. If the amount of aflatoxin contamination is not dependent on the amount of fungal infection, then all peanuts that are identified to be infected with *A. flavus* and/or *A. parasiticus* must be rejected regardless of the amount of infection. If there is dependence, then, a further study must be conducted to determine how much fungal infection is enough to produce an aflatoxin content of 20 ppb.

The traditional methods of detecting and identifying *Aspergillus* infection in peanut and other crops rely on cultural and microscopic techniques. However, these methods are time-consuming, laborious, and require significant training in laboratory mycology because the aflatoxigenic species resemble the species used in food fermentation such as *A. oryzae* and *A. sojae* (1; 11; 13; 14; 21). Conventional PCR, quantitative real-time polymerase chain reaction (qPCR), and other DNA-based detection methods were developed to resolve the concerns with cultural and microscopic techniques. Conventional PCR is advantageous due to its high sensitivity in detecting specific fungal species even in a mixed population. However, its end-point quantification via the amplification of bands in agarose gel is not sufficient for fungal quantification. The intensity of the amplified bands produced in the agarose gel does not necessarily correlate in a linear way to the amount of target DNA present in the beginning of the reaction. This primarily happens because conventional PCR enters a plateau phase after large amounts of PCR products were produced usually due to the exhaustion of one or more substrate(s). As a modification, plotting the amount of amplified product at every cycle of the reaction, as is done by qPCR, is more appropriate. The qPCR method can be performed using different chemistries such as DNA-associating dyes (SYBR® Green) or fluorescently labelled sequence-specific oligoprobes (Taqman® oligoprobes). In using SYBR Green, the dye binds to

double-stranded DNA leading to an increase in fluorescence as the target sequence accumulates during PCR cycles. In Taqman oligoprobes, a probe is constructed containing two fluorescent dyes (a reporter and a quencher). When intact, the close proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye. The probe fluoresces after the target sequence is identified and the polymerase separates the two dyes. When the fluorescence due to SYBR green or the reporter dye in the Taqman probe exceeds a threshold above the background, a cycle threshold (Ct) value is produced. This Ct value is assimilated into a standard curve of known DNA quantities to infer the amount of target DNA concentration (3; 12; 16; 18; 25).

The qPCR approach has already been used to quantify the amount of *Aspergillus* in several commodities including wine grapes, wheat flour, corn, pepper, and paprika. These studies reported that qPCR can be used to predict possible toxigenic risk even with a very low level of infection (4; 23; 25). A study conducted by Passone *et al.* (22) also demonstrated that the amount of DNA representing one conidium of *A. parasiticus* can be detected.

Several genes and enzymes involved in aflatoxin biosynthesis have been targeted in qPCR assays. These include *aflR, aflS, nor-1, norA, ver-1,* and *omtA* (10; 20) which are located in a gene cluster region of approximately 70 kb in both *A. flavus* and *A. parasiticus* genomes (30; 31). The *aflR* gene was identified as the major pathway regulatory gene that activates the transcription of most aflatoxin structural genes (2; 24). Adjacent to *aflR* in the fungal gene cluster is *aflS* (formerly known as *aflJ*) which was used as the target gene for this experiment. Studies using *aflS* showed that this gene is involved in the regulation of the transcription of structural genes and a possible co-activator of *aflR* (24; 30). The disruption of *aflS* in *A. flavus* led to the non-production of metabolites in the aflatoxin pathway (17). The expression of *aflR*

was also enhanced in *A. parasiticus* transformants when the *aflS* region is present as compared to when it is not (6).

6.3 Materials and Methods

6.3.1 Peanut sample collection and preparation

Ground kernel samples from four peanut genotypes (C76-16, Florida-07, 554CC, and A72) were used for the development of the qPCR assay. These genotypes were planted at the Gibbs Farm, in Tift County, GA and were inoculated with aflatoxigenic strains of *A. flavus* (NRRL 3357) and *A. parasiticus* (NRRL 2999) at approximately 60 days after planting. Fungal infection and aflatoxin contamination were enhanced by exposing the plants to end-season drought for six weeks. Drought conditions were carried out by covering the whole trial with a movable rainout shelter that prevented any form of plant irrigation and increased the soil temperature at the same time. After harvest, the aflatoxin content of the peanut samples was quantified via the standard Vicam fluorometry method. Briefly, representative portions (100 g) of shelled peanuts were added with 10 g of NaCl and 200 ml of methanol/water (80:20 v/v), homogenized using a Waring blender at high speed for 1 minute, and filtered through Whatman paper. Five ml of the filtrate was diluted with 20 ml HPLC water then re-filtered. Ten ml filtrate was purified with Vicam immunoaffinity columns (Vicam Aflatest, MA) containing aflatoxin-specific (B_1, B_2, G_1 and G_2) monoclonal antibody and washed with 10 ml HPLC water before eluting the aflatoxin with 1 ml methanol. The eluted fraction was diluted twice with HPLC water and measured with the Vicam fluorometer (Vicam Series 4 Fluorometer). Samples that were contaminated with high (above 400 ppb) and low (below 10 ppb) aflatoxin levels were identified and stored in -80^{o}C until further use.

6.3.2 Extraction of fungal DNA from ground peanuts

Approximately 240 mg of ground peanuts were used for the isolation of fungal genomic DNA using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). As a revision to the kit's original instructions, the amount of reagents used in the initial steps were tripled to accommodate the 240 mg sample. This increase in sample amount and reagents was performed with the objective of collecting more fungal mass with increased amount of peanuts. All steps after running the lysate through the QIAshredder spin columns were done following manufacturer's instructions. The concentration of the obtained DNA was quantified with PicoGreen® (Invitrogen, Eugene, OR) using a fluorometer (Hoefer TKO-100). All the samples were diluted equally to have DNA concentrations of 10 ng/µl.

Genomic DNA was also extracted from *A. flavus* (NRRL 3357) and *A. parasiticus* (NRRL 2999) for the generation of standard curves. These fungi were separately cultured in 50 ml Potato Dextrose Broth and incubated in an orbital shaker at 200 rpm for four days at 25^{o}C. The fungal mycelia were harvested through filtration then ground with mortar and pestle in liquid nitrogen. Approximately 100 mg of the resulting mycelial powder was used for the isolation of total genomic DNA using the DNeasy Plant Mini Kit following manufacturer's instructions.

6.3.3 Sample screening through conventional PCR

Peanut samples were screened for *A. flavus* and *A. parasiticus* infection through conventional PCR (GeneAmp® PCR System 9700) using primers 2250 (aflS_s: 5'-GTTTCC TCGTGCAGACAGAAGCTAAGG-3') and 2251 (aflS_as: 5'AAGTGATGCGTGCGCGTA GATGCAGG-3'). This primer pair encloses an amplicon of 122 bp and targets the *aflS* gene.

The PCR reactions were prepared in 10 µl solutions containing 0.8 µl DNA, 5.20 µl HPLC water, 1 µl 10X Taq Buffer, 1 µl 10% PVP, 0.08 µl 25mM dNTPs, 0.6 µl 25mM $MgS0_4$, 0.1 µl BSA, 1 µl primers, and 0.8 µl Taq polymerase using the following thermocycling conditions: 95^{o}C for 5 minutes, 35 cycles of 94^{o}C for 30 s, 60^{o}C for 30 s, 72^{o}C for 30 s, and 72^{o}C for 2 min. Samples with positive amplicons corresponding to 122 bp in 2% agarose gel were subjected to qPCR in order to quantify the amount of fungal infection. Screening was continued until three extractions for each genotype having high and low aflatoxin contaminations showed positive fungal infection.

6.3.4 Quantification through qPCR

The amounts of fungal infection in the screened peanuts were quantified through qPCR using the same primers that were mentioned above. The qPCR assays were performed and monitored using both a Smart Cycler® II and a Light cycler® 480 (Roche Applied Science System) using SYBR Green. Amplification was performed using a total reaction volume of 10 µl containing 2.5 µl sample DNA, 2 µl PCR-grade water, 0.5 µl 5mM primers, and 5µl SYBR Green master mix subjected to one cycle of 95^{o}C for 2 min and 40 cycles of 95^{o}C for 15 s, 60^{o}C for 15 s, and 72^{o}C for 1 min.

6.3.5 Detection limit of A. flavus and A. parasiticus

In order to determine the lowest amount of fungal DNA detectable by the qPCR assay using primers 2250/2251, known dilutions of fungal DNA ranging from 1 pg to 10 ng were prepared and subjected to the qPCR reaction volumes and thermocycling conditions mentioned above.

6.3.6 Standard curves

For quantification purposes, three standard curves were generated from: (a) serial dilutions of *A. flavus* (NRRL 3357) DNA ranging from 1 pg to 90 ng; (b) serial dilutions of *A. parasiticus* (NRRL 2999) DNA ranging from 1 pg to 90 ng; and, (c) peanut DNA spiked with serial dilutions of *A. flavus* ranging from 20 pg to 25 ng. All concentrations were amplified using primers 2250/2251. The curves were generated by plotting the Ct values against the starting quantity of the template of each dilution (log_{10}). Afterwards, the amplification efficiencies were calculated from the slopes of the standard curve.

6.3.7 Data analysis

The difference between the aflatoxin content (log_{10}), obtained Ct values, and amount of fungal infection of peanut samples with high and low aflatoxin levels were analyzed using one-way PROC ANOVA procedure in SAS ver. 9.2 (SAS Institute, Cary, NC). The correlation between aflatoxin content and fungal DNA concentration was also obtained.

6.4 Results

6.4.1 Aflatoxin extraction

The aflatoxin content of the peanut samples ranged from 3-1,200 ppb. Samples with aflatoxin contents of 3-8 ppb were chosen to represent low contamination level while 420-1,200 ppb was chosen for high contamination level (Table 33). Data analysis showed a significant difference ($P \geq 0.05$) between high and low aflatoxin levels.

6.4.2 Standard curves

Standard curves were generated from known dilutions of *A. flavus* and *A. parasiticus* ranging from 1 pg to 90 ng and amplified using primers 2250/2251 (Figures 9-10). Both standard curves produced correlation coefficients (R^2) of 0.99 indicating high linear correlations between template DNA and Ct values. The slopes of the standard curves were -3.38 and -3.30 for *A. flavus* and *A. parasiticus*, respectively, yielding very reliable respective amplification efficiencies of 99% and 101%. The obtained negative slopes indicate that higher amounts of template DNA corresponded to progressive lower Ct values. These results confirmed that the assay is sensitive and accurate for the detection of the *aflS* gene.

6.4.3 Detection and quantification of A. flavus and A. parasiticus in peanut seeds

The presence of *A. flavus* and *A. parasiticus* in the peanut seeds was initially screened through the production of amplicons corresponding to 122 bp on 2% agarose gel (Figures 9-10). Three extractions per genotype under high and low aflatoxin levels that were identified to have positive fungal infection were obtained. Results of the study prove the capability of the developed qPCR assay to detect fungal DNA in peanut seeds. The screened samples were subjected to qPCR for the quantification of fungal infection. The obtained fungal concentration per sample is shown in Table 33.

The specificity of the primers used in the study was already evaluated by Dr. Ye Chu at NESPAL, Tifton, GA and showed that the primers target only *A. flavus* and *A. parasiticus*. Results of the detection limit assays for both fungi showed that the lowest amount of detectable fungal DNA using primers 2250/2251 is 10 pg (Figures 13-14). The concentrations of 1 pg were no longer detected. When spiked into peanut DNA, the serial dilutions of *A. flavus* ranging from

20 pg to 25 ng were successfully detected (Figure 15). No amplifications were detected from the no template control (NTC) indicating that no primer dimers were formed. The derivative melting curves of the assay produced a single peak indicating that only single amplicons were produced.

Statistical analysis showed that samples with high aflatoxin contamination have significantly lower Ct values ($P \geq 0.05$) and higher fungal infection ($P \geq 0.10$) as compared to samples with low aflatoxin contamination (Table 33). No significant difference was observed in the Ct values and amount of fungal infection across peanut genotypes. Correlation analysis showed a moderate correlation (0.56) between the obtained amount of fungal infection and the measured aflatoxin contamination in the peanut seeds.

6.5 Discussion

This study demonstrated the ability of the developed qPCR assay to quantify the amount of *A. flavus* and/or *A. parasiticus* in peanut seeds using species-specific primers that target the *aflS* gene. The development of rapid and sensitive detection methods like this qPCR assay for the identification of potential aflatoxigenic species in peanuts intended for food and feeds can be helpful to estimate the potential health risks associated with them (28). The assay used an increased sample size of 240 mg of ground peanut seeds, which is thrice higher than is usually being used in DNA extraction assays. This was done in order to have better chances of obtaining fungal biomass. An amount of ~80 mg of ground seeds were initially used for preliminary experiments but were not very successful in detecting fungal biomass. Unlike previous *in vitro* studies that used artificially inoculated seeds in smaller quantities, it was more challenging to obtain fungal biomass from the field-inoculated ground samples obtained from a 1.5 m two-row

plot as not all seeds would be infected by *Aspergillus* nor would they be contaminated with aflatoxin.

The ability of the assay to detect as low as 10 pg of pure fungal DNA or 20 pg when spiked into peanut DNA demonstrates the sensitivity of the assay. However, proper sample collection must be employed for the assay to be successful.

The high amplification efficiencies of the assay confirmed the sensitivity and accuracy of the assay to detect the *aflS* gene. This gene is among the 25 genes comprising the aflatoxin gene cluster region of approximately 70 kb in both *A. flavus* and *A. parasiticus* genomes (32). The aflatoxigenic strains of *A. flavus* and *A. parasiticus* contain the complete gene cluster, while the whole or portions of the cluster is absent in atoxigenic species and strains (15; 19). In the aflatoxigenic strain NRRL 21882 used in Aflaguard®, the portion of the gene cluster which include the *aflS* gene is deleted (19). Thus, this assay can also be useful to distinguish the presence of aflatoxigenic strains in peanut seeds from those of Aflaguard®, which is the registered biological control agent used in peanut (9). It should, however, be noted that the *aflS* gene can still be present in the aflatoxin gene cluster of some atoxigenic strains of *A. flavus* including those of AF36 (registered biological control agent in cotton) (5; 19).

Results of this study showed a significant moderate correlation (r = 0.56) between the amount of fungal infection and aflatoxin contamination in peanut seeds conforming to the report of Mideros *et al.*(18). These authors reported that fungal infection estimated by qPCR can be used to infer aflatoxin concentration and that aflatoxin concentration should reflect the levels of fungal biomass. Since there was significant correlation between aflatoxin contamination and fungal infection, it would be beneficial to identify how much fungal infection in a peanut seed is

sufficient to produce an aflatoxin content of 20 ppb (the regulatory limit for crops to be accepted for direct human and animal consumption).

6.6 Summary and Conclusions

The study demonstrated the capability of the developed qPCR assay to quantify the amount of *A. flavus* and/or *A. parasiticus* infection in peanut seeds wherein fungal DNA as low as 10 pg can be detected. As a result, this assay can serve two possible uses: (a) detect minute fungal infection that is invisible to the eye; (b) and, preliminary test to detect aflatoxigenic *A. flavus* and *A. parasiticus* even before aflatoxin production. Situations may occur when peanut seeds are infected with aflatoxigenic fungi that has not yet produced aflatoxin at the time of aflatoxin testing but has the ability to produce aflatoxin when exposed to favorable conditions during transport or storage. Detecting these fungi before entering the food chain would be important.

A moderate correlation between the amount of fungal infection and aflatoxin contamination was detected. Therefore in the future, it would be beneficial to identify how much aflatoxigenic fungal infection in a peanut seed is sufficient to produce an aflatoxin content of 20 ppb. If this amount would be identified, it can be possible to use qPCR either to confirm or serve as an alternative to more expensive and tedious aflatoxin extraction and quantification methods. As reported by Mideros *et al*. (18), the cost of running samples using qPCR in a well-equipped laboratory is cheaper than immunocapture aflatoxin quantification such as the Vicam AflaTest. It was, however, identified that the *aflS* gene can still be present in some atoxigenic strains of *A. flavus* (19). Thus, it would also be useful to identify other target genes that can discriminate

between aflatoxigenic and toxigenic strains and develop more specific qPCR assays for the detection of aflatoxigenic strain in peanut seeds.

6.7 References

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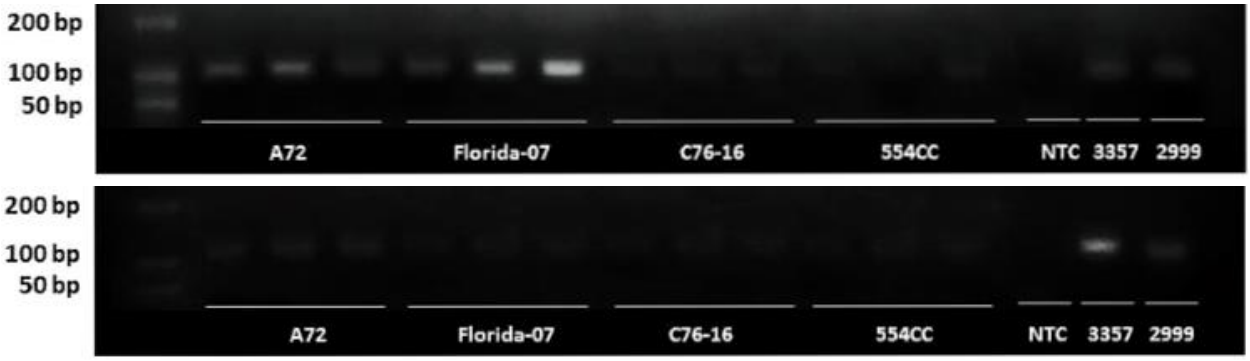
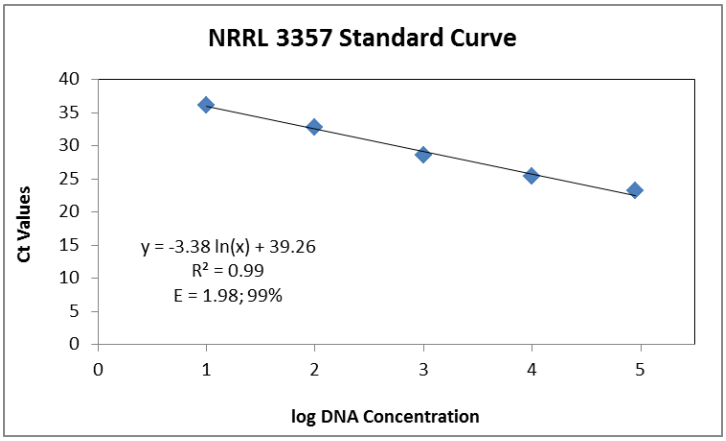
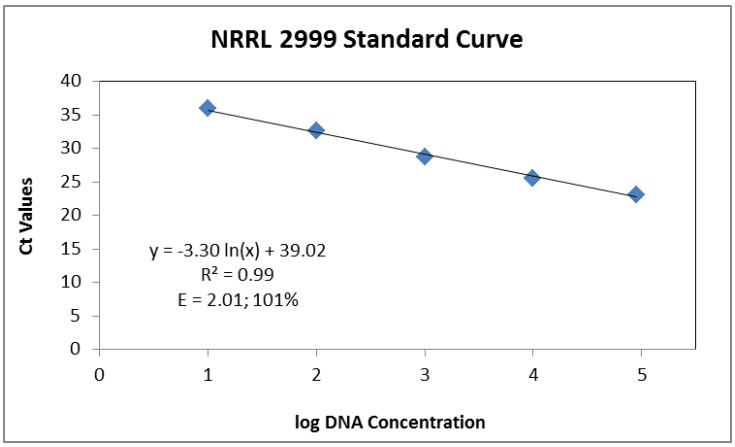
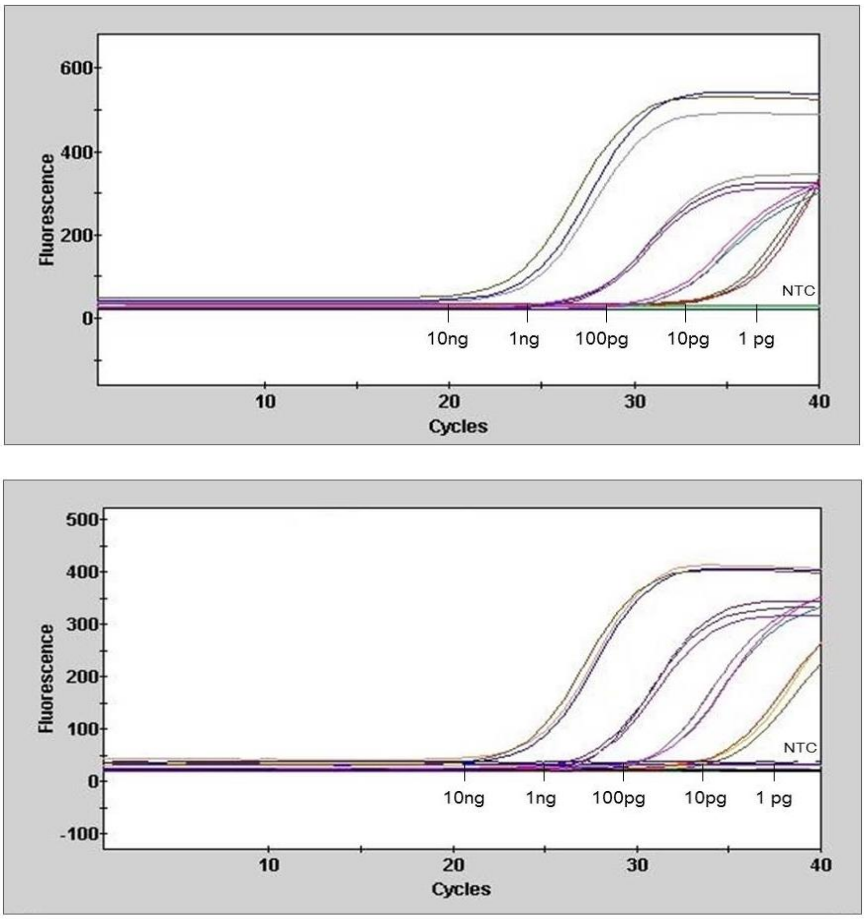


Figures 9-10. Screening of peanut samples for fungal infection. Peanut samples identified to have high (top) and low (bottom) aflatoxin levels were screened for the presence of *A. flavus* and/or *A. parasiticus* using primers 2250/2251 through conventional PCR. The figure shows three sample extractions showing positive fungal infection per peanut genotype under high and low aflatoxin levels. Each PCR reaction contained 0.8 µl template DNA in 10 µl volumes, diluted equally to have DNA concentrations of 10 ng/µl. PCR products were amplified in 2% agarose gel. NTC = non-template control; 3357 = NRRL 3357 (*A. flavus*); and, 2999 = NRRL 2999 (*A. parasiticus*)





Figures 11-12. Standard curves of NRRL 3357 (*A. flavus*) and NRRL 2999 (*A. parasiticus*). Curves were generated from qPCR by plotting the threshold cycle (Ct) vs $\log_{10}$ initial template DNA amplified with primers 2250/2251. Initial DNA concentration ranged from 1 pg to 90 ng of each isolate. E = amplification efficiency; R^2 = correlation coefficient.



Figures 13-14. Amplification curves of the different DNA concentrations of *A. flavus* (NRRL 3357, top) and *A. parasiticus* (NRRL 2999, bottom) ranging from 1 pg to 10 ng detected by qPCR.

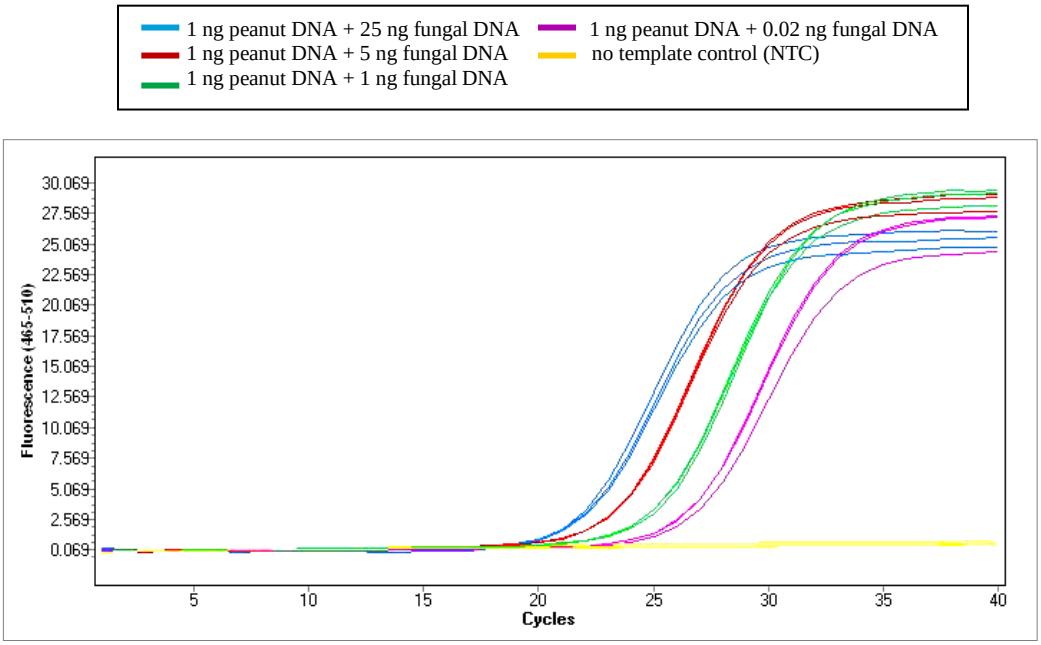


Figure 15. Amplification curves obtained from a fixed amount of peanut DNA spiked with varying amounts of fungal DNA (NRRL 3357) amplified with primers 2250/2251 targeting the *aflS* gene.

Table 33. Quantitative real-time PCR assay results.

AFLATOXIN LEVEL	GENOTYPE	AFLATOXIN CONTENT (ppb)	AFLATOXIN CONTENT (ppb, log_{10})	Ct VALUES	FUNGAL DNA (ng, sqrt)	FUNGAL DNA (ng/g of peanut)
HIGH	A72	1200.0	3.079	31.156	0.124	0.255
	Florida-07	890.0	2.949	30.606	0.101	0.206
	C76-16	420.0	2.623	27.073	1.036	2.124
	554CC	460.0	2.663	31.467	0.316	0.647
	Mean	742.5	2.829 A*	30.075 B*	0.394 A**	0.808 ± 1.51
LOW	A72	3.0	0.477	32.455	0.053	0.109
	Florida-07	4.8	0.681	34.334	0.022	0.045
	C76-16	8.0	0.903	35.107	0.012	0.025
	554CC	3.4	0.531	34.465	0.014	0.029
	Mean	4.8	0.648 B*	34.090 A*	0.025 B**	0.052 ± 0.06

Means with different letters in a column are significantly different at P≤0.05 (*) or P≤0.10 (**) as determined by Fisher LSD test

CHAPTER 7

SUMMARY AND CONCLUSIONS

The best way to manage pre-harvest aflatoxin contamination in food and feeds would be to integrate good agricultural practices which include the use of aflatoxin-resistant genotypes, proper identification of fungal species for the development of proper management strategies, and efficient aflatoxin detection strategies which implement efficient sampling and sample preparation methods (1; 2). These factors were incorporated in the study.

Chapter 2 shows the drought-coping abilities of seven peanut genotypes (Tifguard, Tifrunner, Florida-07, 554CC, NC3033, C76-16, and A72) as evaluated using visual drought stress ratings, chlorophyll fluorescence (PI_{ABS}, Fv/Fm, and PHI_{EO}), SCMR, CT, NDVI, stomatal conductance, and pod yield. Results show that Tifguard and Tifrunner had better performance than the rest of the genotypes indicative of their being good candidates for incorporation into plant breeding programs that aim to develop pre-harvest aflatoxin resistance. Significant correlations were obtained between aflatoxin contamination and visual drought stress ratings, SCMR, CT, CTD, and NDVI. Thus, selection for these drought-tolerance traits can be used for reduced pre-harvest aflatoxin contamination. These evaluation methods are easily measurable and economically reasonable, which can be helpful in improving breeding programs while reducing the cost of developing resistant lines or cultivars.

Chapter 3 reveals how 99 isolates belonging to *Aspergillus* section *Flavi* obtained from peanut samples collected from Texas, Alabama, and Georgia of US; Haiti; and Philippines were

identified through morphological characterization, conventional DNA sequencing, and repetitive-sequence-based PCR (rep-PCR) DNA fingerprinting. Morphological observations showed that colony surface color and conidial wall texture of 7-day old cultures grown in CYA and incubated at 34^{o}C can be helpful in screening species belonging to *Aspergillus* section *Flavi*. Species identification based on morphology can be much cheaper as compared to the cost of reagents used in molecular methods. However, this method can be time-consuming and requires adequate mycological background and skills to be successful. Conventional DNA sequencing offered the advantage of giving more specific results. The use of primers that target the beta tubulin (Tub2F/Tub2R) were not able to discriminate among *Aspergillus flavus, A. parasiticus,* and *A. oryzae.* On the other hand, the combined use of primers that target the internal transcribed region section (ITS) (ITS5/ITS4) and translation elongation factor (TEF1-α) (TEF983F/ TEF2218R) were able to identify the isolates into species. Rep-PCR performed to be the most sensitive approach, identifying 93.33% of the isolates as *A. flavus*, 4.44% as *A. parasiticus,* and 2.22% as *A. oryzae*. Results of dendrogram did not show a distinct clustering of isolates according to geographical location. However, isolates from Georgia, Texas and Alabama mostly clustered together whereas isolates from Haiti clustered with a majority of isolates from Philippines. Three isolates from Georgia formed a group with Aflaguard® suggesting that these can be possible variants of Aflaguard® which can be explored as biological control agents against aflatoxigenic strains.

In Chapters 4-6, three management approaches involved in the detection of aflatoxin in peanuts were studied. These composed of determining the effect of sample size in aflatoxin detection, assessment of the ability of immunochromatographic test strips to perform well under

tropical temperatures, and testing the applicability of qPCR in the detection of *A. flavus* and *A. parasiticus* in peanut seeds.

The sample size experiments in chapter 4 show that the standard method of subsampling peanut samples from 300 g into 100 g for aflatoxin detection can be reduced into subsampling of 100 g into 25 g. This sample size (100 g subsampled into 25 g) produced the narrowest confidence interval of aflatoxin values measured from 25 samples of contaminated peanuts. The reduction in original sample size (100 g instead of 300 g) can save a significant amount of peanut to be taken for aflatoxin analysis. This can be beneficial in situations when peanut harvest is limited and/or if more than one sampling is required. The reduction subsample size (25 g instead of 100 g) will give enormous savings for the cost of chemicals used in aflatoxin quantification. The direct sampling of 25 g is not recommended due to the risk of either hitting or missing kernels with very high aflatoxin content which can lead to unreliable results.

The assessment of the performance of immunochromatographic test strips in detecting aflatoxin levels as based on the 20 ppb cut-off limit in chapter 5 presented a high correspondence (95%) with the use the fluorometry method. The test strips incubated at continuous high (34^{o}C) and fluctuating (34^{o}C for 8 hours and 25^{o}C for 16 hours) temperatures yielded accurate results for 32 and 47 weeks (around 8 and 12 months) for AflaCheck™ and AgraStrip®, respectively. Based on the results of the study, these test strips are recommended for use in the qualitative detection of aflatoxin in peanut production areas or clinical laboratories that lack specialized equipment like the fluorometer or in tropical locations where refrigeration is not a part of normal storage practice. These test strips, however, are not recommended for use if exposed to high and fluctuating temperatures beyond 32 and 47 weeks for AflaCheck™ and AgraStrip®, respectively.

The ability of the qPCR assay using species-specific primers that target the *aflS* gene to quantify the amount of *A. flavus* and/or *A. parasiticus* infection in peanut seeds was demonstrated in chapter 6. This assay can detect fungal DNA as low as 10 pg. This qPCR assay could be used for the purpose of detecting minute fungal infection that is visually invisible to the eye, detecting *A. flavus* and *A. parasiticus* infection even before they produce aflatoxin in peanuts seeds, and confirming or serving as an alternative to more expensive and tedious aflatoxin detection methods. Based on the significant correlation between the amount of fungal infection and aflatoxin contamination, it would be beneficial to identify how much fungal infection in a peanut seed is sufficient to produce an aflatoxin content of 20 ppb (the regulatory limit for crops to be accepted for human consumption).

7.1 References

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