GENETIC ANALYSIS OF AFLATOXIN AND AGRONOMIC TRAITS IN GROUNDNUT (*ARACHIS HYPOGAEA* L.)

BY

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DECLARATION

I declare that the work in this dissertation entitled "GENETIC ANALYSIS OF AFLATOXIN AND AGRONOMIC TRAITS IN GROUNDNUT (*ARACHIS HYPOGAEA* L.)" has been carried out by me in the Department of Plant Science. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Muhammad Ahmad YAHAYA ________________ ________________

Signature Date

CERTIFICATION

This dissertation entitled "GENETIC ANALYSIS OF AFLATOXIN AND AGRONOMIC TRAITS IN GROUNDNUT (*ARACHIS HYPOGAEA* L.)" by Muhammad Ahmad YAHAYA meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this dissertation to my parents who have been and will always be my source of inspiration and to the memory of my fallen Heroes, Late Professor Ahmad Falaki and Professor Balarabe Tanimu, may your gentle souls rest in peace, Ameen.

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ABSTRACT

Groundnut (*Arachis hypogaea* L.) is one of the major crops susceptible to *Aspergillus flavus* infection and subsequent aflatoxin contamination which adversely affects its production and utilization in the small holder setting. Although there are several management strategies that may reduce aflatoxin contamination of groundnuts, the pre-eminent strategy for the prevention of aflatoxin is to develop host resistance to *A. flavus*. The objectives of this study were to determine the level of resistance to *A. flavus* and aflatoxin accumulation among selected groundnut genotypes, to assess the mode of gene action controlling resistance to aflatoxin accumulation and agronomic traits and to estimate heritability for resistance to *A. flavus* and aflatoxin accumulation. Eight groundnut varieties comprising of three *A. flavus* resistant lines and five susceptible varieties were obtained from Institute for Agricultural Research, Samaru. The genotypes were crossed in a 3 x 5 North Carolina (NC II) design II fashion to generate 15 F_1 hybrids. The F_1 seeds were selfed to F_2 and then evaluated alongside the eight parents for twelve quantitative characters using randomised complete block design (RCBD) with three replications at the Department of Plant Science screen-house under artificial *A. flavus* inoculation. Analysis of variance revealed significant ($P \leq 0.05$) differences among the groundnut genotypes for all traits except for 100-kernel weight and shelling percentage. The mean performance of the parents showed that SAMNUT 22 (2.50 ng/g), ICGV 91317 (6.31 ng/g) and ICGV 91324 (12.23 ng/g) recorded lowest aflatoxin accumulation value of less than 20.00 ng/g and the crosses: SAMNUT 26 x ICGV 91317 (0.8 ng/g), SAMNUT 26 x ICGV 91328 (1.2 ng/g), SAMNUT 25 x ICGV 91324 (3.92ng/g), SAMNUT 22 x ICGV 91328 (4.4 ng/g) and SAMNUT 22 x ICGV 91324 (12.3 ng/g), as the best

for resistance to *A. flavus* infection and aflatoxin accumulation under the United States Food and Drug Administration, which sets the limit of aflatoxin in food and feeds at 20 *ng/g*. The general (GCA) and specific (SCA) combining abilities were significant ($P \leq 0.05$) for most characters indicating the role of both additive and non-additive gene effects in the expression of most characters. High broad-sense heritability values were obtained for days to 50% flowering (88.9%), plant height (84.0%), haulm weight (86.8%), kernel infection (85.3%) and aflatoxin accumulation (99.7%). The parental genotypes, SAMNUT 23, SAMNUT 24, SAMNUT 25, SAMNUT 26, ICGV 91324, ICGV 91328 were good general combiners for aflatoxin B_1 accumulation. Among the progenies, SAMNUT 22 x ICGV 91324 and SAMNUT 23 x ICGV 91317 with positive SCA effects for haulm weight and negative SCA effects for kernel infection and aflatoxin accumulation. *A. flavus* infection among the parents and progenies correlates poorly to aflatoxin contamination. The study revealed that non-additive gene action was more important than additive gene action for resistance to *A. flavus* infection, while additive gene action was more predominant for resistance to aflatoxin accumulation. The information provided from this study could be utilized in planning breeding programme for the development of groundnuts with improved pod yield, haulm yield, resistance to *A. flavus* infection and aflatoxin accumulation.

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CHAPTER ONE

1 Introduction

Groundnut (*Arachis hypogaea* L.) is an annual legume which is also known as peanut. It is the 13th most important food crop of the world. Today, groundnut is the world's 4th most important source of edible oil and the 3rd most important source of vegetable protein in the world. Groundnut is cultivated in more than 100 countries in 6 continents and covers an area of 26.4 million hectares worldwide with a total production of 41.3 million metric tons, and an average productivity of 1.4 metric tons per hectare (FAO, 2014). Major groundnut producers in the world are: China, India, Nigeria, USA, Indonesia and Sudan. Developing countries account for 96% of the global groundnut area and 92% of the global production (FAO, 2014). Groundnut kernels are consumed directly as raw, roasted or boiled kernels or oil extracted from the kernel is used as culinary oil. Groundnut seeds contain high quality oil (50%), easily digestible protein (25%) and carbohydrates (20%). It is also used as animal feed and industrial raw material. These multiple uses of groundnut make it an excellent cash crop for domestic markets as well as for foreign trade in several developing and developed countries (Pandey *et al*., 2012).

Problem statement

Groundnut is affected by several diseases like leaf spots, collar rot, rust, bud necrosis and stem necrosis (Prasada *et al.*, 2012). Apart from these, aflatoxin contamination is one of the major problems, produced in the infected groundnut seeds by *Aspergillus flavus* Link ex fries and *Aspergillus parasiticus* Speare, particularly at the end of

season under drought conditions (Fountain *et al.*, 2014). Aflatoxins, especially the most potent aflatoxin B_1, are secondary metabolite produced by *A. flavus.* They are highly carcinogenic, immunosuppressive agents, highly toxic and fatal to humans and animals particularly affecting liver and digestive track (Wild and Gong 2010). These health problems are more severe in African communities due to exposure to aflatoxins throughout their lives (Wild and Gong 2010). There have been increasing reports of aflatoxin contamination in freshly harvested groundnuts in several countries of sub-Saharan Africa. Recently, 22–54% of groundnut samples collected during 2009 and 2010 from different groundnut growing areas in Nigeria showed >20 ng g^{-1} of aflatoxins (Waliyar *et al.*, 2016). This contamination renders the commodity unfit for human consumption and unacceptable for trade in high-value markets. Therefore, aflatoxin contamination in groundnut as well as in maize has been considered as a major non-tariff barrier to international trade since agricultural products that exceed the permissible levels of contamination (4 to 20 ng/g) are banned. About $1.2 billion in commerce is lost annually due to aflatoxin contamination, with African economies losing $450 million each year (IITA, 2013).

Following the outbreak of aflatoxicosis and the enormous economic loss in the poultry industry of Britain in 1961, the Federal government of Nigeria, in order to protect her export trade initiated screening studies to determine the extent of aflatoxin contamination of groundnut and groundnut products (Halliday and Kazaure, 1967). McDonald and Harkness (1965) found aflatoxins in groundnut samples from Zaria, Kano and Mokwa, in Northern Nigeria. Bassir (1969) isolated aflatoxin B_1 from various mouldy food materials offered for sale in Ibadan markets. Since then, toxigenic fungi and mycotoxins have been found in various foods and feedstuffs in many regions of Nigeria. Thus, mycotoxigenic fungi belonging to not less than forty

five fungal genera and about twenty different mycotoxins have been detected in Nigerian foods and foodstuffs (Ezekiel *et al*., 2012). Nigeria has experienced high recorded aflatoxin exposure levels in humans and has also reported the highest estimated number of cases of hepatocellular carcinoma (HCC-liver cancer) attributable to aflatoxins in the whole world (Liu and Wu, 2010). Due to these health risks, many countries have established strict regulations regarding the permissible levels of aflatoxins in food and feed. For the Nigeria, the limit for all foods is 20 ng/g of total aflatoxins (B_1, B_2, G_1 and G_2) except milk, which has a limit of 0.5 ng/g of aflatoxin M_1 (Ezekiel *et al*., 2012).

Justification

In Nigeria, groundnut farmers rely on cultural practices (such as irrigation, early planting, rapid drying after harvest) and lately bio-controls to manage *A. flavus* infection and to reduce aflatoxin contamination. Irrigation has been shown to reduce preharvest aflatoxin contamination in groundnut and maize (Waliyar *et al*., 2015). The biocontrol strategy through the application of atoxigenic strains of *A. flavus* to compete with toxigenic *A. flavus* in the field has been shown to significantly reduce aflatoxin contamination in the African countries (Probst *et al.*, 2011). In general, good cultural and management practices as well as biocontrol can reduce, but not eliminate preharvest aflatoxin contamination as some of the management practices are not always available or cost-effective (Waliyar *et al.*, 2015). Therefore, there was a major effort to identify germplasm with natural resistance to *A. flavus* infection (preharvest resistance) with diminished accumulation of aflatoxin in the past four decades. Attempts have identified potentially resistant groundnut genotypes for pre-harvest aflatoxin contamination (Anderson *et al*., 1995; Upadhyaya *et al.,* 2004; Waliyar *et*

al., 2016). Three groundnut genotypes such as ICGV 87084, ICGV 87094 and ICGV 87110 were identified to be resistant to *A. flavus* and aflatoxin contamination as evaluated in Niger, Senegal and Burkina Faso in West Africa (Waliyar *et al*., 1994). Further improved groundnut germplasm lines such as ICGV 91278, ICGV 91283 and ICGV 91284 were registered as resistant to *A. flavus* seed infection (Upadhyaya *et al*., 2000). However, undesirable agronomic characteristics such as poor shelling outturn and late maturity (Upadhyaya *et al*., 2004) associated with these genotypes, hinder their direct use as commercial aflatoxin resistant groundnut varieties in Nigeria. The effort to incorporate resistant traits from these germplasm into commercial background has been a challenge due to lack of a quick, inexpensive, and reliable means to evaluate resistance (Moreno and Kang, 1999), and a poor understanding of host resistance mechanisms. Genetic improvement of quantitative characters in groundnut through different breeding programs desires the information on the nature and magnitude of gene effects. The genetic potential of groundnut can be predicted and measured by the estimates of genetic effects. Based on this, various breeding strategies can be formulated towards the genetic improvement for resistance to *A. flavus* infection, aflatoxin accumulation and other agronomic characters. It is in the light of the above that this study was undertaken with the following objectives:

i. To determine the level of resistance to *Aspergillus flavus* and aflatoxin accumulation among selected groundnut genotypes and their F_2 progenies
ii. To assess the mode of gene action controlling resistance to kernel infection by *A. flavus* and agronomic traits
iii. To estimate heritability for resistance to *A. flavus* and aflatoxin accumulation

CHAPTER TWO

2 Literature Review

2.1 Groundnut

The cultivated groundnut (*Arachis hypogaea* L.) is an allotetraploid (2n=2x=40) with "AA" and "BB" genomes. All species, except the cultivated species *A. hypogaea* and *A. monticola* in the Section *Arachis*, and certain species in Section Rhizomatosae, are diploid (2n=2x=20). The diploid progenitors; *A. duranensis* and *A. ipaensis*, contributed "AA" and "BB" genomes, respectively, to the cultivated groundnut (Kochert *et al*., 1996). A single hybridization event between the diploid progenitors followed by chromosome doubling about 3500 years ago lead to origin of cultivated groundnut. Southern Bolivia and Northern Argentina are thought to be centre of origin of this crop (Kochert *et al*., 1996). The centre of diversity of the genus includes Western Brazil, Bolivia, Paraguay, and Northern Argentina (Gregory *et al.,* 1980).

Features of a groundnut plant are shown in Fig 2.1 (Nigam *et al.,* 1983). Main stem develops from a terminal bud of the epicotyl and two cotyledonary laterals grow on opposite sides; Tetrafoliolate leaves, with leaflets on the main stem differing in shape and size from those on lateral branches.

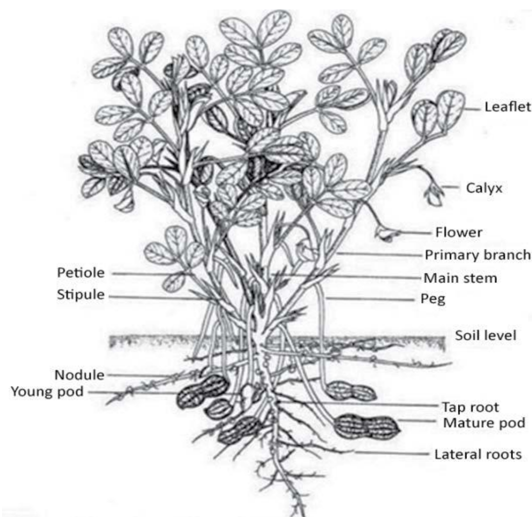

Fig. 2.1. A stylized groundnut plant

Groundnut is a self-pollinated crop with cleistogamous flowers, but natural hybridization can occur to small extent where bee activity is high (Nigam *et al*., 1983). Flowers, simple or compound, are borne in the axils of leaves and never at the same node as vegetative branch. One or more flowers may be present at a node. The stigma becomes receptive to pollen about 24-hours before anthesis and remains so for about 12 hours after anthesis, and the dehiscence of anthers takes place 2-3 hours prior to opening of the flower in the morning. Fertilization occurs about 6-hours after pollination. Depending upon the prevailing temperatures, the peg or gynophore carrying the ovary and fertilized ovule on its tip appears in 6-10 days and grows to enter the soil (positively geotropic) where it develops in to pods. The tip orients itself horizontally away from tap root. Groundnut grows well in well-distributed rainfall of at least 500 mm. The growth and development is largely influenced by temperature in groundnut and the optimum air temperature is between 25° and 30°C.

2.2 Global Groundnut Production

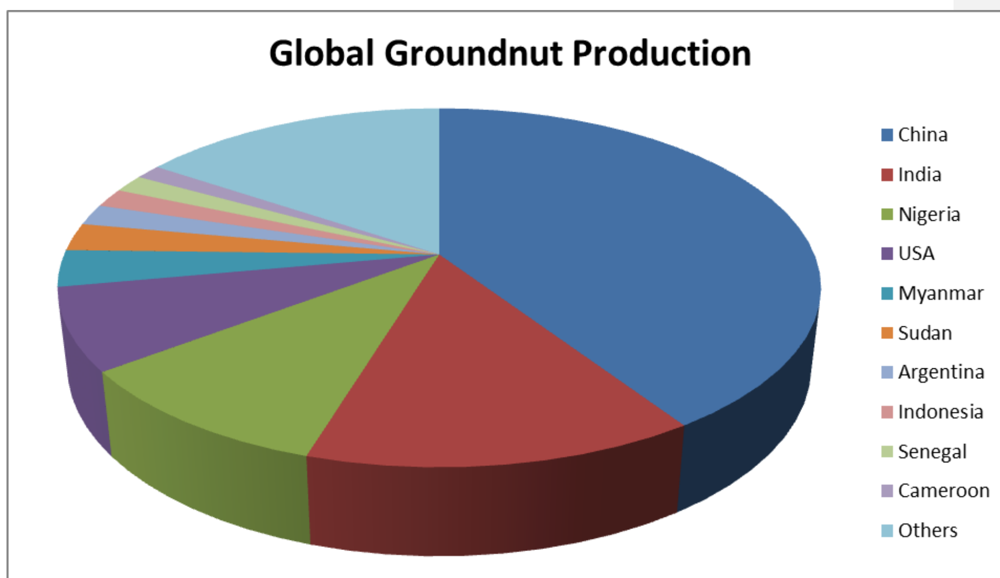

Fig. 2. 2: Global Groundnut Production (FAO, 2014)

Nigeria is the largest groundnut producing country in West Africa accounting for 51% of the production in the region. The country produces 10% and 39% of the World and Africa's total production respectively (FAO, 2014).

2.3 The Genus Aspergillus

The genus *Aspergillus*, a member of the phylum Ascomycota, includes more than 185 known species. Several members of *Aspergillus* section Flavi produce aflatoxin. These includes *Aspergillus flavus* and *Aspergillus parasiticus*, as well as several less common taxa including *Aspergillus nomius, A. tamarii, A. pseudotamarii, A. minisclerotigenes* and *A. bombycis* (Cary *et al*., 2005). *Aspergillus* species are indigenous to soils in Nigeria and soil is the primary source of the inoculum (Horn, 2007). Spores of the fungi are present virtually everywhere because they are airborne,

but specific environmental conditions are necessary for the fungus to contaminate crops. Specifically, Aspergillus grows when temperature fluctuates between 18°C and 33°C and the relative humidity is above 50 percent.

2.4 Aflatoxins

Aflatoxins are a group of structurally related toxic secondary metabolites produced mainly by certain strains of *A. flavus* and *A. parasiticus*. *A. flavus,* in particular, is a common contaminate in agriculture (Bennett and Klich, 2003). The four major aflatoxins are called B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography (Bennett and Klich, 2003). The chemical structures of the four types of aflatoxins are presented below (Bhatnagar *et al*., 1993).

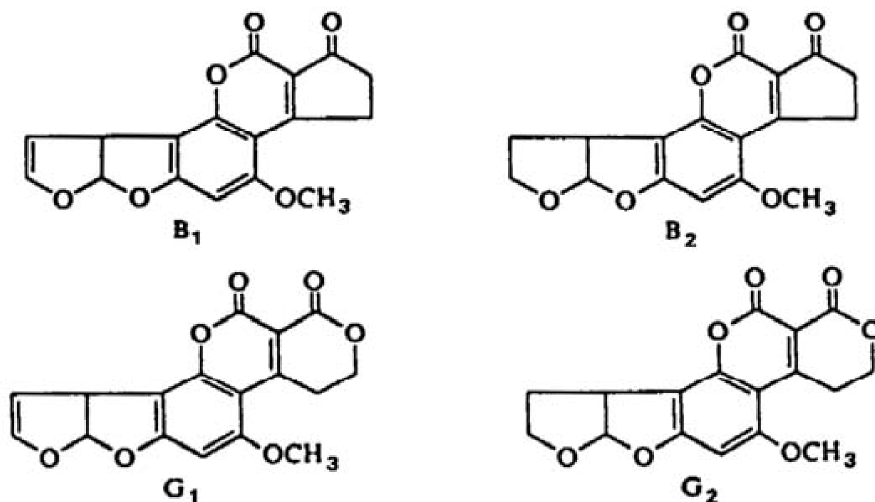

Fig. 2. 3. Chemical structures of aflatoxins B_1, B_2, G_1 and G_2

2.5 Effects of aflatoxin to humans and livestock

Sufficient evidence that AFB_1 and mixtures of B_1, G_1 and M_1 are proven carcinogens has been provided by the International Agency for Research on Cancer which classifies them as Group 1 carcinogens (IARC, 1993). Prolonged exposure to doses of 50 micrograms aflatoxin B_1/kg/day has clinically significant effects on humans. No animal species has been found to be immune to the effects of aflatoxins (Murphy *et al.*, 2006). Aflatoxicosis is the disease associated with the ingestion of aflatoxins. Outbreaks of aflatoxicosis have been reported in Kenya, India and Thailand. For example, in western India in 1974, 108 persons among 397 people affected, died from aflatoxin poisoning (Krishnamachari *et al.*, 1975). A more recent incident of aflatoxin poisoning occurred in Kenya (CAST, 2003). The outbreak which occurred in Kenya is one of the largest and most acute outbreaks of aflatoxicosis ever documented, with 317 cases reported and 125 deaths recorded (CDC, 2004). Investigations revealed that the outbreak was due to the consumption of maize contaminated with extremely toxic AFB_1. The case-control studies carried out by Lewis *et al*. (2005) showed that aflatoxicosis in the affected area was associated with eating home-grown maize stored under humid conditions. During the outbreak, individual daily exposure of AFB_1 was estimated to be 50ng/day (Probst *et al.*, 2011). McDonald and Harkness. (1965) reported that groundnuts cultivated in the Northern parts of Nigeria were contaminated with aflatoxin levels of up to 2000ng/g. Salifu (1978) studied fungal and mycotoxin contamination of sorghum in Nigeria, and reported that aflatoxin levels ranged from 10 to 80ng/g. In a recent report by Ezekiel *et al*. (2012), about 90% (26/29) of groundnut cake samples had aflatoxin concentrations exceeding the maximum limit of 20 ng/g set by NAFDAC.

2.6 Effect of environmental factors on aflatoxin synthesis

The most significant environmental factors that influence aflatoxin synthesis are carbon and nitrogen sources, pH, temperature, water activity, and plant metabolites (Bhatnagar *et al*., 2004). Amino-acids such as tryptophan inhibit the synthesis of aflatoxin whereas tyrosin encourages it (Wilkinson *et al*., 2007). The presence of lipids induces aflatoxinogenesis (Yu *et al*., 2005). Among the organic factors affecting biosynthesis, carbon and nitrogen are the major ones (Luchese and Harrigan, 1993). In addition, highest levels of aflatoxin are produced when the fungus invades the seed embryo, where the highest levels of simple sugars (glucose and sucrose) are present compared to other parts of the seed, where complex carbohydrates predominate (Woloshuk *et al*., 1997). Aflatoxin biosynthesis has been known to be induced by simple carbohydrates, such as glucose and sucrose (Payne and Brown, 1992). Interestingly, a hexose utilization gene cluster is located adjacent to the aflatoxin biosynthetic cluster and may affect expression of aflatoxin genes (Yu *et al*., 2000). Pools of amino acids in the plant, dependent on nitrogen, are reported to be important in regulation of toxin formation (Bhatnagar *et al*., 1986). Nitrate suppression of aflatoxin synthesis in some Aspergillus isolates has been well documented (Niehaus and Jiang, 1989), whereas nitrogen supplied as ammonium in media supports toxin formation. AreA binds to the tetranucleotide recognition site, GATA, in the promoters of aflR and aflJ and affects their expression (Ehrlich and Cotty, 2002). Certain strains of *A. flavus* respond differently to nitrate than do other strains, and these differences correlate with differences in the number of GATA sites near the aflJ transcription start site (Ehrlich and Cotty, 2002).

Fungi are capable of growing over a wide pH range. Under drought stress, physiological pH shifts in plants could occur (Reddy *et al*., 1979). It has been

established that aflatoxin synthesis optimally occurs in the pH range of 3.4–5.5. Biosynthesis is high in acidic mediums while it is inhibited in basic conditions (Cotty, 1988). For *A. parasiticus*, the growth in water is faster with a pH ranging from 5.5 to 6.5 (Al-Gabr *et al*., 2013).

The ideal temperature for aflatoxin production is 29–30 ^{0}C (Payne, 1998). Aflatoxin production is significantly decreased at temperatures below 25 ^{0}C, but is completely inhibited at 37 ^{0}C or above is inhibited due to the attack of transcription genes aflR and aflS, whereas under the conditions of dryness, the production of the aflatoxins is high (Yu *et al*., 2011). Drought stress can increase the percentage of seed infected. The secondary plant metabolites play a key role in the synthesis of aflatoxins (Green-McDowelle *et al.,* 1999). For example, the presence of the octanal causes a reduction of 60% of the fungal growth with a rate of increase in the production of aflatoxins of 500% (Criseo *et al*., 2001). However, hydrolysable tannins considerably inhibit the biosynthesis of aflatoxins (Mahoney and Molyneux, 2004). Some antioxidants such as the phenolic compounds, ascorbic acid and caffeic acid decrease, in an important way, the aflatoxinogenesis, without any effect on the growth of the fungi (Kim *et al*., 2006).

2.7 Resistance-Screening Methods

Much of the effort to study the host resistance to aflatoxin has been directed toward identifying groundnut genotypes resistant to *A. flavus* infection and aflatoxin production. An effective screening technique would be helpful for identification of source of resistance and could aid in studying resistant mechanisms.

2.7.1 Screening for seeds resistance to *A. flavus* invasion

Mixon and Rogers (1973) developed a laboratory inoculation method for screening groundnut genotypes for resistance to *A. flavus* invasion and colonization of rehydrated seeds and identified two resistant Valencia-type genotypes (PI 337394F and PI 337409). Several researchers also identified some resistant genotypes and genotypes with 15% seeds colonized were regarded as resistant.

2.7.2 Screening for resistance to pre-harvest aflatoxin contamination

The *in vitro* seed inoculation screening in the laboratory do not correlate well with the field results (Blankenship *et al.,* 1985), which has stimulated considerable research to develop field inoculation techniques for screening groundnut genotypes for pre-harvest resistance.

Many approaches have been used to inoculate the groundnut plant or the soil to increase the *A. flavus* population densities and to enhance the contact between *A. flavus* and the groundnut pod (Wilson and Stansell, 1983). Common methods used to deliver *A. flavus* inoculums are aqueous suspensions of *A. flavus* conidia, cracked and *A. flavus* infected corn and organic-matrix infested *A. flavus* (Anderson *et al.,* 1995). Holbrook *et al*. (1994) developed a large-scale field screening technique to directly measure field resistance to pre-harvest aflatoxin contamination that uses subsurface irrigation in a desert environment to allow an extended period of drought stress in the pod zone while keeping the plant alive. Without subsurface irrigation, groundnut plants died and their seeds rapidly dehydrated in the soil before contamination could occur. Using this method, 15 accessions showed 70 to 90% reductions in aflatoxin contamination in comparison to susceptible accessions in three environments. Large-scale field screening for resistance to pre-harvest aflatoxin contamination was also

enhanced by the development of a consistently successful and reproducible field inoculation technique (Will *et al*., 1994).

Anderson *et al*. (1996) developed a procedure for greenhouse screening of individual plants of pods from single plant for resistance to invasion by aflatoxigenic fungi and subsequent aflatoxin production by completely isolating the pods from the root zone and imposing drought-stress only on pegs and pods. High levels of fungal infection and high amount of preharvest aflatoxin accumulation were observed by mid-bloom inoculating with *A. parasiticus* contaminated cracked corn and imposing drought period of 40-60 days.

2.8 Constitutive components in seeds related to resistance

Structure of seed coat: As seed coat is the outermost layer of groundnut kernels, several researchers have attempted to characterize features relative to resistance (Zhou *et al*., 1999). LaPrade *et al.* (1973) suggested that resistance could be attributed to seed coat thickness and/or permeability. In other experiments, Taber *et al*. (1973) observed that kernels of resistance genotypes had smaller hila, more compact arrangement of palisade-like layer of testa and a thicker waxy surface compared to susceptible genotypes. Zambettackis (1975) also reported considerable diversity in the testa structure of groundnut lines viewed with electron and light microscope. A method of classifying these differences was given as a guide to measurement of resistance to *A. flavus*. The role of wax and cutin layers in groundnut seeds' coat associated resistance to invasion and colonization by *A. flavus* was studied by Liang *et al*. (2002). The results showed that wax contents of resistant genotypes were significantly higher than susceptible cultivars. The resistant kernels had a thicker and

coarser waxy deposit on seed coat surfaces than the susceptible genotypes under scanning electron microscope. Removal of wax with chloroform and removal of cutin with KOH and cutinase or both (Liang *et al*., 2001) can increase the susceptibility of groundnut seeds. The bioassay of wax in vitro showed that there were no significant test-by-treatment interactions. These results indicated wax and cutin layers of groundnut seed coat might only play a role of physical barrier in resistance to *A. flavus* invasion and colonization.

Phytoalexin accumulation: Phytoalexins are antibiotic secondary metabolites produced by plants in response to injury and invasion by some pathogens and appear to be involved in disease resistance (Sobolev *et al*., 2007). Resveratrol is one of the phytoalexin compounds found in groundnut seeds (Sanders *et al*., 2000). Liang *et al*. (2006) compared the synthesis capacity for resveratrol between the resistant and susceptible groundnut seeds after inoculation with *A. flavus*. The results showed that the accumulation of resveratrol in resistant genotypes was increased at 3 days after inoculation, but susceptible genotypes did not reach the same levels until 4 days after inoculation.

Antifungal proteins: Protein profiles of 15 groundnut genotypes revealed that the trypsin inhibitor and lipid transfer protein were present at relatively high concentration in resistant genotypes (Liang *et al*., 2004). Both proteins exhibited strong bioactivity against the growth of *A. flavus.* In another investigation, the difference of total seed protein in resistant and susceptible groundnut genotypes was investigated by proteomic approaches (Liang *et al.,* 2004). The major qualitative difference between resistant genotypes and susceptible genotypes is that resistant genotypes contained three unique proteins P1 (22.5 kD, pI 4.1), P2 (22.5 kD, pI 8.2) and P3 (23.8 kD, pI 5.9), while susceptible genotypes contained one unique protein

P4 (23.5 kD, pI 7.0). Another protein P5 (22.5 kD, pI 7.3) also was found in concentration tenfold more in resistant vs. susceptible genotypes. The peptide sequences of spots P-1 and P-3 are identical to legumin A precursor from *Vicia narbonensis* L. The peptide sequences of spots P-2 and P-4 are the same and identical to glycinin from *A. hypogaea*. The polymorphic protein peptides distinguished by 2-D PAGE may be used as markers for identification of resistant groundnut lines. In addition to constitutive seed antifungal proteins, there were significant differences of two pathogenesis-related proteins (chitinase and β-1-3-glucanase) between the resistant and susceptible genotypes after inoculation with *A. flavus* (Liang *et al.*, 2005). The activities of endo-chitinase and β-1-3- glucanase increased earlier in resistant than in susceptible genotypes after invasion by *A. flavus*, while more isoform bands of β-1-3-glucanase were observed in resistant than in susceptible genotypes. The purified chitinase can significantly inhibit spore germination and hypha growth of *A. flavus* in vitro, while thin-layer chromatography analysis of the hydrolytic product from β-1-3-glucanase and hypha of *A. flavus* revealed the presence of enzymatic hydrolytic oligomer products (Liang *et al.,* 2005).

2.9 Resistance to Seed Invasion and Colonization by *Aspergillus flavus*

Mixon and Rogers (1973) first suggested that use of groundnut cultivars resistant to seed invasion and colonization by *A. flavus* could be an effective means of preventing aflatoxin contamination. The existence of seed resistance was a logical assumption, considering that seeds with damaged testae are more easily and rapidly invaded by the fungus than are seeds with intact testae, and colored testae conferred greater resistance to invasion by *A. flavus* than white or variegated testae (Carter,1973).

2.10 Resistance to Pod Infection by *Aspergillus flavus*

The groundnut shell has logically been considered as a barrier to penetration by *A. flavus*, as seeds from pods with, damaged shells are more frequently contaminated with aflatoxin than those from intact pods (McDonald and Harkness 1965). Zambettakis (1975) reported that two cultivars, Darou IV and Shulamit, had lower levels of pod infection by *A. flavus* than other cultivars field tested in Senegal. Varietal differences in pod infection were confirmed in subsequent studies, and the differences in resistance appeared to be linked to varietal differences in pod shell structure (Zambettakis *et al*., 1981). They also reported a significant correlation between natural pod infection and seed infection by *A. flavus* in various genotypes tested in Senegal from 1976 to I979 (Zambettakis *et al*., 1981). Considering the concept of the existence of pod shell resistance to *A. flavus*, Kushalappa *et al.* (1976) and Mixon (1980) examined the effects of pod inoculation with *A. flavus* on shell infection and subsequent seed infection in various genotypes in the laboratory. They concluded that resistance to pod infection was highly variable and appeared to be caused by the presence of antagonistic microflora. Mixon (1980) found that in some genotypes seeds were colonized or infected by the test fungus in pods which did not show colonies of *A. flavus* on their surfaces, while in others seeds were not colonized or infected in pods which showed one or more colonies of *A. flavus*.

2.11 Resistance to *A. flavus* Seed Infection and/or Aflatoxin Contamination in the Field

Over the years, about 2500 groundnut accessions have been screened for their resistance to *A. flavus* seed infection in a sick plot under imposed drought conditions at ICRISAT Mali Centre, and sources of resistance to pod wall, seed coat and

cotyledon identified in the genotypes. The 17 genotypes identified as resistant (<4.0ng/g AFB_1 content) were as follows: ICGV 89092, ICGV 91289, ICGV 00362, ICGV 86168, ICGV 02313, ICGV 91283, ICGV 06423, ICGV 99240, ICGV 07220, ICGV 91324, ICGV 01258, ICGV 91278, ICGV 93305, ICGV 91317, ICGV 89106, ICGV 91304 and ICGV 89115 (Osiru and Waliyar, 2013).

In a recent evaluation of 34 *A. hypogaea* genotypes over 4 year (2008-2011, Waliyar *et al*., 2016) at the ICRISAT Sahelian Center in Sadore, Niger, 31 accessions were resistant (10% seed infection compared to 70% for susceptible control JL 24). These included ICGs 13603, 1415, 14630, 3584, 5195, 6703 and 6888, over six years (2008-2013) consistently accumulated very low levels of aflatoxin (<4ng/g). These seven accessions could be potential sources for understanding the resistant mechanisms and can be further used in developing resistant cultivars or introgressing resistance in popular released varieties.

The value of resistance sources depends upon levels and stability of their resistances. Many genotypes have shown high levels of resistance against pre-harvest seed infection, *In-Vitro* seed colonization (IVSC), and aflatoxin production, significant G x E interaction remains a major issue in screening for aflatoxin resistance.

2.12 The relationships between *Aspergillus flavus* infection and aflatoxin accumulation

Among the factors contributing to *in planta* aflatoxin production are genotype (Mehan *et al.,* 1986), environmental factors (Sander *et al.,* 2000), inadequate pre-harvest and storage practices (Hell *et al.,* 2003), the presence of storage insect pests (Dowd, 2003), and the occurrence of other fungi (Hill *et al.,* 1985). The combination of some or all of these factors may ultimately results in increased risk of aflatoxin production

in field crops, stored food and feed (Cardwell and Henry, 2004). In tropical agricultural systems, the threat of aflatoxin contamination is high, since most of the factors that favour both the fungal and toxin development are more prevalent. The determinant factor for *A. flavus* infection in a groundnut field is the presence of primary inoculum. The presence of *A. flavus* propagules in their infective form during pod filling, harvesting, and during storage is an essential factor for groundnut contamination by aflatoxin. Horn (2003) showed that the higher the inoculum of *A. flavus* in a field, the higher the risk of groundnut becoming contaminated (Jaime-Garcia and Cotty, 2004).

Pearson's correlation coefficients and spearman's rank correlation coefficients between ear rot ratings and aflatoxin content in maize ranged from 0.35 to 0.49 and 0.22 to 0.50 in a study conducted by Campbell and White (1995). Pearson's correlation coefficients were nonsignificant or relatively low due to random variation between ear rot ratings and aflatoxin content. Some ears in the plant had ear rot ratings greater than or equal to five, but have 0*ng/g* aflatoxin content, while some plants with ear rot ratings greater than or equal to four, but aflatoxin values greater than 1000ng/g. In general, the study concluded that visual ratings of ears were not particularly good measure of the entire range of aflatoxin values. Tucker *et al.* (1986) reported moderate to high correlation between kernel infection and aflatoxin content. Similarly, poor correlations are reported between aflatoxin content, colonization of seed or shells and population densities of *A. flavus* in the soil (Girdthai *et al*., 2010; Rahmianna *et al.,* 2015).

2.13 Genetics of resistance to *A. flavus* infection and aflatoxin accumulation

Very little is known about the inheritance of resistance to pre-harvest seed infection, *in vitro* seed colonization (IVSC), or aflatoxin production. A few published reports give information on broad sense heritability (low to moderate) and combining ability of resistance sources (Rao *et al*., 1989; Utomo *et al*., 1990; Upadhyaya *et al*., 1997; Xue *et al.,* 2004, Ozimati *et al*., 2014). Utomo *et al.* (1990) reported broad-sense heritability estimates in F_2 derived F_6 populations from two crosses, AR-4 / NC 7 and GFA-2 / NC 7. AR-4 and GFA-2 are IVSCAF-resistant genotypes, and NC 7 is a susceptible cultivar. The heritability estimates from those two crosses were 55 and 63%, respectively, for seed colonization, 27 and 33% for pre-harvest seed infection, and 23 and 21% for aflatoxin production. Upadhyaya *et al.* (1997) on the other hand reported heritability estimates of 56 to 87% for pre-harvest seed infection. In another study, Arunyanark *et al.* (2010) reported heritability for seed infection which ranged from (h^2=0.30 to 0.51), while those of aflatoxin contamination (h^2=0.30 to 0.51). Ozimati *et al.* (2014) reported high broad-sense heritability for seed infection (85% - 90%) and aflatoxin contamination (96%).

General combining ability (GCA) of a genotype is the mean performance in all its crosses when expressed as deviation from the mean of all crosses. It is the average value of all crosses having this genotype as one parent, the value being expressed as a deviation from the overall mean of crosses. Any particular cross, then, has an expected value, which is sum of the general combining abilities of its two parental lines. The cross may deviate from this expected value to a greater or lesser extent. This deviation is called specific combining ability (SCA) of the two lines in

combination. In statistical terms, the general combining abilities are main effects and the specific combining ability is an interaction (Olfati *et al*., 2012).

A study on combining ability of IVSCAF-resistance using line x tester analysis at ICRISAT (IC) found J 11 to have non-significant general combining ability effects (Upadhyaya *et al*., 2000). However in a diallel study, significant reciprocal effects were noticed in some crosses indicating maternal influence on testa structure (Rao *et al*., 1989). Yoopum *et al.* (2005) reported significant GCA mean squares for the parents and significant SCA mean squares for the crosses and concluded that both additive and non-additive gene-action was involved in the inheritance of *A. flavus* and aflatoxin contamination resistance in groundnuts. The significant GCA and SCA effects suggested that the trait was controlled by both additive and non-additive gene action, however the Baker‘s ratios (0.2 and 0.3 in the respective Sets) revealed that the trait under study was predominately conditioned by non-additive gene action. The low Baker‘s ratio highlighted the importance of SCA variance, and hence the importance of dominance and/or epistatic gene effects in increasing resistance to aflatoxin contamination (Griffing, 1956). This ratio is in congruence with the heritability estimates of 0.1 and 0.2 in the two sets. These values are defined as being low. The predominance of the non-additive gene action implies that non–fixable dominance deviation and epistatic effects are likely to hinder improvement through simple pedigree selection, which is commonly followed in groundnuts (John *et al*., 2011).

Resistance to *A. flavus* colonization and aflatoxin contamination was demonstrated to be quantitative and heavily influenced by environmental interactions (Fountain *et al.*, 2014). Therefore, recent breeding studies focused on the discovery and characterization of quantitative trait loci (QTL) for aflatoxin resistance were forced to

consider the environment in obtaining phenotypic data, and have faced numerous challenges in identifying consistent QTL for aflatoxin resistance. Willcox *et al.* (2013) utilized an F_2 mapping population in maize derived from Mp313E x Va35 (resistant x susceptible) to identify 20 QTLs with combined phenotypic variance explained (PVE) of 22–43%. However, when the mapping populations were grown in multiple environments, only 11 QTLs were found to be consistent with a combined PVE of 2.4–9.5%. An earlier study by Brooks *et al.* (2005) also examined a population derived from Mp313E for the presence of QTL for aflatoxin resistance. These studies demonstrate that resistance to *A. flavus* is not conferred by a single gene and is highly quantitative in nature. In addition, given the relatively low level of PVE provided by each QTL, it is likely that many QTL with low PVE (<10%) contribute to aflatoxin resistance and may be indicative of the polygenic nature of resistance and the involvement of multiple physiological and morphological traits in the overall resistance phenotype (Fountain *et al.*, 2014).

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CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Experimental Site

The experiment was conducted at the screen-house of the Department of Plant Science and Mycology Laboratory of the Department of Crop Protection, Institute for Agricultural Research (IAR), Ahmadu Bello University, (A.B.U) Zaria (Latitude $11^0$11'N and $7^0$38'E), Northern Guinea Savannah Zone in Nigeria. The research was carried out during the 2015/2016 rainy seasons.

3.2 Genetic Materials

The genetic materials used in the study comprised of eight genotypes; five commercially grown varieties of groundnut (SAMNUT 22, SAMNUT 23, SAMNUT 24, SAMNUT 25 and SAMNUT 26) developed by IAR and were used as female parent in a cross with three groundnut genotypes (ICGV 91317, ICGV 91324, ICGV 91328) previously evaluated by ICRISAT and known for their resistance to A. *flavus* infection (Osiru and Waliyar, 2013) as male parent (Table 3.1). The parental lines were crossed in a 3 x 5 North Carolina mating design II as described by Comstock and Robinson (1948) to generate 15 F_1 hybrids. The F_1s seeds were selfed to F_2 to obtain enough seed for evaluation as suggested by Hallauer *et al.* (1988) for self-pollinating crops.

3.3 Population Development

The genetic materials were sown in polyvinyl chloride (PVC) pots measuring 36 cm in diameter and 50 cm in height in August, 2015. Fifteen pots were assigned to each of the five female parents and ten pots were assigned to each of the male parents.

Table 3.1: Description of genetic materials used in this study

Genotype	Description
Samnut 22	It is medium maturing variety with a vegetative cycle of 110 to 120 days. It is susceptible to Aspergillus flavus. Resistant to groundnut rosette disease (GRD), tolerant to *cercospora* leaf spot. The seed colour is tan. The oil content is 45%. It is adapted to Sudan and Guinea savannah. Potential pod yield is 2.4 tons /hectare while haulm yield is 4.0 tons/hectare
Samnut 23	It is an early maturing variety with vegetative cycle of 90 to 100 days. It is susceptible to *Aspergillus flavus*. Resistant to GRD and foliar diseases. The seed colour is red with oil content of 53%. It is adapted to Sudan and Sahel savannah. Potential pod yield is 2.2 tons/ha. Potential haulm yield is 1.3 tons/ha.
Samnut 24	It is an extra early maturing (about 75 days) variety with high grain and moderate fodder yield. It is resistant to GRD. Yield average 1.78tons/ha. Suited to Northern guinea and Sudano Sahelian savannah.
Samnut 25	It is an early maturing variety with vegetative cycle of 80 to 100 days. It is resistant to GRD. It stays green up to harvest. Dual purpose cultivar. It is adapted to Sudan and Sahel savannah. Potential pod yield is 3-4 tons/ha with huge potential haulm yield.
Samnut 26	It is an early maturing variety with vegetative cycle of 80 to 100 days. It is resistant to GRD. It stays green up to harvest. Dual purpose cultivar. It is adapted to Sudan and Sahel savannah. Potential pod yield is 3-4 tons/ha with huge potential haulm yield.
ICGV 91317	High pod and haulm yield. Good tolerance to drought. Resistant to *Aspergillus flavus*
ICGV 91324	High pod and haulm yield. Good tolerance to drought. Resistant to *Aspergillus flavus*
ICGV 91328	High pod and haulm yield. Good tolerance to drought. Resistant to *Aspergillus flavus*

Source = IAR, ABU Zaria

Staggered planting was done to synchronize flowering among the genotypes. A total of four healthy seeds were sown in each pot. The pots were watered regularly and plants protected from pests such as Spider mites (*Tetranychus* spp.), Aphids (*Aphis craccivora*) and Leaf spots (*Cercospora* spp.) by spraying with Punch chemical which is a combination of Acaricide, insecticide and nematicide.

3.4 Hybridization activities

Hybridization exercise began in September, 2015 and lasted for three weeks. During hybridization, care was taken to avoid contamination of genotypes as well as injury to the selected buds. The emasculation exercise was carried out between 1630hrs – 1830hrs depending on the prevailing environment (Nigam *et al.,* 2009). Pollination was done in the morning (0600hrs – 0730hrs). In both activities coloured nylon threads were used to mark the emasculated and pollinated buds. One nylon thread denoted that the bud had been successfully emasculated and two threads meant that the bud had been pollinated.

Harvesting of the crosses started 70 days after the last pollination. All the pods (mature and immature) originating from the nodes that had two nylon threads on their upper internodes were picked as hybrid pods. All selfed pods from the female plants were discarded. The hybrid pods removed were immediately dried for 15 days at ambient air temperatures in well labelled muslin bags to avoid direct exposure to the sun. The dried pods were then shelled and caution was taken to prevent any damage of the seed components (testa, cotyledons and embryo).

Figure 3.1. Hybridization activities in the screen-house

3.5 Evaluation and Screening activities

The 8 parents and 15 F_2 s were laid out in a randomized complete block design (RCBD) with three replications in April, 2016 at the screen-house of the Department of Plant Science. A total of four healthy seeds from each genotype were sown at 1 to 2 cm depth in dark loamy soil in each pot. Two pots were allocated for the segregating populations and a pot for the parents. Two healthy and vigorous seedlings in each pot were retained after germination. The screening of the 8 parents and their 15 F_2 crosses for *Aspergillus flavus* resistance was carried out in the screen house as described by Anderson *et al*. (1995). The method involved inoculation with toxigenic strains of *Aspergillus flavus* inoculums. This was carried out four times, that is at 37, 51, 65 and 79 days after sowing (DAS) starting on 2nd June, 2016. The modified method developed by Will *et al*. (1994) was used and this involved the use of autoclaved sorghum seed as a carrier for the fungus. A total of 3.5 g of inoculum per plant was incorporated into the soil. The *A. flavus* strains used were isolated from cultures maintained at the Mycology Laboratory, Department of Crop Protection. The inoculation technique helped to reduce the inherent variability of pre-harvest aflatoxin contamination (Holbrook *et al.,* 2008).

Drought conditions were imposed by stopping watering at 70 DAS. However, one lifesaving watering was done before harvesting. Harvested pods were dried to 7% moisture and hand sorted to remove visibly damaged pods. Harvested groundnuts pods were hand shelled and stored in a dry place in the groundnut improvement laboratory, Department of Plant Science (Holbrook *et al.,* 2008).

3.5.1 Scoring for *A. flavus* Colonization

For ascertaining seed infection by *A. flavus*, individual seeds were scored for severity of colonization of *A. flavus* on modified scale of 1-5 and averaged over the number of kernels in the replication (Oluret, 2012). The kernel infection score was transformed using Log_{10} transformation to normalized values before subjecting the data to analysis of variance (Table 3.2).

3.6 Analysis and Quantification of Aflatoxin

3.6.1 Preparation of groundnut seed extracts

One hundred grams of groundnut kernels were made into powder using a blender and twenty grams was taken from this powder and mixed with 100 ml 70% methanol (v/v-70 ml absolute methanol in 30 ml distilled water) containing 0.5% KCl. This mixture was added into a blender and blended until a homogenous paste was formed. The paste was transferred to a conical flask, sealed with parafilm and shaken for 30 min at 300 rpm using mechanical shaker. This was then filtered through Whatman No. 4 filter paper. The liquid portion was collected and stored at $4^{o}C$ prior to analysis. Similarly, one toxin free groundnut sample extract was prepared which was used as standards' dilution as well as control.

Table 3.2: The modified rating scale for severity of *A. flavus* on kernels

Scale	Disease reaction	Description
1	Highly resistant	0 % No visible mycelial growth
2	Resistant	1-20% surface coverage of mycelial growth on the kernels
3	Moderately resistant	21-50% surface coverage of mycelial growth on the kernels
4	Susceptible	51-70 % surface coverage of mycelial growth on the kernels
5	Highly susceptible	71-100% surface coverage of mycelial growth on the kernels

Source: Oluret (2012)

3.6.2 Enzyme Linked Immuno-Absorbent Assay (ELISA) procedure for the AFB1 detection

AFB1-BSA conjugate, goat anti-rabbit immunoglobulin G –alkaline phosphatase (IgG-ALP) conjugate, ρ-nitrophenyl phosphate and bovine serum albumin (BSA) purchased from Sigma (St Louis, USA) and microtitre plates (Maxi-sorp F96) were from Nunc (Nalge Nunc International, Denmark). All other chemicals were reagent grade and/or chemically pure. Polyclonal antibodies for AFB_1 (Thirumala-Devi *et al*., 1999) were purchased from International Crops Research Institute for the Semi- Arid Tropics, Patancheru, India.

The detection of AFB_1 from the groundnut genotypes was estimated by deploying the method of Reddy *et al*. (2009). The limit of detection (LOD) of the assay was 0.01 *ng/g*. ELISA microtitre wells were coated with 100 ngml^{-}1 of AFB1-BSA in sodium carbonate buffer (pH 9.6; 150 μl/well) and left overnight at 4°C. These plates were then washed in PBST, added with 0.2% BSA and allowed to stand at 37°C for 1 h. ELISA plates were again washed with PBST and added with 100 μl AFB1 standards ranging from 25 ng to 10 pgml^{-1}. Pre-incubation was carried out with 50 μl antiserum diluted in PBST-BSA (1: 6000) and held for 45 min at 37°C. Filtrate samples extracted from groundnut seed with aqueous methanol-KCl as described earlier were added to wells at 1: 10 dilution in PBSTBSA. Goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase were used at a 1: 4000 dilution to detect rabbit antibodies attached to AFB1-BSA. ρ-Nitrophenyl phosphate was used as a substrate at 0.5 mg/ml. Absorbance was recorded at 405 nm with an ELISA plate reader (Biorad-680) after incubation at 28°C for 45 - 60 min. Standard curves

were obtained by plotting log10 values of AFB1 dilutions at A_{405}. AFB1 ($ngml^{-1}$) in samples was determined from the standard curves as:

AFB1 ng/g of groundnut = [aflatoxin ($ngml^{-1}$) in sample x buffer (ml) x extraction solvent (ml)]/sample weight (g) (Reddy *et al*., 2009)

3.7 Data Collection

Days to 50% flowering: Number of days to 50% flowering was recorded from date of sowing till when half of the plants in each pot had flowered.

Plant height (cm): The plant height was measured in cm at maturity using a thread and meter ruler. The measurement was taken from the ground level (base) to the tip of the plant.

Days to maturity: The number days that the plants took to attain maturity was recorded from the day of sowing.

Number of pods per plant: The number of pods per plant was counted from each of the 3 plants in each pot at harvest.

Pod weight per plant (g): The pods obtained from each plant after harvest was weighed using a digital balance device and recorded in grams.

Number of kernel per plant: The number of kernels per plant was counted from each of the 3 plants in each pot at harvest.

Kernel weight per plant (g): The pods from each plant were threshed and the skernels obtained weighed using a digital balance device and recorded in grams.

Hundred- kernel weight (g): One hundred (100) sound kernels were counted from the threshed pods weighed and measured in gram.

Shelling percentage: The harvested pods were shelled by hand and the shelling percentage was determined using the following equation:

$$\frac{\text{weight of kernel(g)}}{\text{weight of pod(g)}} \times 100$$

Haulm weight (g): The dried haulm obtained from each plant after harvest was weighed using a digital balance device and recorded in gram.

3.8 Data analysis

The data collected was subjected to analysis of variance (ANOVA) using General Linear Model procedure of Statistical Analysis System (SAS) package (SAS, 2002) and where there was significant difference between treatment means, Fisher's protected Least Significant difference (LSD) test was used for comparison (Gomez and Gomez, 1984).

The statistical model used for RCBD analysis was:

$$y_{ij} = \mu + r_i + g_j + e_{ij}$$

Where: y_{ij} = Observed effect for i^{th} replication and j^{th} genotypes

μ = grand mean of the genotype

r_i = effect due to the ith replication

g_j = effect due to j^{th} genotype

e_{ij} = effects due to the residual or random error of the experiment

Table 3.3. Form of Analysis of Variance for one environment for fixed model

Source of variation	df	MS	EMS
Replication	$(r-1)$	-	
Genotype	$(g-1)$	MS_2	$\sigma_e^2 + \left[\frac{r}{(g-1)}\sum g^2\right]$
Error	$(r-1)(g-1)$	MS_1	σ_e^2

Where g= number of genotype, $\sum g^2$ =genotypic variance of effect, r= number of replication, σ_e^2 =error variance, df= degree of freedom, MS= observed mean squares, EMS= expected mean squares.

3.8.1 North Carolina II Analysis of Combining Ability and Gene Action

Mean squares were partitioned into difference due to male parents and female parents, which were attributed to general combining ability (GCA), and differences due to male x female interaction, which was attributed to specific combining ability (SCA).

The following linear model was used:

$$y_{ijk} = \mu + m_i + f_j + (mxf)_{ij} + r_k + \ell_{ijk}$$

Where,

y_{ijk} = observed trait value, μ = mean effect, m_i = effect of the ith male, f_j = effect of the jth female, $(mxf)_{ij}$ = effect of interaction between i^{th} male and j^{th} female, r_k = effect of kth replication and ℓ_{ijk} = experimental error

The genetic variance of effect was estimated by equating the variances to the respective mean squares from the ANOVA Table shown below.

Table 3.4: Form of ANOVA of NCD II for Model I (Comstock and Robinson, 1948)

Source of Variation	Df	MS	EMS
Replication	$(r-1)$		
Males (M)	$(m-1)$	MS_m	$\hat{\sigma}_e^2 + \left[\frac{rf}{m-1}\right]K_{male}^2$
Females (F)	$(f-1)$	MS_f	$\hat{\sigma}_e^2 + \left[\frac{rm}{f-1}\right]K_{female}^2$
M x F	$(m-1)(f-1)$	MS_{f*m}	$\hat{\sigma}_e^2 + \left[\frac{r}{(m-1)(f-1)}\right]K_{crosses}^2$
Error	$(r-1)(mf-1)$	MS_e	$\hat{\sigma}_e^2$
Total	$mf(r-1)$		

K_{male}^2 = is the quadratic form (analogous to a variance component but referring to a fixed effect) derived from the mean square of male GCA

K_{female}^2 = is the quadratic form (analogous to a variance component but referring to a fixed effect) derived from the mean square of female GCA

$K_{crosses}^2$ = is the quadratic form of SCA.

The components of gene effect for fixed model were calculated as follows:

Males $$K_{male}^2 = \frac{MS_m - MS_e}{rf/(m-1)}$$

Females $$K_{female}^2 = \frac{MS_f - MS_e}{rm/(f-1)}$$

Male x female interaction $$K_{crosses}^2 = \frac{MS_{f*m} - MS_e}{r/(m-1)(f-1)}$$

3.8.2 Proportional contribution of males, females and their interactions to total variance

The proportional contributions males, females and their interaction to the total variance of effects were calculated in accordance with Singh and Chaudhary (1985) method;

Contribution of males= $\frac{SS(Males)}{SS(Crosses)} x100$

Contribution of females= $\frac{SS(Females)}{SS(Crosses)} x100$

Contribution of $(f\,X\,m)$ = $\frac{SS(fxm)}{SS(Crosses)} x100$

3.8.3 Estimation of general combining ability (GCA) and specific combining ability (SCA) effects

Estimation of GCA effects:

(a) **Females**: $g_i = \frac{x_i..}{rm} - \frac{x...}{rfm}$

Where, $x_i..$ =total of ith females over males, $x...$ = Grand total, f = number of females m = number of males r = number of replications

(b) **Males**: $g_m = \frac{x_j..}{rf} - \frac{x...}{rfm}$

Where,

$x_j..$ = Total of jth males over females, $x...$ = Grand total, f = number of females, m = number of males, r = number of replications

Estimation of SCA effects:

$$s_{ij} = \frac{x_{ij}}{r} - \frac{x_i..}{rf} - \frac{x._j.}{rm} - \frac{x...}{rfm}$$

Where,

s_{ij} = Specific combining ability, f = number of females, m = number of males, r = number of replications, $x_i.$ = Total of all the hybrids containing and i^{th} female, $x._j.$ = Total of all hybrids containing j^{th} male, x_{ii} = Total of all hybrids containing an ith female and j^{th} male and $x_{...}$ = Grand total of crosses

The significance of the GCA (male), GCA (female) and SCA effects were estimated on the method as described by Singh and Chaudhary (1985);

(a) Significance of GCA effects:

$$t = \frac{GCA}{SE_{gca}(female)} \qquad \text{Where, } SE_{gca(female)} = \left(\frac{M_e}{rxm}\right)^{1/2}$$

$$t = \frac{GCA}{SE_{gca}(male)} \qquad \text{Where, } SE_{gca(male)} = \left(\frac{M_e}{rxf}\right)^{1/2}$$

Where; M_e= error mean square,

r = number of replications, f= number of females, m=number of males and SE= Standard error

(b) Significance of SCA effects

$$t = \frac{SCA}{SE_{sca}(femalesxmales)} \qquad \text{Where, } SE_{sca} = \left(\frac{M_e}{r}\right)^{1/2}$$

Where;

M_e= error mean square and r= number of replications

The importance of GCA and SCA was assessed using the equation $2K^2_{GCA_m} + 2K^2_{GCA_f} / 2K^2_{GCA_m} + 2K^2_{GCA_f} + K^2_{SCA}$, modified from Baker (1978) by Hung and Holland, 2012, in which K^2_{GCA} is the quadratic form (analogous to a variance component but referring to a fixed effect) derived from the mean square of GCA effect and K^2_{SCA} is the quadratic form of SCA effects since the total genetic variation among single-cross progeny is equal to twice the GCA component plus the SCA component. The closer this ratio is to unity, the greater the predictability of a specific hybrid's performance based on GCA alone.

3.9 Heritability

A model with all terms considered random was fitted for the purpose of estimating entry mean heritability for each character. The GCA and SCA variances were estimated as described by Singh and Chaudhrary (1985) and Hallauer *et al*. (1988) as follows:

$$\sigma^2_{GCA_f} = \frac{MS_f - MS_{f*m}}{rm}$$

$$\sigma^2_{GCA_m} = \frac{MS_m - MS_{f*m}}{rf}$$

$$\sigma^2_{SCA} = \frac{MS_{f*m} - MS_e}{r}$$

Therefore, the broad-sense heritability was estimated by the formulae suggested by (Ruming, 2004) as follows:

Broad-sense heritability $$H_b = \frac{\hat{\sigma}^2_{GCA_f} + \hat{\sigma}^2_{GCA_m} + SCA_{fm}}{\hat{\sigma}^2_{GCA_f} + \hat{\sigma}^2_{GCA_m} + SCA_{fm} + \hat{\sigma}^2_e}$$

The heritability percentage was categorized following Robinson *et al.* (1949) method being Low (0-30%), Moderate (30-60%), and High (>60%).

CHAPTER FOUR

4 RESULTS

4.1 Mean Squares from ANOVA

The mean squares from the analysis of variance for the 23 characters of groundnut evaluated under artificial *Aspergillus flavus* inoculation at Samaru are presented in Table 4.1. Results showed highly significant ($P \leq 0.01$) differences between the genotypes for all the characters studied except for days to maturity which was significant at $P \leq 0.05$; and 100 kernel weight and shelling percentage which showed non-significant ($P > 0.05$) difference.

4.2 Mean Performance of growth, yield characters and aflatoxin contamination

The mean for the twelve characters measured among the 23 groundnut genotypes is given in Table 4.2. The genotypes showed considerable variation for the different characters. Among the eight parents used in this study, SAMNUT 23, ICGV 91324 and ICGV 91328 showed the lowest *per se* performance for days to 50% flowering and days to maturity (31 days, 89 days; 31 days, 89 days and 29 days, 89 days respectively). These three genotypes matured early and ranged from 29 to 36 days for days to 50% flowering and 89 to 91 days for days to maturity. The F_2 cross, SAMNUT 24 x ICGV 91324 recorded the lowest *per se* performance for days to 50% flowering (29 days) and days to maturity (88 days). Plant height among the parents ranged from 40cm (SAMNUT 25) to 59 cm (SAMNUT 21) with a mean of 52 cm. Among the crosses, plant height ranged from 44 cm for SAMNUT 26 x ICGV 91328 to 60 cm for SAMNUT 22 x ICGV 91328.

Table 4. 1: Mean squares of analysis of variance for aflatoxin accumulation and other agronomic traits among 23 groundnut genotypes evaluated under artificial *A. flavus* inoculation at Samaru in 2016

Source of variation	Degree of freedom	**Mean Squares**											
		Days to 50% flowering	Plant height (cm)	Days to maturity	Number of pod per plant	Weight of pod per plant (g)	Number of seed per plant	Seed weight per plant (g)	100 seed weight (g)	Shelling percentage	Haulm weight (g)	Kernel Infection (log transformed)	Aflatoxin B_1 accumulation
Replication	2	5.28	13.35	3.96	2297.52**	400.83	13911.58**	996.39	379.66**	322.37**	12.87	0.01	15.88**
Genotype	22	20.62**	96.63**	1.95*	1431.54**	1551.81**	2667.78**	456.20**	93.11	172.26	1877.32**	0.22**	3736.51**
Error	44	0.84	7.66	0.96	372.58	647.39	518.5	144.39	85.93	129.75	17.82	0.01	2.46

** Highly Significant and * Significant at 0.01% and 0.05% levels of probability, respectively

Number of pods per plant across the 23 genotypes had a mean of 66 pods and ranged from 29 pods (SAMNUT 22) to 66 pods (SAMNUT 24) among the parents and 37 pods (SAMNUT 22 x ICGV 91317) to 107 pods (SAMNUT 26 x ICGV 91317) among the crosses. Pod weight among the parents ranged from SAMNUT 22 (31.3 g) to ICGV 91324 (92.3 g). Among the crosses, SAMNUT 22 x ICGV 91317 recorded the least pod weight per plant (48.0 g) and highest weight was recorded by SAMNUT 26 x ICGV 91317 (118.7 g).

Kernel weight per plant among the parents ranged from 16 to 53 g. Among the progenies, SAMNUT 22 x ICGV 91317a and SAMNUT 25 x ICGV 91324 recorded the least kernel weight of 22 g and SAMNUT 22 X ICGV 91328 recorded the highest kernel weight of 62 g. The mean for hundred seed weight was 43g across all the genotypes and ranged from 36g (ICGV 91328) to 55 g (SAMNUT25) among the parents. Among the progenies, SAMNUT 25 x ICGV 91317 recorded the lowest value of 31 g for hundred seed weight and the highest value of 48 g was recorded by SAMNUT22 x ICGV91317 and SAMNUT25 x ICGV91328. Results for shelling percentage revealed that the parents ICGV91317 recorded the highest value (70.8%) followed by ICGV91328 (68.5%) and the least (52.2%) was recorded by SAMNUT 22. Among the progenies, the shelling percentage ranged from 40.3 to 66.3%. Haulm weight among the parents ranged from 46.7 to 104.3 g with SAMNUT 22 being the highest producer of haulm among the parents. Among the progenies, haulm weight varied from 52 g (SAMNUT 22 x ICGV 91317) to 128 g (SAMNUT 23 x ICGV 91317), with a general mean of 91.85g. For kernel infection, the values ranged from 10.00 to 57.00 (1.00 to 1.69) with SAMNUT 22 recording the highest value for kernel infection (57.00) among the parents. Among the progenies, kernel infection varied

from 11.67% (1.06) for SAMNUT 26 x ICGV 91324 to 57.00% (1.75) for SAMNUT 25 x ICGV 91328. The mean for aflatoxin B_1 accumulation across the 23 genotypes was 36.49 ng/g. Among the parents, the lowest aflatoxin B_1 accumulation was recorded by SAMNUT22 (2.50 ng/g) and the highest value was recorded by SAMNUT25 (98.15 ng/g).

Based on the data, *A. flavus* infection poorly correlated to aflatoxin contamination and followed the equation of $y = -2.93x^2 + 29.34x - 5.22$ with $R^2 = 0.154$ (Fig. 4.1) among the parental genotypes. Among the progenies, SAMNUT 26 x ICGV 91317 recorded the lowest aflatoxin B_1 accumulation of 0.79 ng/g and the highest value was recorded by SAMNUT22 x ICGV91317 (87.11 ng/g). Similarly, *A. flavus* infection among the progenies correlates to aflatoxin contamination and followed the equation of $y = -0.19x^2 + 0.95x + 36.50$ with $R^2 = 0.113$ (Fig. 4.2).

Table 4. 2: Mean performance of 23 groundnut genotypes for aflatoxin accumulation and other agronomic traits evaluated at Samaru in 2016

Genotypes	Days to 50% flowering	Plant height (cm)	Days to maturity	Number of pods per plant	Pod weight per plant (g)	Number of Kernels	Kernel weight per plant (g)	100 seed weight	Shelling percentage (%)	Haulm weight (g)	Kernel Infection (Log transformed)	Aflatoxin B_1 accumulation (ng/g)
Parents												
SAMNUT22 (P1)	36	59	91	29	31.3	39	16	41	52.2	104.3	50.00	2.50
SAMNUT23 (P2)	31	50	89	54	52.7	81	35	44	66.7	59.7	25.00	45.96
SAMNUT24 (P3)	34	44	89	66	62.0	87	36	42	59.8	87.7	10.00	67.37
SAMNUT25 (P4)	34	40	89	31	41.0	42	22	55	59.4	86.6	13.33	98.15
SAMNUT26 (P5)	35	49	89	54	59.0	78	35	46	59.2	64.0	46.33	95.49
ICGV91317 (P6)	35	49	89	51	52.7	93	37	41	70.8	46.7	44.33	6.31
ICGV91324 (P7)	31	56	89	63	92.3	113	53	53	61.8	61.8	16.67	12.23
ICGV91328 (P8)	29	52	89	54	56.0	106	38	36	68.5	69.0	10.00	88.75
Progenies												
P1 x P6	35	47	89	37	48.0	46	22	48	47.9	52.0	40.33	87.11
P1 x P7	35	57	89	91	103.0	127	56	46	55.7	115.8	37.67	12.33
P1 x P8	30	60	89	95	92.7	136	62	46	66.3	104.5	17.67	4.40
P2 x P6	35	59	89	85	93.0	131	46	36	54.4	128.0	21.00	19.93
P2 x P7	35	58	90	75	84.0	108	46	43	55.2	119.7	46.67	12.32
P2 x P8	36	50	89	60	52.0	91	32	36	59.5	119.5	12.67	82.98
P3 x P6	35	52	89	64	92.3	110	45	40	48.7	54.3	13.33	15.75
P3 x P7	29	54	88	77	75.3	107	46	44	60.9	105.0	39.67	27.51
P3 x P8	30	48	89	73	81.7	133	54	41	66.0	111.2	13.33	43.12
P4 x P6	34	55	89	96	85.0	138	47	31	50.1	105.2	20.00	76.07
P4 x P7	36	47	91	44	53.3	61	22	36	40.3	88.9	44.67	3.92
P4 x P8	35	47	89	88	93.7	112	54	48	57.2	107.1	57.00	11.53
P5 x P6	35	57	88	107	118.7	118	49	43	48.5	107.3	43.67	0.79
P5 x P7	28	59	90	48	57.7	84	36	42	61.4	111.3	11.67	23.47
P5 x P8	36	44	88	85	87.7	114	46	40	52.7	103.0	12.67	1.20
Mean ± SE	33.0±0.78	52.0±1.35	89.0±0.80	66.0±3.58	72.4±4.11	98.0±3.89	41±2.83	43±2.48	57.5±2.75	91.9±1.67	30.17±3.25	36.49±1.022
CV	2.73	5.33	1.10	29.11	35.15	23.20	29.59	21.81	19.80	4.60	18.67	4.30

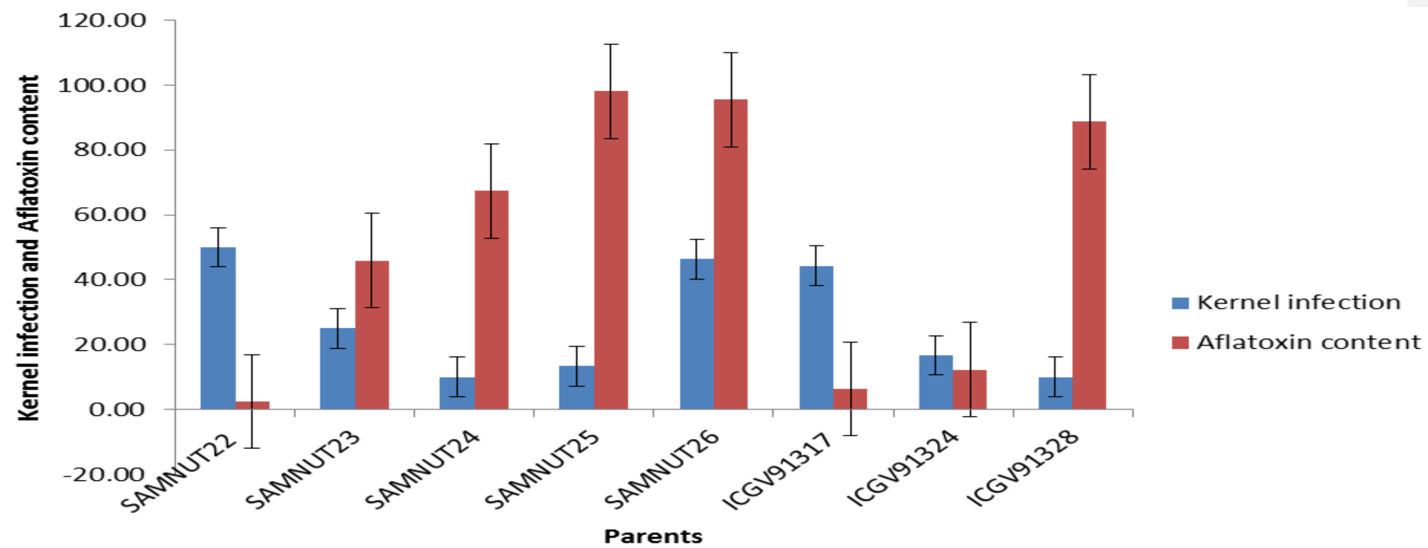

Fig. 4.1: *Aspergillus flavus* infection and aflatoxin accumulation among the parental groundnut genotypes evaluated under artificial *A. flavus* inoculation at Samaru in 2016

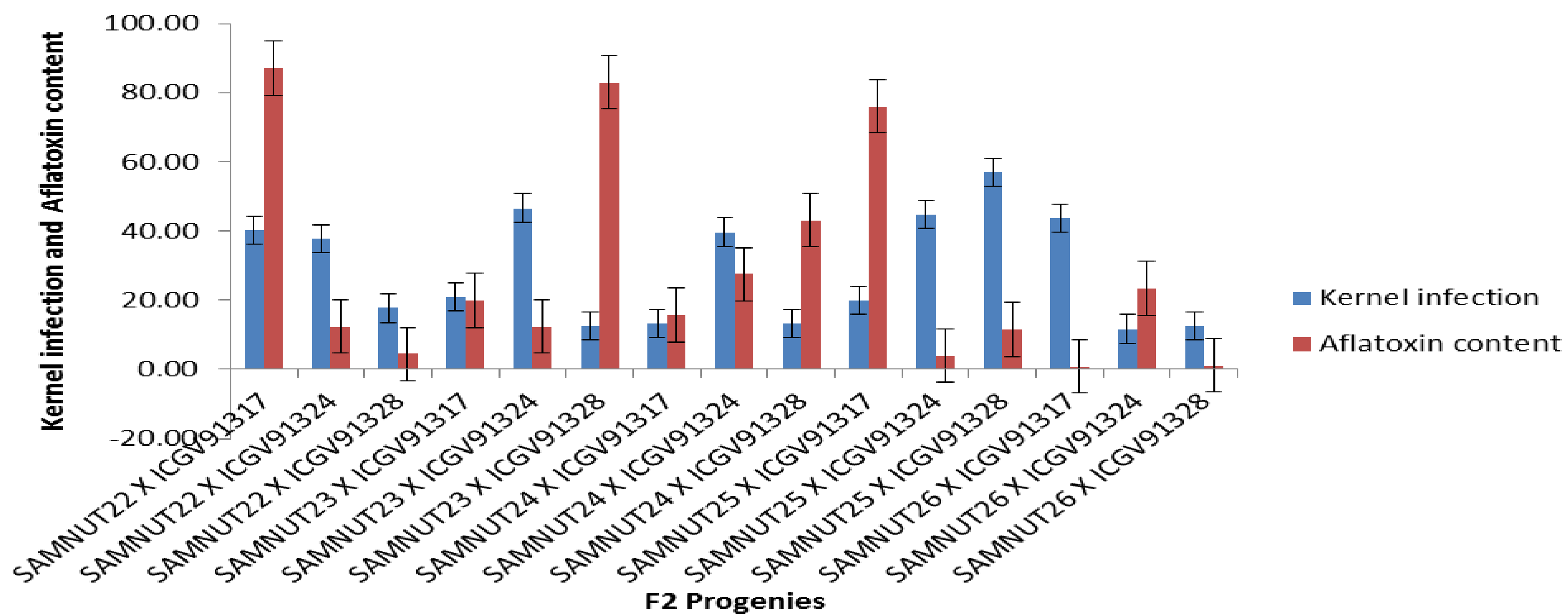

Fig. 4.2: *Aspergillus flavus* infection and aflatoxin accumulation among the hybrid groundnut genotypes evaluated under artificial *A. flavus* inoculation at Samaru in 2016

4.3 Combining Ability and Heritability Estimates

The mean squares for combining ability for the 23 characters of groundnut evaluated under artificial *Aspergillus flavus* inoculation at Samaru are presented in Table 4.3. The mean squares due to female were significant ($P \leq 0.05$) for all the characters studied except days to maturity and shelling percentage (Table 4.3). The mean squares due to both males and females were highly significant ($P \leq 0.01$) for 50% flowering, plant height at maturity, kernel infection and aflatoxin B_1 accumulation. Mean squares due to females were highly significant ($P \leq 0.01$) for number of pods per plant, weight of pod per plant, seed weight per plant and haulm weight but not significant ($P > 0.05$) for mean squares due to males. The female x male mean squares were found to be highly significant ($P \leq 0.01$) for days to 50% flowering, plant height, haulm weight, kernel infection and aflatoxin B_1 accumulation.

High Baker's ratio (close to unity) was recorded by number of pods per plant (0.75), weight of pod per plant (0.72), number of seed per plant (0.73), seed weight per plant (0.83), aflatoxin B_1 accumulation (0.52), and days to 50% flowering (0.51). Low Baker's ratio was recorded by plant height at maturity (0.39), days to maturity (0.22), shelling percentage (0.34), kernel infection (0.28) and haulm weight (0.24).

High broad-sense heritability estimates were recorded for kernel infection (85.3%) and aflatoxin B_1 accumulation (99.7%). Moderate broad-sense heritability estimates were recorded for number of pods per plant (32.5%) and 100-seed weight (37.8%) while low values were recorded for pod weight per plant (27.0%) (Table 4.3).

Table 4.3: Mean squares for combining ability for agronomic traits and aflatoxin accumulation among groundnut genotypes evaluated under artificial *A. flavus* inoculation at Samaru in 2016

Source of variation	Degree of freedom	Days to 50% flowering	Plant height (cm)	Days to maturity	Number of pod per plant	Weight of pod per plant (g)	Number of seed per plant (g)	Seed weight per plant (g)	100 seed weight (g)	Shelling percentage	Haulm weight (g)	Kernel Infection (Log transformed)	Aflatoxin B_1 accumulation
Rep	2	3.62	6.81	1.4	1302.03	812.47	13765.49	1606.07	74.73	573.8	17.27	0.03	13.09
Female	4	46.06**	107.92**	1.36	2940.83**	3090.41**	4961.64*	1103.97**	84.21*	189.91	1566.74**	622.19**	3185.17**
Male	2	6.49**	107.62**	0.62	31.49	188.07	1270.02	104.6	105.97*	103.42	35.08	917.27**	6283.25**
Female x Male	8	15.66**	72.00**	1.62	800.27	587.68	1069.24	142.27	58.59	152.99	1697.21**	761.49**	1580.22**
Error	30	0.96	5.44	1.11	556.80	635.40	1439.51	216.27	27.11	169.28	63.55	40.40	3.12
$K^2_{GCA_{female}}$		20.04	45.55	0.11	1059.57	1091.12	1565.39	394.53	25.38	9.17	668.08	258.57	1414.24
$K^2_{GCA_{male}}$		0.74	13.62	-0.07	-70.04	-59.64	-22.60	-14.89	10.52	-8.78	-3.80	116.91	975.72
$K^2_{SCA_{femXmal}}$		39.20	177.51	1.36	649.24	-127.26	-987.38	-197.33	83.96	-43.44	4356.41	1922.90	4205.60
Baker's ratio		0.51	0.39	0.22	0.60	0.72	0.73	0.83	0.41	0.34	0.24	0.28	0.52
Broad-sense heritability (%)		88.90	84.00	6.30	32.50	27.00	18.30	26.90	37.80	2.80	86.80	85.30	99.70

** Highly Significant and * Significant at 0.01% and 0.05% levels of probability, respectively

4.4 Proportional Contributions of Females, Males and their Interactions to total variance

The proportional contribution of females, males and their interactions to the variations in total sum of squares of progenies for aflatoxin accumulation and agronomic traits is presented in Table 4.4. The female parents contributed significantly greater variations than the male parent to all the characters studied indicating the predominance of maternal effects for these characters. The sum of contributions of both male and female parents were greater than the contributions of the progenies in the variations observed for days to 50% flowering (61.1%), weight of pod per plant (73.0%), 100 seed weight (53.6%) and aflatoxin accumulation (66.7%) while the progenies contributed more than the parents for the characters: days to maturity (66.1%), shelling percentage (55.9%), haulm weight (68.2%) and kernel infection (58.5%).

Table 4. 4: Proportional contribution of females, males and their interaction to total variance of effects of groundnut genotypes evaluated under artificial *A. flavus* inoculation at Samaru in 2016

Character	Contribution (%) due to		
	Female	Male	Interaction
Days to 50% flowering	57.13	4.02	38.84
Plant height (cm)	35.30	17.60	47.10
Days to maturity	27.60	6.33	66.06
Number of pod per plant	64.53	0.35	35.12
Weight of pod per plant (g)	70.88	2.16	26.96
Number of seed per plant	64.14	8.21	27.65
Seed weight per plant (g)	76.62	3.63	19.75
100 seed weight (g)	33.10	20.83	46.07
Shelling percentage	34.68	9.44	55.88
Haulm weight (g)	31.47	0.35	68.18
Kernel Infection	23.90	17.61	58.49
Aflatoxin B_1 accumulation	33.57	33.11	33.31

4.5 Combining Ability Effects

4.5.1 General combining ability effects

The estimates of general combining ability effects are presented in Table 4.5. SAMNUT 22 recorded highly significant ($P \leq 0.01$) positive GCA effects for days to 50% flowering (2.22) while highly significant ($P \leq 0.01$) negative GCA effects were recorded by SAMNUT 25 (-2.56), SAMNUT 24 (-2.33) and ICGV 91324 (-0.76) for days to 50% flowering. Highly significant ($P \leq 0.01$) positive GCA effects were recorded by ICGV 91317 (3.04) for plant height while very low and highly significant ($P \leq 0.01$) negative GCA effects were recorded by SAMNUT 22 (-4.72). Non-significant difference was recorded for days to maturity. However, SAMNUT 24 recorded the lowest negative GCA effect (-0.47) for days to maturity. Significant positive GCA effects was recorded for number of pods per plant by SAMNUT 24 (18.00 and) ICGV 91317 (1.64) while SAMNUT 22 recorded the lowest highly significant negative GCA effects (-28.22) for number of pods. Highly significant ($P \leq 0.01$) positive GCA effects were recorded for pod weight by ICGV 91317 (2.93) while SAMNUT 22 (-41.84) and ICGV 91324 (-3.93) recorded highly significant ($P \leq 0.01$) negative GCA effects for pod weight. Highly significant ($P \leq 0.01$) positive GCA effects were recorded for kernel infection by SAMNUT 23 (7.20), ICGV 91328 (5.13) and ICGV 91317 (3.87) while SAMNUT 25 (-13.47) and ICGV 91324 (-9.00) recorded highly significant ($P \leq 0.01$) negative GCA effects for kernel infection. Highly significant ($P \leq 0.01$) positive GCA effects were recorded for aflatoxin B_1 accumulation by ICGV 91317 (11.77), SAMNUT 23 (10.25 and SAMNUT 25 (2.35) while SAMNUT 26 (-19.68) and ICGV 91324 (-12.25) recorded highly significant ($P \leq 0.01$) negative GCA effects for aflatoxin B_1 accumulation.

Table 4.5: General combining ability effects of parents for twelve quantitative traits of groundnut genotypes evaluated under artificial *A. flavus* inoculation at Samaru in 2016

	Days to 50% flowering	Plant height (cm)	Days to maturity	Number of pod per plant	Weight of pod per plant (g)	Number of kernel per plant	kernel weight per plant (g)	100 kernel weight (g)	Shelling percentage	Haulm weight (g)	Kernel Infection	Aflatoxin B_1 accumulation (ng/g)
Female												
SAMNUT22 (P1)	2.22**	-4.72**	0.53	-28.22**	-30.09**	-41.84**	-19.02**	-1.48	-3.90	-16.43**	3.76	6.45**
SAMNUT23 (P2)	1.33**	-1.36	-0.24	6.11	15.13	8.49	7.31	3.18	-5.85	-9.10**	7.20**	10.25**
SAMNUT24 (P3)	-2.33**	4.11**	-0.47	18.00*	14.36	12.60	8.31	3.28	9.51*	1.27	4.87*	0.63
SAMNUT25 (P4)	-2.56**	2.56**	-0.02	-6.33	-3.76	8.38	1.20	-1.51	5.12	17.01**	-13.47**	2.35**
SAMNUT26 (P5)	1.33**	-0.59	0.20	10.44	4.36	12.38	2.20	-3.48*	-4.87	7.25*	-2.36	-19.68**
S.E±	0.326	0.777	0.351	7.866	8.402	12.647	4.902	1.735	4.337	2.657	2.119	0.589
Male												
ICGV91317 (P6)	0.44*	3.04**	0.18	1.64*	2.93**	2.02*	2.33**	2.34**	-19.73**	1.26**	3.87**	11.77**
ICGV91324 (P7)	-0.76**	-1.03**	-0.22	-1.09	-3.93**	8.02**	0.53	-2.89**	-18.58**	-1.70**	-9.00**	-12.25**
ICGV91328 (P8)	0.31*	-2.01**	0.04	-0.56	1.00	-10.04**	-2.87**	0.55	38.31**	0.44	5.13**	0.48
S.E±	0.147	0.228	0.153	0.724	0.748	0.918	0.572	0.340	0.538	0.421	0.376	0.198

** Highly Significant and * Significant at 0.01% and 0.05% levels of probability, respectively

Table 4.6: Specific combining ability effects for twelve quantitative traits of groundnut genotypes evaluated under artificial *A. flavus* inoculation at Samaru in 2016

Crosses	Days to 50% flowering	Plant height (cm)	Days to maturity	Number of pod per plant	Weight of pod per plant (g)	Number of kernel per plant	Kernel weight per plant (g)	100 kernel weight (g)	Shelling percentage	Haulm weight (g)	Kernel Infection	Aflatoxin B_1 accumulation (ng/g)
P1 x P6	-1.22*	-4.13**	-1.07	-11.64	-6.04	-21.69	-5.78	5.63	-4.06	-34.87**	0.06	30.05**
P1 x P7	1.31*	2.84*	0.00	14.09	4.82	16.98	6.02	-1.24	6.34	32.06**	0.00	0.07
P1 x P8	-0.09	1.29	1.07	-2.44	1.22	4.71	-0.24	-4.39	-2.28	2.81	-0.35	-5.56**
P2 x P6	-0.33	0.86	0.04	8.69	3.73	8.64	2.22	-1.29	5.62	21.63**	0.05	-7.97**
P2 x P7	0.87	1.78	0.11	-16.24	-0.07	-14.69	-7.31	-1.49	-2.53	-33.58**	0.23	-16.60**
P2 x P8	-0.53	-2.64	-0.16	7.56	-3.67	6.04	5.09	2.78	-3.09	11.94*	-0.21	4.05**
P3 x P6	-1.33*	0.26	0.27	0.47	-5.82	13.87	7.22	-0.65	0.85	-6.90	-0.22	-18.38**
P3 x P7	-1.80**	-1.99	0.00	-15.13	-16.29	-21.13	-6.98	2.45	-5.60	3.39	0.25	-4.32**
P3 x P8	3.13**	1.72	-0.27	14.67	22.11	7.27	-0.24	-1.81	4.75	3.51	0.10	-6.64**
P4 x P6	3.56**	0.79	-0.18	14.8	12.62	13.09	-1.33	-6.12*	-6.62	14.35*	0.07	25.29**
P4 x P7	-0.58	-6.83**	-0.11	5.53	8.16	9.09	8.13	4.22	3.89	-6.19	0.15	-34.10**
P4 x P8	-2.98**	6.04**	0.29	-20.33	-20.78	-22.18	-6.80	1.90	2.72	-8.17	0.22	18.31**
P5 x P6	-0.67	2.21	0.93	-12.31	-4.49	-13.91	-2.33	2.42	4.21	5.79	0.09	9.87**
P5 x P7	0.20	4.20**	0.00	11.76	3.38	9.76	0.13	-3.94	-2.11	4.31	-0.15	14.61**
P5 x P8	0.47	-6.41**	-0.93	0.56	1.11	4.16	2.20	1.52	-2.10	-10.10*	-0.31	-8.69**
S.E±	0.564	1.346	0.609	13.624	14.553	21.905	8.491	3.006	7.512	4.603	3.670	1.020

** Highly Significant and * Significant at 0.01% and 0.05% levels of probability, respectively

4.5.2 Specific combining ability effects

The estimates of specific combining ability effects are presented in Table 4.6. Positive highly significant SCA effects were recorded for days to 50% flowering by SAMNUT 24 x ICGV 91328 and SAMNUT 25 x ICGV 91317 while negative significant SCA effects were obtained by SAMNUT 22 x ICGV 91317, SAMNUT 24 x ICGV 91317, SAMNUT 24 x ICGV 91324 and SAMNUT 25 x ICGV 91328. For plant height at maturity, positive and highly significant SCA effects was recorded for SAMNUT 25 x ICGV 91328 while highly significant and negative SCA effects were recorded by SAMNUT 22 x ICGV 91317 and SAMNUT 25 x ICGV 91324. For haulm weight, the crosses SAMNUT 26 x ICGV 91317 and SAMNUT 26 x ICGV 91324 recorded positive and highly significant SCA effects while SAMNUT 26 x ICGV 91328 recorded negative highly significant SCA effects. Kernel infection SCA effects were non-significant for the entire crosses; however, the crosses SAMNUT 22 x ICGV 91328 and SAMNUT 26 x ICGV 91328 had very low negative SCA effects for kernel infection. The SCA effects for Aflatoxin B_1 accumulation were highly significant ($P<0.01$) for all crosses except SAMNUT 22 x ICGV 91324. Highly significant ($P<0.01$) positive SCA effects for Aflatoxin B_1 accumulation were obtained by SAMNUT 22 X ICGV 91317 (30.05) and SAMNUT 23 x ICGV 91328 (4.05), SAMNUT 25 x ICGV 91317 (25.29), SAMNUT 25 X ICGV 91328 (18.31), SAMNUT 26 x ICGV 91317 (9.87), and SAMNUT 26 x ICGV 91324 (14.61) while negative highly significant SCA effects were recorded by SAMNUT 22 x ICGV 91328 (-5.56), SAMNUT 23 x ICGV 91317 (-7.97), SAMNUT 23 x ICGV 91324 (-16.60), SAMNUT 24 x ICGV 91317 (-18.38), SAMNUT 24 x ICGV 91324 (-4.32), SAMNUT 24 x ICGV 91328 (-6.64), SAMNUT 25 x ICGV 91324 (-34.10) and SAMNUT 26 x ICGV 91328 (-8.69).

CHAPTER FIVE

5 Discussions

Plant breeding research with self-pollinating individuals has been traditionally performed with single crosses between any two parents, followed by development of segregating populations. This method generally results in reasonable amount of genetic variability needed for selection and attaining homozygosity. In this study; a 3 x 5 North Carolina design II model I was used to develop segregating population and evaluated using RCBD with 3 replications. Significant differences among the groundnut genotypes were observed for all the characters studied demonstrating that the genotypes are highly variable, which further suggest that progress could be made through selection. The presence of genetic variability encourages selection, because selection on its own does not create variability (Izge *et al.*, 2005). The low range and variation in maturity days of the groundnut genotypes was possibly due to narrow genetic base of groundnut. Earliness being an important adaptation trait to a wide range of cropping systems in semi-arid tropics may provide escape from terminal drought's damage which decreases the chances of aflatoxin contamination in groundnut. This is in agreement with the findings of Holbrook *et al.* (2000) who suggested that extremes of weather, as well as late maturity favours fungal infection and increases aflatoxin contamination. Pods are the most important component of the groundnut crop, because they are used to produce many products (oil, cake, flour, animal feed). The wide variability observed for pod weight implied that there is ample opportunity for selection for improvement of this important economic character (Shuting *et al.*, 1993). Most of the crosses produced more pods per plant as compared

Comment [HU1]: How? Affect in what way positive or negative? Explain

to the parents. This showed that genes controlling pod yield in the parents contributed favourably in the crosses. It also showed that the parents that differ in their genetic background combined well and produce promising crosses. Similar findings were reported by Jogloy *et al.* (2011) and Shamsideen (2016). The significant differences for haulm weight among the parents and for most of the crosses suggests variability for this trait among the genotypes which provide opportunity for breeders to make selection for the improvement of dual-purpose (pod and haulm yield) groundnut whereby the seeds could be used for human consumption or further processing and the haulms provide good-quality livestock roughage after the crops have been harvested. This result is similar to the findings of Waliyar *et al.* (2016) who reported significant genotypic difference for haulm yield in groundnut.

The means for the parents and crosses indicated that none of the genotypes screened was free of *A. flavus* infection as reflected in the kernel infection indicating the toxigenic nature of the isolate. Based on the scale provided by Oluret (2012), the kernel infection score for *A. flavus* grouped the genotypes by their response to the infection into three groups; resistant, susceptible and highly susceptible. The varied reactions to pre-harvest aflatoxin contamination in the parental groundnut genotypes proved none to be free from aflatoxin accumulations. However, ICGV 91328 bred for resistance to aflatoxin production, accumulated high amount of aflatoxin while SAMNUT 22 which was believed to be susceptible, recorded very low value for aflatoxin accumulation. This suggests that *A. flavus* infection and aflatoxin production in groundnut is significantly influenced by environmental parameters such as temperature (29 – 30^{0}C) and soil moisture especially end of the season drought. High G x E interaction effects and inconsistencies among genotypes offering resistance to aflatoxins has been reported under different climatic conditions (Anderson *et al*. 1995,

Comment [HU2]: Were there any cases of transgressive segreagant among the crosses? Discuss if there is !!!

Comment [HU3]: Explain this finding? Response: Findings explained!!!

Waliyar *et al.,* 2016). Earlier studies have established that severe drought promoted growth and persistence of *A. flavus* population in soils, thereby resulting in high aflatoxin levels in crops (Arunyanark *et al.* 2010). Similarly, genotypes with low *A. flavus* infection surprisingly produced high aflatoxin. The biosynthesis of the aflatoxin B_1 requires several steps and it is perhaps affected by the intervention of several environmental factors (stress, quorum sensing and protein signalling pathway). Amino-acids such as tyrosin encourage the synthesis of aflatoxin. The presence of the lipids induces the aflatoxinogenesis. Similar findings were reported by Wilkinson *et al*., (2007). Among the organic factors affecting biosynthesis, carbon and nitrogen are the major ones. In addition, simple sugars such as glucose and fructose support this biosynthesis (Payne, 1998). Concerning the physical factors, the optimal temperature of biosynthesis is between 28 ^{0}C and 35 ^{0}C. Under the conditions of dryness, the production of the aflatoxins is high. For pH, biosynthesis is high in acidic media while it is inhibited in basic conditions (Cotty 1988). The secondary plant metabolites play key roles in the synthesis of aflatoxins. For example, the presence of the octanal causes a reduction of 60% of the fungic growth with a rate of increase in the production of aflatoxins of 500% (Criseo *et al*., 2001). Four genotypes accumulated low aflatoxins of up to $4 ng/g$, which is in accordance with the European Commission standards (of total aflatoxin) for human consumption (Wilson and Otsuki 2001). Similarly, 10 genotypes from this study were within the permissible limits (15 ng/g of total aflatoxins) of countries such as South Africa, Australia, Canada and Gulf Corporation Council (GCC) countries like Saudi Arabia, Kuwait and Qatar. Similarly, a total of thirteen genotypes had toxin levels within the limits of United States Food and Drug Administration (FDA) and National Agency for Food and Drug Administration (NAFDAC) safety limits of $20 ng/g$. The rest of the

genotypes were with aflatoxin levels >20 ng/g. However, in African countries, the legislation on aflatoxin limits are restricted only to the export crops and thus, there is significant risk of rural poor being exposed to aflatoxins constantly with serious health impacts (Wagacha and Muthomi, 2008).

Correlation coefficient analysis helps to determine the nature and degree of relationship between any two measurable characters; it resolves the complex relationship between characters into simple form of association (Channayaa, 2009). When correlation exists between any two characters, a selection for one character will lead to corresponding change in the other correlated character. Based on the association study of this research, *A. flavus* infection correlated poorly to aflatoxin contamination, which explained why the presence of *A. flavus* in groundnut seeds does not automatically mean the presence of aflatoxin, but rather, that a potential for contamination exists. Conversely, the absence of *A. flavus* does not guarantee that groundnut seeds are free from aflatoxin. Other studies conducted by Zablotowich *et al*. (2007), Gridthai *et al.* (2010) and Rahmianna *et al*. (2015) also mentioned that the condition of *A. flavus* colonization and fungal growth on seeds are poorly correlated to aflatoxin production. The fungal growth and aflatoxin contamination were the consequence of an interaction among the fungus, the host and the environment. Nigam *et al*. (2009) also advanced the theory further from their study that aflatoxin contamination is a rare event and occurs only when all the conditions in and around a geocarposphere are favourable.

Based on the genetic analysis of *A. flavus* contamination and aflatoxin accumulations, the significant mean genetic variation for female, male and female x male suggested the contribution of both additive and non-additive types of gene actions involved in

the inheritance of the traits studied. These results are in agreement with the findings of Yoopum *et al.* (2005) in groundnut and Asea *et al*. (2012) in maize. Baker's ratio close to unity observed for aflatoxin B_1 accumulation indicates that GCA is a useful guide to progeny performance in general, so that evaluations based on a single representative male parent should be sufficient for initial hybrid selections. Specific combining ability is important enough, however, the initial screenings to identify better parents should be followed by evaluations with multiple males, as there is an important component of hybrid resistance that cannot be predicted from data on a different male. In contrast, the predictive ratio for kernel infection was close to zero, indicating the importance of non-additive gene action for the inheritance of this trait and testing susceptible parents in combination with multiple resistant parents will be required to identify superior progenies (Hallauer *et al.*, 1988). These findings are in agreement with those reported by Ozimati *et al. (*2014) in groundnut (aflatoxin content) and Hung and Holland (2012) in maize (fumonisin content) but contradicts the findings of Xue *et al.,* (2004), Arunyanark *et al*. (2010) and Habaninema *et al.* (2012) in groundnut (aflatoxin content).

Comment [HU4]: What is the breeding implications of these findings?

Heritability estimates provide information about the transmissibility of the quantitative character of economic importance and are essential for an effective crop breeding strategy. The breeder makes rapid progress where heritability is high using selection methods that are dependent solely on the phenotype. However, where heritability is low, the method of selection based on families and progeny testing are more effective and efficient (Acquaah, 2007). High estimates of broad-sense heritability recorded for *A. flavus* infection and aflatoxin B_1 accumulation implied the possibility for genetic improvement of these characters. The magnitude of heritability estimated was influenced by the variability between genotypes used, the extent to

which a particular character is affected by the prevailing environment conditions and the method used to obtain the estimate (Osman and Khidir, 1974). The high broad-sense heritability signifies the preponderance of additive gene action in the inheritance of these characters. These findings are in agreement with those obtained by Ozimati *et al.* (2014) and Hamblin and White (2000) who agreed that non-additive gene action in some cases to be more important than additive gene action for inheritance of resistance to *A. flavus* infection while they reported additive gene action to be more important than non-additive gene action for inheritance of resistance to aflatoxin accumulation. However, the findings of this study contradict those of Mudenda, (2013), Bala, (2012) and Xue *et al.,* (2004) who reported predominantly non-additive genetic variance for inheritance of resistance to *A. flavus* infection and aflatoxin production.

Comment [HU5]: Recorded for which charters?

The results of the proportional contribution of the parents and their progenies to the total variation indicated that the female parents contributed more to variation for most measured characters. This suggests the influence of maternal effects in the variation observed in the characters and indicates that when developing breeding populations for resistance to *A. flavus* infection, the choice of a maternal parent is very important.

The proportional contribution of the parents was higher than their progenies for resistance to aflatoxin accumulation, thus confirming the preponderance of additive gene action for the expression of this character. Similarly, the proportional contribution of the progenies was higher than those of their parents for resistance to kernel infection indicating the importance of non-additive effects for the expression of this character. Because this study was based on a limited number of selected parents, the inferences are applicable to these populations alone. However, the preponderance of evidence from this study clearly demonstrates the importance of non-additive

effects in the inheritance of resistance to *A. flavus* infection and additive effects in the inheritance of resistance to aflatoxin contamination. The importance of non-additive and additive effects, as measured by the proportional contribution of the parents and their interactions to the total variation, is reflected in the Baker's ratio estimates for resistance to *A. flavus* infection and aflatoxin contamination. This finding contradicts that of Ozimati *et al.* (2014) who reported Baker's ratio close to unity for resistance to *A. flavus* infection and Baker's ratio close to zero for resistance to aflatoxin contamination.

Based on GCA effects, the best parents for producing progenies with resistance to kernel infection and aflatoxin contamination were SAMNUT 26 and ICGV 91324. The significance of negative GCA effects for both female and male parental lines suggested a substantial contribution by the parents to variability among crosses for resistance to fungal infection by accumulating favourable alleles through selection to attain genetic improvement. Li, (2014) also reported negative GCA effects for resistance to fungal infection.

The three progenies with negative SCA effects for kernel infection and aflatoxin contamination: SAMNUT 22 x ICGV 91328, SAMNUT 24 x ICGV 91317, and SAMNUT 26 x ICGV 91328 were identified as the most promising genotypes in breeding programme for resistance these traits.. These progenies originated from parents with negative and positive GCA values. This suggests the difficulty in predicting the *A. flavus* resistance level of the progenies based on GCA alone and should necessitate testing of specific male x female combinations. Arunga *et al*. (2010) found that the SCA effect alone has limited value for parental choice in breeding programmes. They, therefore, suggested that the SCA effects should be used in combination with other parameters, such as hybrid means and the GCA of the

respective parents such that a hybrid combination with both high mean and favourable SCA estimates and involving at least one of the parents with high GCA, would tend to increase the concentration of favourable alleles; which is desired by any breeder. Furthermore, it was observed that crosses involving one good combiner and one average or poor combiner showed negative SCA effects. For example, SAMNUT 22 and ICGV 91328 had poor GCA values for kernel resistance, while their cross had negative and desirable SCA effects. Hannan *et al*. (2007) observed that some parents exhibited a similar phenomenon in studies on tomato (*Lycopersicum esculentum* Mill.). This manifestation of progenies having reactions not related to the parent's attributes introduced a different dimension in the inheritance of groundnuts to resistance to aflatoxin contamination. John *et al*. (2011) and Ayo-Vaughan *et al*. (2013) observed similar phenomena in groundnuts and cowpeas, respectively and attributed it to genetic interaction between favourable alleles and between unfavourable alleles contributed by both parents. This suggests that inheritance of this trait is not simple and that inter-allelic interactions due to epistasis could be responsible. Specific combining ability effects values provide important information about the performance of the hybrid relative to its parents. However, SCA effects are not very important for crops like groundnuts that are highly self-pollinated and difficult to produce commercial hybrids, a point advanced by Kimani and Derera, (2009) while working on beans; self-pollinated counterpart of groundnut.

CHAPTER SIX

6 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Aflatoxin is a secondary metabolite produced by *Aspergillus flavus* that grows on seed coat (testa) of groundnut. Aflatoxin contamination in groundnut is one of the major barriers in international trade and a serious food safety issue throughout the world. The availability of groundnut genotypes resistant to *A. flavus* infection and/or aflatoxin contamination is urgently needed for enhanced economy of producers' and consumers' safety. This research was conducted to study the inheritance of resistance to *A. flavus* infection, aflatoxin contamination and other agronomic traits in groundnut. The parental materials used in this study comprises of eight parents (three males and five females) selected based on their resistance status to *A. flavus* infection and aflatoxin contamination. The parents were mated in a North Carolina mating design II Model I in the screen house, generating 15 F_1s which were advanced to F_2 to provide enough seeds for evaluation. The 8 parents and their 15 F_2 progenies were evaluated in the screen house of the Department of Plant Science, Institute for Agricultural Research (IAR) Samaru, Ahmadu Bello University Zaria, during the 2015/2016 season. The experiment was laid out in a randomized complete block design with three replications. Data were collected on some agronomic traits along with *A. flavus* infection score and aflatoxin accumulation. Data were analysed using SAS (2002) following RCBD and NCII design model I.

Significant genetic variations were observed for most of the characters studied under artificial *A. flavus* inoculation indicating the presence of appreciable variability among the genotypes which is a pre-requisite for any crop improvement programme. The results of this study also showed progenies that were resistant to *A. flavus* infection and aflatoxin contamination with superior agronomic traits such as good pod and haulm yield.

The entry mean broad-sense heritability estimates were moderate to high for most studied characters. This gave an indication of the importance of additive gene effects governing these characters and selection for genetic improvement would be worthwhile. Broad sense heritability was high for haulm weight, kernel infection and aflatoxin B_1 accumulation. This revealed that variation were transmitted to the progeny and indicated potential for developing *A. flavus* resistant varieties with improved agronomic features, through selection of desirable progenies in succeeding generations. The crosses, SAMNUT 26 x ICGV 91317 (0.8 ng/g , 118.7g, 107.3g pod and haulm weight), SAMNUT 26 x ICGV 91328 (1.2 ng/g , 87.7g, 103.0g pod and haulm weight), SAMNUT 22 x ICGV 91328 (4.4 ng/g , 92.7g, 104.5g pod and haulm weight) and SAMNUT 22 x ICGV 91324 (12.3 ng/g , 103.0g, 115.8g pod and haulm weight) uniquely had low levels of pre-harvest aflatoxin contamination, high pod and haulm yield and good shelling outturn.

Comment [HU6]: No results indicated here, why? Please show some results

General combining ability made significant and important contributions to hybrid variations for both *A. flavus* infection and aflatoxin contamination. The analysis revealed that additive and non-additive gene actions were important for the inheritance of resistance to *A. flavus* infection, aflatoxin accumulation and improved agronomic characters. This was further confirmed by Baker's ratio which was less

than unity for some characters and close to unity for others. Parents that exhibited combining abilities in the desired direction could be useful in hybridization programmes. However, parents that are good combiners do not always produce best hybrid combinations. For this reason, the specific combining abilities of the progenies should be considered in selecting the best parents. General combining ability of parents showed that SAMNUT 22 was a good general combiner for days to 50% flowering and aflatoxin B_1 accumulation. SAMNUT 25 is a good general combiner for haulm weight and kernel infection. SAMNUT 26 is a good general combiner for haulm weight, kernel infection and aflatoxin B_1 accumulation. The male parent, ICGV 91324 is a good general combiner for kernel infection and aflatoxin B_1 accumulation. The progenies showed great deal of specific combining ability for kernel infection, aflatoxin B_1 accumulation and other agronomic characters.

6.2 Conclusions

It is concluded from this study that sufficient genetic variability exists for aflatoxin contamination resistance and other agronomic characters among the groundnut genotypes used. Twelve genotypes were identified as unique, with low levels of *A. flavus* infection and pre-harvest aflatoxin contamination coupled with important agronomic traits. The most promising genotypes are SAMNUT 26 x ICGV 91317 (0.8 ng/g, 118.7g, 107.3g pod and haulm weight), SAMNUT 26 x ICGV 91328 (1.2 ng/g, 87.7g, 103.0g pod and haulm weight), SAMNUT 22 x ICGV 91328 (4.4 ng/g, 92.7g, 104.5g pod and haulm weight) and SAMNUT 22 x ICGV 91324 (12.3 ng/g, 103.0g, 115.8g pod and haulm weight). These materials are considered as resistant to *A. flavus* infection and aflatoxin contamination.

Comment [HU7]: What was their status on aflatoxin accumulation?

The study revealed further that non-additive gene action was more important than additive gene action for resistance to *A. flavus* infection, while additive gene action was predominant for resistance to aflatoxin accumulation.

The parents SAMNUT 22 was identified as a good general combiner for days to 50% flowering and aflatoxin B_1 accumulation. SAMNUT 25 is a good general combiner for haulm weight and kernel infection. SAMNUT 26 is a good general combiner for haulm weight, kernel infection and aflatoxin B_1 accumulation. The male parent, ICGV 91324 is a good general combiner for resistance to kernel infection and aflatoxin B_1 accumulation. Cross combinations such as; SAMNUT 26 x ICGV 91317, SAMNUT 26 x ICGV 91328, SAMNUT 22 x ICGV 91328 and SAMNUT 22 x ICGV 91324; were also identified with good specific combining ability.

Broad sense heritability estimates were high for *A. flavus* infection (85.3%) and aflatoxin accumulation (99.7%), implying low environmental effects in their overall phenotypic expressions.

6.3 Recommendations

From the study, it is recommended that the promising segregating lines should be further advanced by single seed descent or backcrossing to fix these characters and develop agronomically superior groundnut genotypes that are highly resistant to aflatoxin contamination.

To fully exploit resistance discovered in these lines, identification of DNA markers would be useful in the transfer of the resistance genes to commercially useful backgrounds. To achieve this goal investigation needs to be carried out to discover factors that contribute to resistance such as QTL analysis to locate regions of chromosomes associated with resistant phenotypes.

Progress in aflatoxin resistance breeding has been limited as genotypes tend to exhibit variable performance due to high G x E interaction and lack of reliable screening protocols. It is therefore recommended that advanced screening protocol should be further exploited and also the identified promising genotypes be further evaluated in wider and diverse growing conditions. Such breeding effort may lead to the development of agronomically superior groundnuts that are free of aflatoxin contamination.

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