COMBINING ABILITY STUDY FOR THE DEVELOPMENT OF RESISTANCE TO AFLATOXIN CONTAMINATION BY *Aspergillus flavus* Link ex Fries IN GROUNDNUT (*Arachis hypogaea* L.) GENOTYPES

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF ZAMBIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (PLANT BREEDING AND SEED SYSTEMS).

By

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DECLARATION

I hereby declare that this dissertation entitled "Combining Ability Study for the development of resistance to aflatoxin contamination by *Aspergillus flavus* Link ex Fries in groundnut (*Arachis hypogaea* L.)" genotypes" is the result of my own research work. No part of this dissertation has previously been submitted for a degree or any other qualification. The information derived from literature has been duly acknowledged in the text and explicit references are given.

Signature…….....

Date…….....

APPROVAL

This dissertation of Mable Mudenda meets the regulations governing the award of the degree of Master of Science in Plant Breeding and Seed Systems, and is approved for its contribution to scientific knowledge and literary.

Examiner's Name and signature	**Date**
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DEDICATION

I hereby dedicate this research to my children Mwangala and Chimuka for their unwavering support, I love you very much.

ABSTRACT

Groundnuts are one of the important household food security crops grown in Zambia. The crop provides minerals, fats, protein, vitamins and it also acts as a source of income for resource poor farmers. Production and utilization of groundnuts in the small holder setting is hampered among others, infection by *Aspergillus flavus* and subsequent aflatoxin contamination. 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 *Aspergillus flavus.* A greenhouse study was, therefore, undertaken at ICRISAT in Lilongwe, Malawi to establish the genetic basis of resistance to aflatoxin contamination by *Aspergillus flavus* in groundnut genotypes. Ten parental genotypes consisting of three resistant (55-437, J11, Ah 7223) and seven susceptible (Katete, Luena, Natal common, Chalimbana, Chishango, MGV-4 and MGV-5) were crossed in a 3 x 7 North Carolina Design II. F_1 crosses were evaluated for aflatoxin contamination levels in a Completely Randomized Design with 3 replications and *Aspergillus flavus* colony forming units enumerated from the soil. Significant ($P<0.05$) differences among the crosses for aflatoxin B_1 contamination levels and colony forming units measured as propagule density $(cfu)g^{-1}$ soil were observed. Total aflatoxin B_1 contamination levels ranged from 0.2parts per billion (ppb) to 7.3 ppb, while propagule densities of *Aspergillus flavus* ranged from log(10) 4.11 to 4.78 cfu g^{-1} soil. Combining ability estimates of the parents for aflatoxin contamination registered significant ($P<0.05$) negative GCA effects for 55-437(-0.77), Ah 7223 (-1.46) and Katete (-1.95), while specific combining ability effects (SCA) of the crosses for the same trait were significant ($P<0.05$) and negative for 55-437 x N/Common (-1.43), J11 x Luena (-1.21), J11 x Katete (-1.81) and Ah 7223 x N/Common (-1.81). The narrow sense heritability (h^2) estimates were in the range of 0.1 and 0.2, while the Baker's ratio ranged between 0.2 and 0.3, suggesting the influence of non-additive gene action in controlling the resistance to aflatoxin contamination by *Aspergillus flavus*. The study thus concluded that improvement of resistance to aflatoxin contamination can be achieved via a recurrent selection procedure.

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CHAPTER ONE

1.0 INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an annual legume which is also known as peanut. It is the 13th most important food crop and 4th most important oilseed crop in the world (Reddy *et al.,* 2011) after soybean, cottonseed and rapeseed. Worldwide, there are over 100 countries growing groundnuts and developing countries constitute 97% of global area and 94% of the global production (Ntare *et al*., 2007).

Groundnut is important for small-scale farmers in semi-arid tropic regions, which are characterized by low soil fertility, unpredictable rainfall and rain distribution (Jogloy *et al*., 2005). The groundnut kernels are consumed directly as raw, roasted or boiled and the oil extracted from the kernel is used as culinary oil (Reddy *et al.,* 2011). It is a rich source of minerals, fats, protein and vitamins, supplementing diets where maize, rice, and cassava are the major energy foods (Monyo *et al.,* 2012). Groundnut as a legume is also an important source of income when sold locally or when exported particularly to the European Union (Monyo *et al*., 2012).

Groundnuts in Zambia are cultivated in all provinces (Fig. 1) to varying degrees (Mwila *et al*., 2008). An estimated 721636 households currently grow, consume and trade in groundnuts countrywide (Ross and De klerk, 2012). Groundnut therefore, plays an important role in the Zambian diet and is the second most common field crop grown by smallholder farmers, following maize (Denison, 2011).

The groundnut plant is a legume whose fruit develops below the ground (Armstrong, 2009). The flowers are formed and fertilized above ground, but downward growth of the pegs ensures that the fruit (pods and seeds) develop in the soil (Waliyar and Reddy, 2009). This facilitates the penetration of plant tissues with toxigenic fungi such as *Aspergillus flavus* (Beasley, 2011).

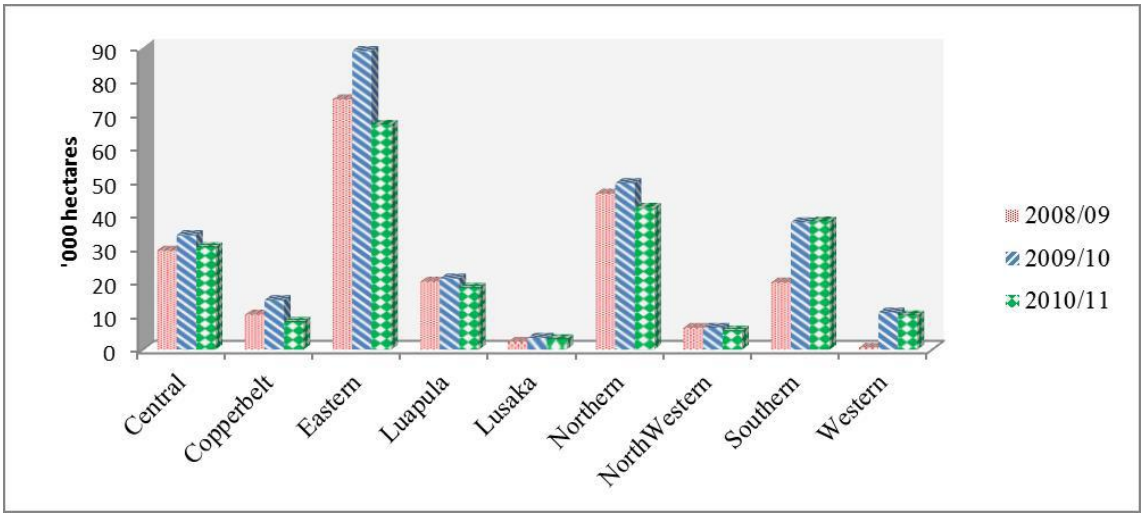

Figure 1: Groundnut production per province (CSO/MAL 2008/09 – 2010/11).

Aflatoxin contamination is one of the biggest problems affecting peanut production. This is because a wide range of environmental conditions in sub-tropical regions often favour the *Aspergillus flavs* infection both in the field as well as in storage (Narasimhulu, 2007). Fungal growth and aflatoxin contamination are the consequence of interactions among the fungus, the host and the environment. The appropriate combination of these factors determines the infestation and colonization of the substrate and the type and amount of aflatoxin produced (Dhanasekaran, *et al*., 2011).

Aflatoxins are extremely toxic and carcinogenic secondary metabolites (Yu *et al*., 2004; Arunyanark *et al*., 2010; Reddy *et al*., 2011) comprising a group of more than fifteen toxins (Waliyar and Reddy, 2009) and their synthesis may be triggered by host stress signals (Holbrook *et al.*, 2008). The term aflatoxin refers to a group of bisfuranocoumarin metabolites (Dhanasekaran *et al*., 2011) isolated from strains of two fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. Therefore, they are referred to as "mycotoxins": This term is derived from "mikes" (Greek word for fungus) and "torsion" (poison) (Waliyar and Ready, 2009; Reddy *et al.*, 2011).

While aflatoxin contamination affects peanut quality the greater problem is that it affects humans and livestock that consume contaminated peanut and peanut products (Arunyanark *et al*., 2010). Aflatoxins have been associated with various diseases, such

as aflatoxicosis, in livestock, domestic animals and humans throughout the world (Reddy *et al*., 2011). The possible presence of aflatoxins in foods and feeds has had a profound effect on the utilization and trade in groundnuts and its products. Many developing countries have been unable to export their groundnuts and groundnut products (Waliyar and Reddy, 2009) due to unacceptable contamination levels.

The toxicity of aflatoxins in food materials has resulted in international agencies to permit the presence of 20 ppb of aflatoxin in food materials as the maximum permissible level for human consumption (Dhanasekaran *et, al.,* 2011; Reddy *et al*., 2011; Holbrook *et al.,* 2008). The maximum allowable level in the United States is 20ppb and the European Union has an allowable level of 4 ppb total aflatoxins and under 2 ppb aflatoxin B_1 (Holbrook *et al.,* 2008). These regulations directed at minimizing human exposure to aflatoxins poses a challenge to producers, handlers, processors and marketers of the contaminated crop (Cotty and Garcia, 2007).

In Zambia very little of the groundnut surplus was exported in 2011 because of aflatoxin contamination (Feed the future strategic report, 2011). The unmanaged aflatoxin levels also present health issues for local consumers (Ross and De klerk, 2012). The aflatoxin contamination and especially the introduction of stricter allowable levels under the *Codex Alimentarius* and the European Union Legislation have seriously undermined the export potential of Zambia to South Africa and beyond (Regional Market overview report, 2005).

Attempts to mitigate the problem of aflatoxins using good post-harvest handling practices such as good produce handling and storage has not proved to be effective because aflatoxin contamination can occur in the field as well as in storage and use of fungicides is also not cost effective for small scale farmers. The practices are further not widely adopted by the small scale farmers in the developing countries, which contribute about 60% of the world groundnut production (Upadhyaya *et al.,* 2000).

Thus it is thought that the use of genotypes resistant to *A.flavus* and to accumulation of aflatoxins is the best option for farmers (Ozimati *et al*., 2012). The development of peanut cultivars with resistance to aflatoxin contamination could, therefore, serve as a valuable tool in addressing the aflatoxin challenge (Mixon and Rogers, 1973; Mehan and

McDonald 1984; Holbrook *et al.*, 2008) and associated economic losses and health hazards (Upadhyaya *et al*., 2000). The management of aflatoxin contamination requires both preventive and remedial approaches starting from sowing and harvesting to processing and storage. Resistant cultivars should be an effective low-cost part of an integrated aflatoxin management program and the most viable economical solution to the aflatoxin problem (Narasimhulu, 2007).

The resistance to aflatoxin-producing fungi in groundnuts has been found to be of three types: resistance to pod infection (pod wall); resistance to seed invasion and colonization (seed coat); and resistance to aflatoxin production (cotyledons) (Mehan, 1989). Sources of all the three types of resistance have been reported in cultivated peanut. Shulamit and Darou IV were identified for resistance to pod infection, PI 337394 F, PI 337409, GFA 1, GFA 2, UF 71513, Ah 7223, J 11, Var 27, U 4-47-7, Faizpur, and Monir 240-30 for resistance to in vitro seed colonization by *A. flavus* (IVSCAF); and U 4-47-5 and VRR 245 for resistance to aflatoxin production (Mehan, 1989). Other sources of resistance identified include ICG 1122, ICG 1173, ICG 1323, ICG 1859, ICG 3263, ICG 3336, ICG 4888, ICG 7633, ICG 9407 and ICG 10933 (Nigam *et al*., 2009).

Knowledge about genetic mechanism, involved in the expression of traits, is helpful in developing these superior genotypes. The information about the relative contribution of components of variation i.e., additive, non-additive and epistasis, is essential for effective plant-improvement. It provides guidelines for plant breeders to select parent lines to be used in breeding programs and to use promising cross combinations for further selection (Jogloy *et al*., 2005). Combining ability analysis is therefore an important technique to understand the genetic potential of parents and their hybrids. General combining ability is associated with genes which are additive in effects while specific combining ability is attributed primarily to deviations from the additive scheme caused by dominance and epistasis (Rojas and Sprague, 1952). Narrow-sense (or additive) heritability ($h^{2)}$ is meant to capture the "additive" contribution of genes to the trait. It is the maximum variance that can be explained by a linear combination of the allele counts. Traditionally h^2 is routinely used to measure progress toward explaining the genetic basis of a trait (Wray and Visscher, 2008).

In light of the above, the present study was formulated with the following objectives.

1.1 Main objective

To establish the basis on which groundnut genotypes resistant to aflatoxin contamination by *Aspergillus flavus* could be developed.

1.2 Specific objective

To determine the gene action and inheritance of resistance to aflatoxin contamination by *Aspergillus flavus* among selected groundnut genotypes.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Groundnuts

Groundnuts or peanuts are annual herbaceous, self-pollinating legume crops which belong to the section *Arachis*, series amphiploidies, family Leguminosea (Gregory *et al.*, 1973) and subfamily Papilionaceae. The distinguishing morphological features of this genus are aerial flowers that give rise to subterranean fruits. There are about 70 species, most of them diploid while two are allotetraploids. Five species have been cultivated, but only *A. hypogaea* has been domesticated and grown extensively for seeds and oil (Stalker, 1992).

Taxonomically, the cultivated peanut *A. hypogaea* is divided into two subspecies, one with two botanical varieties, and one with four. In the subspecies *hypogaea var. hypogaea* (Virginia and Runner market types) and *var. hirsuta*, the varieties have long duration cycle and seeds undergo dormancy While in subspecies *fastigiata* with *var. fastigiata* (Valencia market class) and *var. vulgaris* (Spanish market class), the varieties are early-maturing but generally without fresh seed dormancy (Krapovockas and Gregory, 1994).

2.2 Global groundnut production

Groundnuts are currently grown on over 22.2 million hectares worldwide with a total production of over 35 million tonnes. India and China are the world's largest producers of groundnuts, accounting for over 41% and over 18% of world production respectively.

Millions of small-holder farmers in Sub-Saharan Africa (SSA) grow groundnut as a food and cash crop, accounting for over 9 million hectares of cultivated farmland. Nigeria, Sudan and Senegal are Sub-Saharan Africa's leading producers, and the crop also does well in southern Mali and adjacent regions of Cote D'Ivoire, Burkina Faso and Ghana. Although SSA has 40% of the world total of land under groundnut, its output amounts to only 25% of total world production due to low yield (0.95 tonnes/ha as compared to 1.8 tonnes/ha in Asia) (Integrated breeding platform, 2013).

2.3 Economic importance of groundnuts

Groundnuts are important to millions of small scale farmers in Africa and Asia, particularly women farmers both as a source of protein and income in support of their livelihoods (Setimela *et al.,* 2004). Groundnut kernels are consumed directly as raw, roasted or boiled while oil extracted from the kernel is used as culinary oil. It is also used as animal feed (oil pressings, seeds, green material and straw) and industrial raw material (oil cakes and fertilizer) (Reddy *et al.,* 2011). Milk from groundnuts is a good lactose-free milk-substitute beverage. Groundnut provides over 30 essential nutrients and phytonutrients, including niacin, folate, fibre, magnesium, vitamin E, manganese and phosphorus. In addition, groundnuts and other legumes contribute to soil fertility by fixing nitrogen, which can increase the yields of cereal crops planted in rotation with legumes (Integrated breeding platform, 2013).

2.4 Groundnut production constraints

The main constraints hampering higher yields and quality in Africa are intermittent drought due to erratic rainfall patterns and terminal drought during maturation, with drought-related yield losses running to millions of dollars each year. It is also affected by heavy weed pressure and calcium deficiency (causing unfilled shells or "pops") (Integrated breeding platform, 2013). The groundnut crop is affected by several diseases like leaf spots, collar rot, rust, bud necrosis, stem necrosis rosette virus *etc* (Reddy *et al.,* 2011).

A drought-related quality issue that affects groundnuts is pre-harvest contamination with aflatoxin, a carcinogenic mycotoxin produced in the infected peanut seeds by fungi such as *Aspergillus flavus* Link ex fries and *Aspergillus parasiticus* Speare, particularly at the end of the growing season under drought conditions (Diener *et al.,* 1987). The pre-harvest conditions favoring aflatoxin contamination are high temperatures and drought stress during the last 3 to 6 weeks of the growing season (Cole *et al*., 1989).

2.5 Zambian groundnut production

Groundnuts are the second most common field crop grown by smallholder farmers (Feed the future multiyear strategy report, 2011). The crop is produced by nearly half of the estimated 1.4 million rural smallholder households, making it the second largest, after

maize, in terms of production volume and hectares cultivated. Approximately 8.8% of total land cultivated in Zambia is planted to groundnuts. Groundnuts therefore play an integral role in the livelihoods of the majority of the Zambian population, particularly the rural households (Mukuka and Shipekesa, 2013). In 2009/10, Zambia produced 164,000 MT (valued at $138 million) with an estimated local human consumption of 70,000 MT (valued at $58 million) leaving more than 93,000 MT for other industrial uses (Feed the future multiyear strategy report, 2011).

Most of Zambia's groundnuts are grown in the Central, Copperbelt, Eastern, Luapula and Northwestern provinces. Half the country's groundnuts are produced in the Eastern and Northern Provinces, which generate 30 and 21% respectively of the country's production. In the Eastern Province the highest proportion of small and medium scale farmers grow groundnuts (69%), followed by Northern Province (60%), compared to the country average of 49% (Rose and De klerk, 2012).

Like most groundnut producing countries in Sub-Saharan Africa, Zambia's groundnut yields vary from anywhere between 0.3 and 0.5 MT/ha with occasional highs of 0.7 and 0.8 MT/ha (Mukuka and Shipekesa, 2013). Variations in groundnut yields are typically related to changes in rainfall pattern, varieties planted, seed quality, agronomy practices employed, pest attacks, soil fertility, farming systems/practices (Regional market overview report, 2005).While the hectarage planted is largely influenced by the availability of markets, prices in the previous season, and prices of competing crops. (Mukuka and Shipekesa, 2013).

The varieties grown in the country are mainly used for confectionary and oil extraction purposes. These varieties are adapted to different agro-ecological conditions and have varying characteristics which include high yielding, disease resistance, early/late maturity, drought tolerance, high/low oil content availability and peanut butter making. The varieties grown include Chishango, Katete, MGV-5, MGV-4, Luena, Chipengo, Champion, Chalimbana, Makulu Red, Natal common, MGS-2(M13) and comet (Ross and De klerk, 2012).

The major constraints to production include shelling which is labour intensive, poor and late rains which result in late planting and poor yields, pops (shells containing no

kernels), lack of pesticides, insect pests, lack of seed, lack of knowledge, foraging pigs and unmeasured and unmanaged aflatoxin levels. (Mukuka and Shipekesa, 2013; Ross and De klerk, 2012).

2.6 The Aflatoxin problem in Zambia

The high levels of aflatoxin in groundnuts pose serious threats to human health. Currently, it is not known how much damage aflatoxin has caused among the Zambian consumers (Mukuka and Shipekesa, 2013). Siame and Nawa (2008) mentioned that several reports are now available regarding mycotoxin contamination of maize and other commodities in Zambia. In one report Njapau *et al.*, (1998) reported that substantial amounts of aflatoxin were detected in processed foods from maize and peanuts obtained from a farmer and a supermarket in Central and Lusaka provinces. In another earlier report Njapau and Muzungaile (1993) also recorded high aflatoxin levels during an analysis of feed samples. In maize Kankolongo *et al* (2008) reported fumonisins and aflatoxins ranging from 0.02 to 21.44ppb, and 0.7 and 108.39ppb in 96.4% and 21.4% of samples analysed, respectively. However inspite of the reported high levels of toxins detected and the presence of these toxigenic fungi in some commodities in the country, as well as the existence of ample information that exposure to toxins constitutes a serious threat to human and animal health (Kankolongo *et al*., 2008), farmers and consumers in Zambia are unaware of the health implications of aflatoxins (Mukuka and Shipekesa, 2013).

In Eastern Province, farmers use various traditional methods to dry the groundnuts. The most common methods are where farmers leave the groundnuts in the field or on bare ground at the homestead. About 96% of the farmers in Eastern Province use these two methods (IAPRI/CSO/MAL 2012). Drying on the ground in the field or on bare ground at the homestead exposes the groundnuts to ground moisture. The bare ground drying is a major source of fungal contamination (Okello *et al.* (2010).

Zambia like many countries worldwide control mycotoxins in food and feed through policy regulations and regular monitoring for acceptable levels through the Zambia Bureau of Standards (Kankolongo *et al*., 2008). The Zambia Bureau of Standards has however not fulfilled its mandate in that most domestic products comply only with

rudimentary standards often dealing with surface imperfection and product size (World Bank/USAID report, 2006, Giovannucci *et al*., 2001).

When it comes to storage, harvested groundnuts are commonly stored in shells/pods or what is referred to as unshelled groundnuts. In Eastern Province, about 98.6% of the farmers store their groundnuts in shells/pods (IAPRI/CSO/MAL 2012). Storing groundnuts in this form is recommended because shells offer protection against mould infection. However most farmers sell their produce in shelled form (Ross and Dekerk, 2012), for example in eastern province 80% of the groundnuts are shelled before they are sold (Mukuka and Shipekesa, 2013). This therefore deteriorates the groundnuts very fast because they pick-up moisture and are easily invaded by moulds, insects and rodents (Okello *et al.*, 2010).

Another determining factor of quality in groundnuts is where the groundnuts are stored. To avoid fungal infection, storing groundnuts in a place with lots of air circulation is recommended. In Eastern Province, most of the households store groundnuts either in sacks inside the house and/or in a groundnut granary. Groundnuts stored inside the house in sacks are often not exposed to enough air circulation considering that the houses have very little space. Again, this remains a challenge in controlling the aflatoxin contamination levels (Mukuka and Shipekesa, 2013).

The demand for groundnuts by the confectionery and peanut butter industries, both locally and on the export market is enormous. Statistics indicate that 61,082 MT of shelled groundnuts were traded in Zambia as a whole in 2011 and 37,688 MT in 2010 (CSO, 2011), of this 10,740 MT and 8947 MT respectively were traded in the Eastern Province alone (Mukuka and Shipekesa, 2013). However the major constraint to accessing export markets in Zambia is the control of aflatoxin levels which are not reliably measured and often said to be too high to meet European and South African standards. Zambia is therefore among the lowest exporters of groundnuts in the region (fig. 1) despite having favourable agro-ecological conditions for growing the crop (Ross and De klerk, 2012; Mukuka and Shipekesa, 2013; Regional market overview report, 2005).

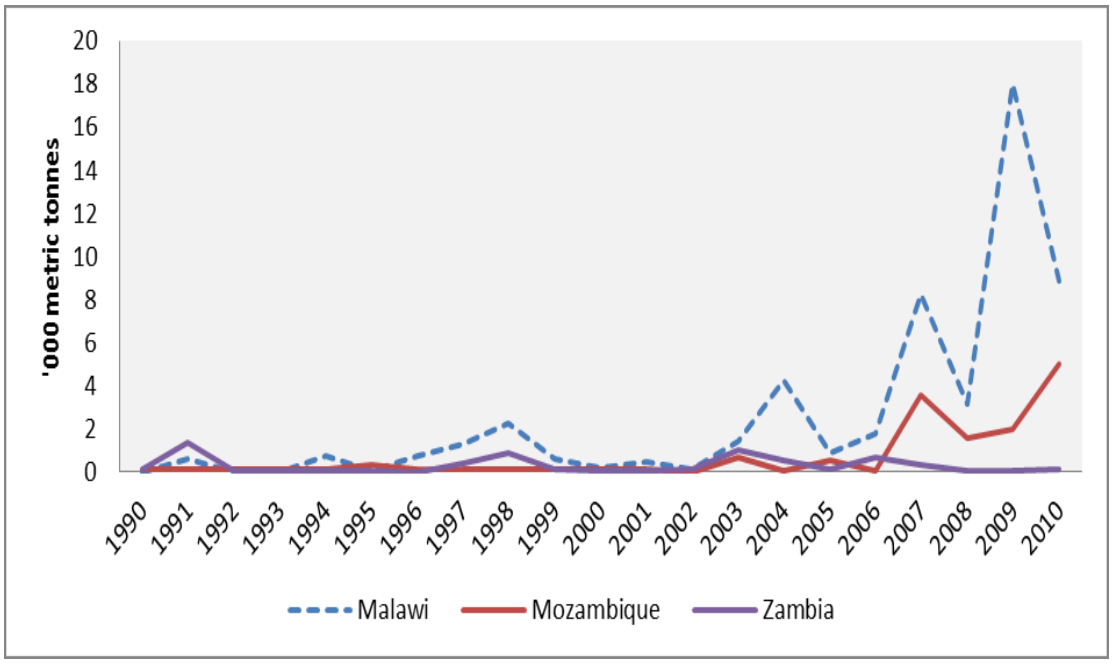

Figure 2: Groundnut Exports from Zambia, Mozambique, and Malawi (FAOSTAT, 2010)

2.7 The *Aspergillus flavus* pathogen.

2.7.1 Description of the pathogen

Aspergillus flavus belongs to the genus *Aspergillus* and some of the most economically important toxigenic species of fungi belong to this genus (CAST, 2003). The genus is named based on the structural similarity of its conidiophore structure to the aspergillum and currently contains over 200 species (Amaike and Keller, 2011). The genus is subdivided into 7 subgenera, which in turn are further divided into sections (Devi *et al*., 2013).

The identification and description of the fungi is based on a range of morphological features (Devi *et al.,* 2013). The *Aspergillus flavus* sterigmata are typically biseriate, conidia conspicuously echinulate, conidiophores heavy walled and coarsely roughened (Reddy *et al*., 2011). The conidia germinate at temperatures between 12^{o}C and 37^{o}C, with a minimum water potential at 270 bars at 45^{o}C. The optimum growth occurs within the broad range of 25-42^{o}C with a minimum of 17-19^{o}C and a maximum of 47-48^{o}C (Domsch *et al*., 2007).

2.7.2 Distribution of *Aspergillus flavus*

The genus Aspergillus has a diverse family of fungi that are worldwide in distribution but primarily occupy subtropical and warm temperate climates (CAST, 2003) however about one third of reports come from India or the temperate regions. The distribution of the fungi in soil is not limited by pH or soil depth. It can be isolated from a depth of 45cm. It has also been found to have a relatively high competitive ability (Domsch *et al*., 2007) especially under conditions of high temperature and low water activity *A. flavus* may become the dominant fungal species in the soil (Payne, 1998). *A. flavus* is however a weak pathogen and natural infection by this fungus is sporadic from one growing season to the next (Windham and Williams, 2007).

2.7.3 *Aspergillus flavus* host range

Aspergillus flavus has a wide host range (Domsch *et al*., 2007). It contaminates a vast array of dietary staples and agricultural products (Farombi, 2005). The commodities affected include oilseeds, cereals, legumes and spice crops (Waliyar and Ready, 2009; Devi *et al.,* 2013). Aflatoxins are also found occasionally in milk, cheese and a variety of other foods and feeds. However milk, eggs, and meat products are contaminated because of the animal consumption of aflatoxin contaminated feed (Dhanasekaran, *et al*., 2011).

2.7.4 Aflatoxin production by *Aspergillus flavus*

The *Aspergillus flavus* fungus is a complex species which produces widely different quantities of aflatoxins (Hua *et al.,* 2004). It forms a polyphyletic assemblage containing isolates of different sclerotial morphotypes based on size (Hua *et al.,* 2004, Cotty 1989) and ability to produce aflatoxins (Jeffrey *et al*, 2006): the typical L strain that produces large sclerotia >400 µm and the S strain, which produces numerous small sclerotia <400µm (Cotty, 1989) described as variety *parvisclerotigenus* (Saito and Tsuruta 1993).

The formation of aflatoxins is influenced by physical, chemical and biological factors. The physical factors include temperature and moisture. The chemical factors include the composition of the air and the nature of the substrate. Biological factors are those associated with the host species (Hesseltine, 1983).

However different theories have been given as to why fungi make aflatoxins (Bhatnagar *et al*., 2000; Lillehoji, 1991). These explanations include the following: Aflatoxin and its precursors are a defence response by fungi to stress; they provide protection from UV damage; they are by products of primary metabolism; they are virulence factors; they increase fungal fitness and they provide protection from predators for reproductive structures such as conidia and sclerotia (Wilkindson *et al.,* 2004).

The production of mycotoxins can be useful to separate strains of the *Aspergillus flavus* group. The *A. flavus* fungus usually only produces aflatoxin B_1 and aflatoxin B_2, but is also capable of synthesising cyclopiazonic acid, a mycotoxin confirmed as being present in the batch of contaminated groundnuts which killed turkey poults in 1960 (Turkey 'X'disease) (Smith, 1991). However many isolates of the *A. flavus* fungus do not produce aflatoxin and are named atoxigenic strains. *A. parasiticus* strains on the other hand produces aflatoxin $B_{I,}$ $B_{2,}$ $G_{I,}$ G_2 (Diener *et al*., 1987) and atoxigenic isolates have rarely been described. B_1 is the most prevalent, most potent carcinogen known (Hua *et al.,* 2004; Farombi, 200 5) followed by aflatoxins G_1, B_2, and G_2 in order of decreasing potency (Waliyar and Reddy, 2009). Although aflatoxins B_1, B_2 and G_1 are common in the same food sample, B_1 predominates (60-80% of the total aflatoxin content). Generally B_2, G_1 and G_2 do not occur in the absence of B_1 (Dhanasekaran, *et al*., 2011).

The aflatoxins produced are known to fluoresce strongly in ultra violet light. The designation of B_1, B_2, G_1 and G_2 represents their blue and green fluorescence in UV light. B_1 and B_2 fluoresces blue, while G_l and G_2 fluoresces green. All aflatoxins absorb UV light in the range of 362-363nm, a characteristic used in preliminary identification (Dhanasekaran, *et al*., 2011).

2.7.5 Effects of Aflatoxins

Aflatoxins have been associated with various diseases, such as aflatoxicosis, in livestock, domestic animals and humans throughout the world (Reddy *et al*., 2011). A prominent threshold effect of aflatoxicosis is immune suppression, manifested by what appears to be exaggerated virulence of a host of pathogens. Liver failure may also be seen (Beasley, 2011) as the liver is the primary site of biotransformation of ingested B_1 (Farombi, 2005). The consumption of aflatoxin contaminated food and incidence of

cancer in humans are therefore positively correlated (Dhanasekaran *et al*., 2011). Because of their mutagenic, teratogenic, and carcinogenic potency, aflatoxins are classified within Group 1, as compound carcinogenic to humans (IARC, 1993).

Acute toxicity due to aflatoxins for any given species of animal is however influenced by such factors as age, size, breed, condition of animal and composition of diet. Young animals tend to be more sensitive than mature animals (Waliyar and Reddy, 2009). Potential interactions with aflatoxins are also of concern. Included are interactions, with other hepatotoxic mycotoxins and with infections, especially by hepatitis virus (Beasley, 2011).

2.8 The Sources of resistance to *Aspergillus flavus* and aflatoxin production.

Genetic improvement for host resistance in peanut to fungal infection and aflatoxin production is among the approaches for integrated management of the problem (Lei-yong *et al*., 2005). However the value of a resistant source depends upon the level and stability of its resistance (Upadhyaya *et al.*, 2000). According to Holbrook, *et al*, (2008) host plant resistance to aflatoxin contamination can be approached by reducing fungal infection and/or reducing aflatoxin biosynthesis.

Rao and Tulpule (1967) were the first to report varietal differences in resistance to aflatoxin production in peanut. In their laboratory study they introduced genotype US 26 (PI 246388) which did not support aflatoxin production when colonized by aflatoxin producing strain of *A. flavus*. Since then other resistant sources have been developed. These sources of resistance include J11, PI 337394F, PI 337409, AR-1, -2, -3, -4, UF 71513, 55-437, Ah 7223, Faizpur 1-5 and Var. 27, these have been confirmed by testing over locations (Rao *et al.,* 1989). All these sources exhibit resistance to IVSCAF, FSCAF or PAC and they have been used extensively in breeding programs to develop desirable cultivars with resistance to *A. flavus* (Mixon1983a, 1983b, 1986).

Genotypes such as Shulamit and Darou IV for resistance to pod infection, PI 337394 F, PI 337409, GFA 1, GFA 2, UF 71513, Ah 7223, J 11, Var 27, U 4-47-7, also Faizpur, and Monir 240-30 for resistance to in vitro seed colonization by *A. flavus* (IVSCAF); and U 4-7-5 and VRR 245 for resistance to aflatoxin production (Upadhyaya *et al*.,

2000). Others such as AR-1, -2, -3, and -4 have been used as resistance sources in Thailand, and drought-resistant line 55-437 has been used in Senegal (Rao *et al*., 1989)

Other sources include ICGV 88145 and ICGV 89104 which were released as improved germplasm lines in India at IC (Rao *et al*., 1995).

2.9 Breeding for *Aspergillus flavus* resistance

Breeding for resistance to *Aspergillus flavus* is predicated on availability of genetic variance for resistance in order to incorporate a gene or genes for resistance (Holbrook et al., 2009). It also depends on the existence of high-level stable resistance sources, reliable assessment methods and an understanding of the inheritance of the traits. The resistance to *A. flavus* is known to be quantitatively inherited and strongly influenced by environmental conditions. The resistance is polygenic and involves the integration of several physiological processes (Holland *et al*., 2003; Melanie, 2006).

2.9.1 Combining Ability.

The information about combining ability is important for making breeding strategies. The concept of Specific Combining Ability (SCA) and General Combining Ability (GCA) was introduced by Sprague and Tatum (1942). The information from combining ability provides guidelines for plant breeders to select parent lines to be used in breeding programs and to use promising cross combinations for further selection

General combining ability (GCA) of the line 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 line 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). Preliminary studies on combining ability using line x tester analysis on F_o seed indicated that Ah 7223 had significant negative GCA effects and therefore had good combining ability for seed-coat resistance. Variety J I1 on the other hand registered non significant GCA effects (Rao *et al*., 1989).

2.9.2 Heritabilty

Heritability is measured in two ways: broad-sense heritability (H^2) and narrow-sense heritability h^2. H^2 measures the full contribution of genes and is the relevant quantity for clinical risk assessment, because it measures our ultimate ability to predict phenotype from genotype, the proportion of phenotypic variance attributable to genetic causes (Wray and Visscher, 2008).

The h^2 on the other hand is meant to capture the "additive" contribution of genes to the trait: It is the maximum variance that can be explained by a linear combination of the allele counts. It is used to measure progress toward explaining the genetic basis of a trait because one can readily calculate the contribution of individual loci to h^2 (Wray and Visscher, 2008).

Estimates of heritability from segregating populations are useful in understanding the genetic consequences of hybridization and inbreeding. They can help the breeder in selecting and utilizing superior individuals from the population (Holland *et al*., 2003; Melanie, 2006).

In a study done at IC ICGV 91283 was reported to be resistant to seed infection in the field and seed colonization after artificial inoculation. The genotype ICGV 91283 was selected from a cross between U4-7-5 a resistant source and JL 24 known to be susceptible to seed infection and seed colonization by *A. flavus*. However the aflatoxin content in this line was not reported (Upadhyaya *et al.*, 2001). The results suggested that a low aflatoxin production trait could be transferred to other lines. Upadhyaya *et al.* (1997) on the other hand reported heritability estimates of 56 to 87% for pre-harvest seed infection. Utomo *et al.* (1990) reported broad-sense heritability estimates in F_2 derived F6 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. There were no significant correlations among types of resistance, and it was concluded that different genes controlled them. In another study done by Arunyanark *et al.,* (2010) heritability for seed infection ranged from (h^2=0.30 to 0.51), while those of aflatoxin contamination (h^2=0.30 to 0.51). In India, the heritability was 60% in a cross involving J 11 (resistant) and OG 43-4-1 (susceptible) and 59% in a cross between two resistant parents, J 11 and Ah 7223 (Upadhyaya *et al.*, 1997).

2.10 Types of resistance to *Aspergillus flavus* and aflatoxin production

The resistance to aflatoxin-producing fungi may be of three types: resistance to pod infection (pod wall); resistance to seed invasion and colonization (seed coat); and resistance to aflatoxin production (cotyledons) (Upadhyaya *et al.,* 2000). The type of resistance that may be present depends on the site at which the genotype is tested or cultivated. The value of a resistant source however depends upon the level and stability of its resistance. Resistance to pod infection has been reported to be highly variable and of a low level. Similarly, IVSCAF-resistance is not absolute and even the best sources show up to 15% seed colonization; only a few lines (J 11, PI 337394 F, and PI 337409) have shown stable resistance. For aflatoxin contamination, resistance levels are not very high (Anderson *et al.,* 1995). Highly significant genotype environment interaction effects have been observed for aflatoxin contamination (Upadhyaya *et al*., 2000).

2.10.1 Resistance to invitro seed colonization by *Aspergillus flavus* (IVSCAF).

The mechanisms of resistance to *Aspergillus* colonization and infection may relate to combinations of physical and chemical characteristics of the testa. However, this type of resistance depends upon the testa being complete and undamaged, and the conditional nature of this type of resistance limits its utility under field conditions. The undamaged shell of the peanut pod is a natural barrier to *A. flavus* invasion. Resistance of peanut pods to *A. flavus* invasion appears to be associated with shell structure (Zambettakis *et al*., 1975), the presence of antagonistic microflora in the shell (Kushalappa *et al*., 1976;

Mixon, 1980), and thick-walled parenchyma cells (Pettit *et al*., 1977). Therefore peanut pod resistance has not been used widely for germplasm screening.

Zambettakis *et al*., (198I) reported highly significant correlations between seed colonization in the laboratory and field infection of seed by *A. flavus* in 101 genotypes tested in several field trials in Senegal. However it should not be assumed that all IVSCAF-resistant genotypes will have resistance to seed infection by *A. flavus* in the field or that all IVSCAF-susceptible genotypes all show susceptibility to field infection by the fungus (Mehan, 1987).

2.10.2 Resistance to field seed colonization (FSCAF) and pre-harvest aflatoxin contamination *(PAC).*

The pre-harvest aflatoxin contamination (PAC) has been identified as the most serious challenge facing the groundnut industry. The evaluations of resistance to pre-harvest aflatoxin contamination are performed in the field where care must be taken to avoid damaging the seed and pod (Holbrook *et al*., 1994). *A. flavus* is not an aggressive pathogen and its ability to invade undamaged pods and seeds is strongly influenced by environmental conditions such as temperature and moisture during pod maturation and whether the soil contains high populations of toxigenic strains of *A.flavus.* Thus, field screening for pod resistant to invasion and aflatoxin production is difficult due to the large variability in *A.flavus* growth under field conditions, even with intensive management of soil water status and inoculum levels (Holbrook *et al*.,1994).

Artificial inoculation helps to ensure uniform testing conditions, which reduces the number of escapes and reduces variation in the data that could mask genetic differences (Holbrook *et al.,* 2009) as it provides uniform and high level inoculum. Mehan (1989) reported that genotypes could be evaluated for resistance to PAC by applying inoculum of an aflatoxigenic strain to soil around developing pods about 4 weeks before harvest. He found that genotypes resistant to IVSCAF had significantly lower levels of aflatoxin than genotypes susceptible to IVSCAF. A large-scale field screening technique was developed by Holbrook *et al*., (1994) to directly measure field resistance to PAC using subsurface irrigation in a desert environment to provide an extended period of drought stress in the pod zone but still keep the plant alive. Anderson *et al*. (1996) was also able

to develop a screening technique for use in the greenhouse. All these methods provide uniform, high-levels of inoculum and environmental conditions favourable to fungal development and aflatoxin production and they are valuable tools in the search for reliable resistant lines.

Nigam *et al*, (2009), reported ten germplasm lines (ICG 1859, ICG 1994, ICG 1326 (JII), ICG 3267, ICG 10094, ICG 3241, ICG 1422, ICG 3251, ICG 9820 and ICG 4160 as having resistance to pre-harvest seed infection or aflatoxin production. Others that were identified include 55-437, Tamnut 74, and PI 365553. Three breeding lines, ICGV 87094, ICGV 87084 and ICGV 87110 were found to be resistant to pre-harvest seed infection when tested in Niger, Senegal and Bukina Faso (Waliyar *et al.,* 1994).

Mehan, (1989) evaluated IVSCAF resistant varieties (UF 71531,J11, 55-437,Ah 7223, Var 27 and U 4-47-7) for resistance to field infection of seed by *A.flavus* and aflatoxin contamination and found them to be 4.2-19.1% lower than susceptible varieties and recommended that cultivar J11 and C 55-437 could be useful in minimizing aflatoxin contamination in some environments.

Blankenship *et al*., (1985) reported that four genotypes (GFA 1, AR 3, UF 77316 and UF 791041) resistant to seed colonization and the cultivar florunner were highly susceptible to aflatoxin contamination when grown under late season drought.

Davison *et al*., (1983) found Sunbelt Runner and Florunner which were reported to be resistant to *A. flavus* seed colonization to be susceptible to aflatoxin contamination. Mehan and McDonald, (1984) reported lower levels of infection and aflatoxin contamination when IVSCAF resistant genotypes (Ah 7223, J11, P1 337394F and UF 71513) were evaluated in the field.

2.10.3 Resistance to aflatoxin production.

This type of resistance does not support production of aflatoxin when seeds are colonized by toxigenic strains of *Aspergillus* spp. A laboratory inoculation method was developed at ICRISAT to screen peanuts for resistance to aflatoxin production (Mehan and McDonald, 1980). The method is similar to the IVSCAF screening procedure, but the seeds' testae were scarified with a sterile needle and seeds were tested for aflatoxin content. This method has been used in several post-harvest screening programs.

Although no germplasm highly resistant to aflatoxin accumulation has been found, genotypes do differ in the concentrations of aflatoxin they support during infection by *A. flavus* (Mehan *et al.,* 1986a, 1991; Dange *et al*., 1989; Ghewande *et al.,* 1989).

2.10.4 Screening genotypes for *Aspergillus flavus* resistance and aflatoxin production.

There are two requirements for developing cultivars with resistance to pre-harvest aflatoxin contamination. First, there must be genetic variance for resistance in order to incorporate a gene or genes for resistance into cultivars. The second requirement is the availability and use of reliable and efficient screening techniques to identify plants containing genes for resistance (Holbrook *et al.,* 2008; Holbrook *et al.,* 2009). An effective screening technique is therefore helpful for identification of sources of resistance and could aid in studying resistant mechanisms (Liang *et al.,* 2006). Artificial inoculation is frequently used when screening germplasm for resistance as it helps to ensure uniform testing conditions, which reduces the number of escapes and reduces variation in the data that could mask genetic differences (Holbrook *et al.,* 2009).

Mixon and Rogers (1973) developed a laboratory inoculation method for screening groundnut genotypes for resistance to *A. flavus* invasion and colonization of rehydrated, mature, sound, stored seeds. They selected two valencia-type genotypes, PI 337394Fand PI 337409, that showed a high level of resistance to in vitro seed colonization by *A.flavus.* Six more breeding lines (GFA 1, GFA 2, AR 1, AR 2, AR 3, and AR 4) were later reported resistant (Mixon, 1986).

Since then thousands of peanut germplasm lines have been screened at ICRISAT and other research institutions (Mehan and McDonald, 1980; Zambettakis *et al.,* 1981; Mixon, 1986; Ghewande *et al*., 1989). At ICRISAT alone 850 germplasm accessions were screened for their reaction to seed invasion and colonization by *A. flavus.* The percentages of seeds of different genotypes with sporulating colonies of *A. flavus* ranged from 6 to 100%. Genotypes with 15% or fewer seeds colonized were regarded as resistant. Resistance of the three genotypes, PI 337394F, PI 337409, and UF 71 513, was confirmed, and six new sources of resistance (Ah 7223, J 1 I, **U** 4-47-7, Var, 27, Faizpur, and Monir 240-30) were identified. It was observed that absolute percentage incidence

of seeds colonized by *A. flavus* varied considerably for specific genotypes within trials in the same season, and between seasons (Mehan *et al*., 1991).

Blankenship *et al* (1985) evaluated groundnut genotypes to *A. flavus* infection under laboratory conditions and found that all were resistant. However, these genotypes when evaluated under field conditions by imposing the drought and temperature conditions were found to be susceptible. While a study conducted three years later by Kiran *et al* (1988) found that high yielding lines were susceptible to invasion by *A. flavus* and aflatoxin contamination after he evaluated 53 groundnut cultivars. The results also indicated that line OG 35-1 showed highest resistance with low yield potential and J-11 showed resistance to aflatoxin production and moderately susceptible to *A. flavus* invasion.

A year later Waliyar and Bockelee-Morvan (1989) reported significant varietal differences in levels of seed invasion by *A. flavus* at harvest. They also showed that under field conditions, resistance was positively correlated with *in vitro* seed colonization. The commercially grown CVS 55-437, 73-30 and 73-33 exhibited moderate to high levels of resistance to infection. The cultivar resistant to seed invasion had lower frequency of *A. flavus* counts in their rhizosphere compared to those of susceptible cultivars.

Desai *et al* (1991) tested 39 different groundnut varieties and breeding lines to *A. flavus* infection and found that tested groundnuts were significantly differed in infection and aflatoxin production, infection and seed colonization were strongly correlated and no correlation was found between infection and aflatoxin content.

Ghewande *et al* (1993) screened 38 groundnut genotypes, under artificially inoculated conditions and reported that there was a significant correlation between infection, colonization and aflatoxin content. However, there was no correlation between sugar content and infection, colonization and aflatoxin content. Waliyar *et al.*, (1994) on the other hand evaluated 25 groundnut lines and reported that 55-437, J-11 and PI337394 were least infected, while ICGV-87084, ICGV-87094, ICGV-87110 were resistant and var-29 showed a high percentage of infection with low aflatoxin contamination.

Anderson *et al* (1995) evaluated 12 potentially resistant genotypes for pre-harvest aflatoxin contamination and found that none of the genotypes were more resistant to pre-harvest aflatoxin contamination than the genotype florunner. In the same year Rao *et al* (1995) assessed the Spanish groundnut germplasms ICGV-88145 and ICGV-89104 for seed colonization by *A. flavus* under artificial inoculation conditions and it averaged 22.2 and 24.0% compared with 15.6%.

Holbrook *et al* (2000) evaluated 20 genotypes of groundnut having drought tolerance and susceptibility. These results indicated that susceptible genotypes had greater pre-harvest aflatoxin contamination and drought tolerant genotypes had less pre-harvest aflatoxin contamination.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental site

The research was conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) at Chitedzi research station ($13^{o}59´S$, $33^{o}38´E$) in Lilongwe Malawi. It was conducted in the 2012/2013 growing season from August 2012 to August 2013. The research was a greenhouse experiment divided into two phases.

3.2 Experimental material

The first phase of the research involved the use of experimental material comprising 10 groundnut genotypes previously evaluated by ICRISAT and known for their resistance and susceptibility to *A. flavus*. The resistant genotypes (Table 1) were Ah 7322, 55-435 and J11, while the susceptible genotypes (Table 2) comprised of Chalimbana, Luena, Chishango, MGV-4, MGV-5, Katete and Natal common. The second phase involved the screening of the 21 F_1 crosses for resistance to *A. flavus*.

3.3 Experimental designs

During hybridization the genotypes were crossed in a 3×7 North Carolina Mating Design II to generate F_1 hybrids. Completely Randomized Design (CRD) with unequal replications was used during the screening of the F_1 crosses.

3.4 Planting and agronomic practices

Plants were grown in PVC (polyvinyl chloride) pots measuring 35cm in diameter. The pots had 4 to 6 holes at the base. Four pots where used for each susceptible genotype and six pots for each resistant genotype. The pots were filled with dark vertisols and the soil had to be air-dried and sieved with a 4mm mesh sieve before use. A total of four, Thiram 80WP treated healthy seeds were sown in each pot at a depth of 1 to 2 cm and the same number of plants was retained after germination. Planting was done on 31st August, 13th and 14th September 2012 to synchronize the flowering in the genotypes and replanting was also done for genotypes that had poor germination. The pots were watered regularly and plants protected from insect pests such as aphids by spraying with karate and

Ascomec 1.8 EC on a weekly basis, while Bravo (Chlorothalonil 54%) was used after germination to protect the groundnuts from diseases such as damping off.

Table 1: Sources of resistance to *A. flavus* used in the study

Source of resistance	Botanical group	PI number	Type of Resistance	Country where used	References
55-437 Cultivar	Spanish bunch	360862	FSCAF	Senegal	Waliyar and Bockelee-Morvan (1989) Mehan, (1989), Diaye (1990), Faye *et al*., 2009
		363058			Zambettakis *et al.* (1981)
		407492	IVSCAF	Senegal	Zambettakis *et al.* (1981)
Ah 7223 Cultivar	Spanish bunch	590343	FSCAF	India	Mehan *et al.* (1986b, 1987)
			IVSCAF	India	Mehan and McDonald (1980) Ghewande *et al.* (1989)
J 11 Cultivar	Spanish bunch		FSCAF	India	Mehan *et al.* (1986b, 1987), Narasimhulu, (2007), Mehan, (1989)
			IVSCAF	India	Mehan and McDonald (1980) Ghewande *et al.* (1989)
				USA	Kisyombe *et al.* (1985)

IVSCAF= *Invitro* seed colonization by *A. flavus* **FSCAF**=Field seed colonization by *A. flavus*

Table 2: Susceptible genotypes used in the study

Genotype	Botanical group	Year released	Potential yield MT/Ha and kernel deiscription	Other characteristics (Altitude, duration, oil content, oleic/Linoleic (O/L) ratio, and recommended uses)	References
MGV-4 (CG 7)	Virginia (cultivar)	1992	2.0-3.0 Large tan kernels	Medium, uniform tan kernels Agro ecological regions I,II and III 120-140 days, 48-50% oil O/L ratio 2.1 Confectionery	Chiyembeka, *et al*, (2000). Ross and De klerk, (2012)
Chishango (ICGV-SM 90704) (Serenut 2)	Virginia (Cultivar)	Approx. 2005	2.0 Medium, uniform, tan-pink	120 days, 47% oil, O/L ratio 1.5 confectionery.	Chiyembeka, *et al*, (2000). Ross and De klerk, (2012)
N/common	Spanish (cultivar)	1976	0.5-1.5	400-1000m, 90- 100 days 45-48% oil, no seed dormancy confectionery.	Herselman, (2003), Ross and De klerk, (2012)
Luena (JL24)	Spanish (cultivar)	1998	1.0-2.0 Small tan kernels	400-1000m, 90- 100 days 48% oil, O/L ratio 1.1, no seed dormancy, confectionery.	Ross and De klerk, (2012)
Chalimbana	Virginia (Cultivar)	1950	2.0 Large, tan kernels	800-1200m, 140- 160days 45-48% oil, O/L ratio 1.6, confectionery.	Ross and De klerk, (2012) Syamasonta, (1992)
MGV-5	Virginia (Cultivar)	Approx. 2008	2.0 Large tan kernels	120 days, 48% oil, O/L ratio 1.5 No seed dormancy confectionery.	Ross and De klerk, (2012)
Katete	Spanish (Cultivar)	-	Small tan kernels	90- 100 days No seed dormancy confectionery.	Unpublished data, 2010

3.5 Hybridization activities

During hybridization proper care was taken to avoid contamination of genotypes as well as injury to the selected buds. The emasculation exercise was carried out between in the afternoons with temperatures ranging from 29 to 40^{o}C. Pollination was done in the morning with temperatures ranging from 20 to 28^{o}C. In both activities 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 was ready for pollination. Since the hybridization activities occurred in the hot season were temperatures were ranging from 29 to 40^{o}C a humidifier was used for 2 to 3 hrs daily to help raise the humidity. The hybridization activities lasted for three weeks from 11th October to 2nd November, 2012.

Figure 3: Hybridization activities in the green house

The harvesting of the crosses was done 70 days after the last pollination from 14th to 16th January 2013. Before harvesting of the plants was done, watering was suspended for three days and during harvesting plants where carefully lifted to avoid pod loss. 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).The hybridization success rate (Fig. 4) was calculated as a ratio of the number of successful hybrid pegs to the number of pollinations made expressed as a percentage.

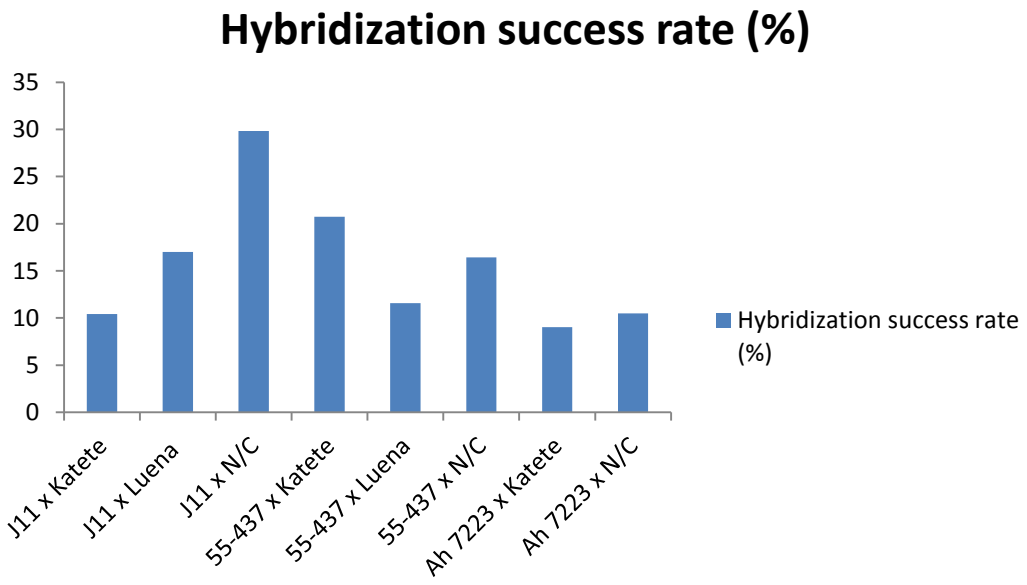

Figure 4: Hybridization success rate for set A and B

3. 6 Screening activities

The second phase of the research was planted on 29th January 2013. This phase involved the screening of 21 F_1 crosses for *Aspergillus flavus* resistance. Before planting the seeds, possible dormancy had to be broken for the Virginia type Chalimbana, Chishango, MGV-5 and MGV-4 using Ethephon (Ethephon 21.7%). Seeds were soaked in Ethephon for 1min at a rate of 1 ml of distilled water per ml of solution (Kapur *et al.*, 1990). A total of four healthy seeds from each cross were sown at 1 to 2 cm in dark

dambo loamy soil in each pot and one pot per replication was used. Three healthy and vigorous seedlings in each pot were to be retained after germination. However this was not the case as the germination was very poor. Therefore all Virginia varieties were removed from the study and in order to meet the NCDII requirements of each male mated to each female, successful crosses were grouped into sets.

3.6.1 Inoculation activities

The Inoculation with toxigenic strains of *A. flavus* was done at mid bloom (70 days) after planting on 9th April, 2013. The modified method developed by Will *et al*., (1994) was used and this involved the use of sorghum as a carrier for the fungus. A total of 3.5 g of inoculum was incorporated into the soil per pot. The sorghum served as a food source for the fungus. The *A. flavus* strains that were used were isolated from Zambian soils under the Aflatoxin Mitigation project under the Feed the Future program. The inoculation technique helped to reduce the inherent variability of pre-harvest aflatoxin contamination (Holbrook *et al.*, 2008).

3.6.1.1 Inoculum preparation and application method.

A total of 300 g of sorghum seed was weighed and placed into a one litre beaker. The beaker and its contents were autoclaved for 15 minutes at 121ºC and also at the same time distilled water was sterilized in a one litre Erlenmeyer flask. The cooled sterilized distilled water was then added to the beaker containing the sorghum completely covering the seeds and was left overnight (24hrs). The following morning the excess water from the sorghum seed was drained off and the beaker was covered with non-absorbent cotton plug and aluminium foil. The beaker and its contents were autoclaved again for 15 minutes at 121^{o}C.

The sterilised distilled water was poured on the growth media with *Aspergillus* strains (a petri dish with Aspergillus culture). The concentrated *A. flavus* spore solution was then diluted by getting 1 ml of the spore solution in 9 litres of sterilized distilled water. The number of spores/ml (total cell count in 4 squares x 2500 x dilution factor) was 2.2 x 10^6 spores/ml using a haemocytometer.

A total of 10 ml of spore solution from the petri dish was used to sprinkle on the 300g of sterilized sorghum seed and the contents agitated once every day for two days. The

inoculated sorghum seed was then incubated at room temp in the fume hood for 5 days for the fungus to fully colonise the seed.

After 5 days the contents of the beaker were poured out and air-dried for two days. The inoculum was then sprinkled on the soil in the flower pots and mixed evenly with the soil, care was taken to ensure the pods were not disturbed or damaged. A total of 3.5g of inoculum was applied per pot. The calculations of the amount of inoculum to apply was based on 375kg/ha inoculum applied in the field (Hoolbrook 2008). The total area used for the experiment was 2.3187m^2.The unused inoculum was maintained at room temperature in sterile plastic bags to be used for future experiments.

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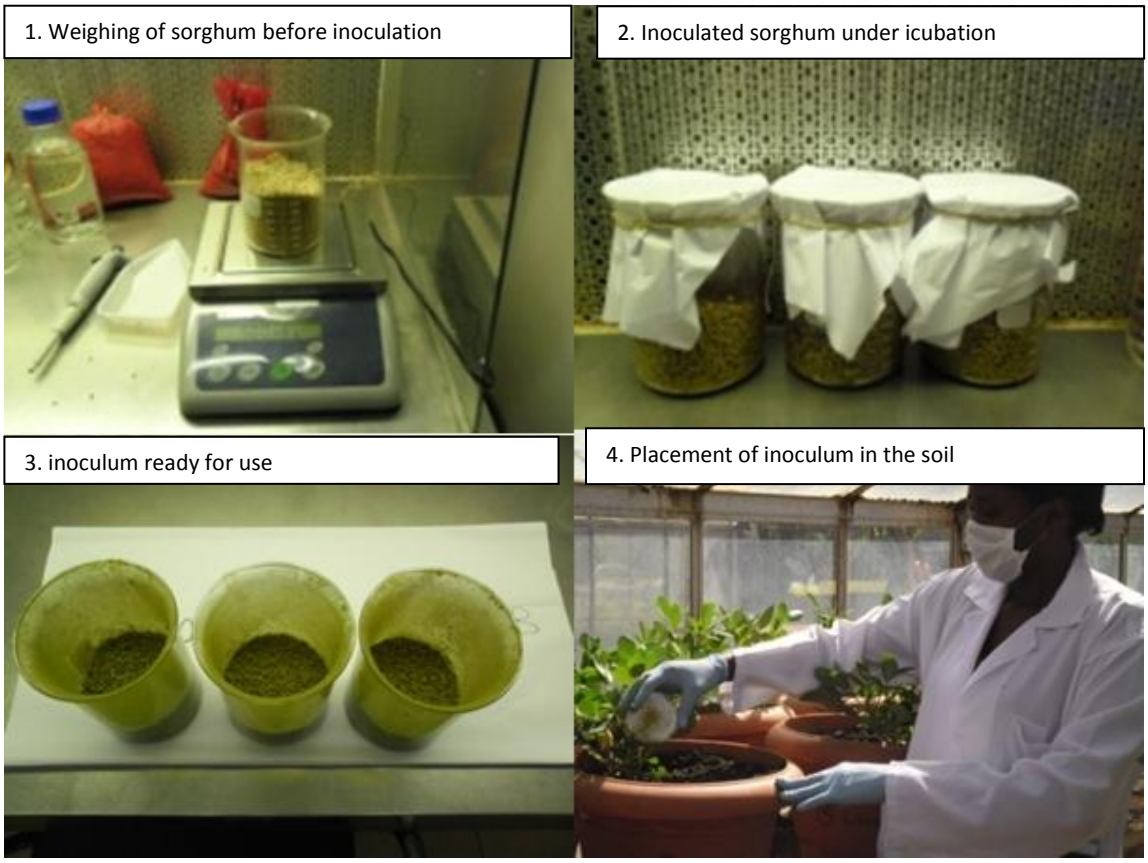

Figure 5: Inoculum preparation in the laboratory and inoculum activities in the green house

3.7 Laboratory activities

3.7.1 Determination of fungal soil populations.

The soil samples were taken prior to harvesting, one kilogram of soil was collected from each pot from a depth of 5cm. The soil was then thoroughly mixed and taken to the laboratory for analysis. From each sample 3.3 g of soil was weighed and vortexed in 10ml of 0.2% water agar. The soil suspension for each sample was dilution plated onto three plates (0.2 ml/plate) of Modified Dichloran-Rose Bengal medium (MDRB) and incubated for 3 days at 37^{o}C. Soil suspensions were dispensed and spread using a spiral plater (Spiral Systems, Bethesda, MD, USA). Colonies of Species from *Aspergillus* section *flavi* were enumerated and identified under stereomicroscope directly from the dilution plates as described by Horn and Dorner (1998). Only colonies exhibiting typical *A. flavus* morphology (Klich, 2002) were counted. Microbial propagule densities (colony-forming units) were calculated on a soil dry weight basis and transformed on a log(10) scale.

Figure 6: Determination of fungal colony forming units in the laboratory

3.7.2 Determination of Aflatoxin concentration levels using ELISA

Aflatoxin was quantified at ICRISAT, Malawi by using an enzyme linked immuno-sorbent assay (ELISA: Transia Society, France) technique (Waliyar *et al*., 1994). For each analysis, 20 g of unsorted groundnut seed was randomly picked and ground in an electric mill. 100ml of 70% methanol solution (v/v) solution containing 0.5% KCL was added to 20g of the ground groundnuts and was ground further. The mixture was then transferred to a 250 ml conical flask on an orbital shaker at 300 rpm for 30 minutes. The mixture was filtered through three whatman filter papers. The supernatant obtained from each sample was used for aflatoxin B_1 analysis by assaying in duplicate wells. The colour intensity obtained during analysis was inversely proportional to the amount of aflatoxins present in the samples. The results were obtained by reading the absorbance of the wells with a micro-plate reader using 450 nm filter.

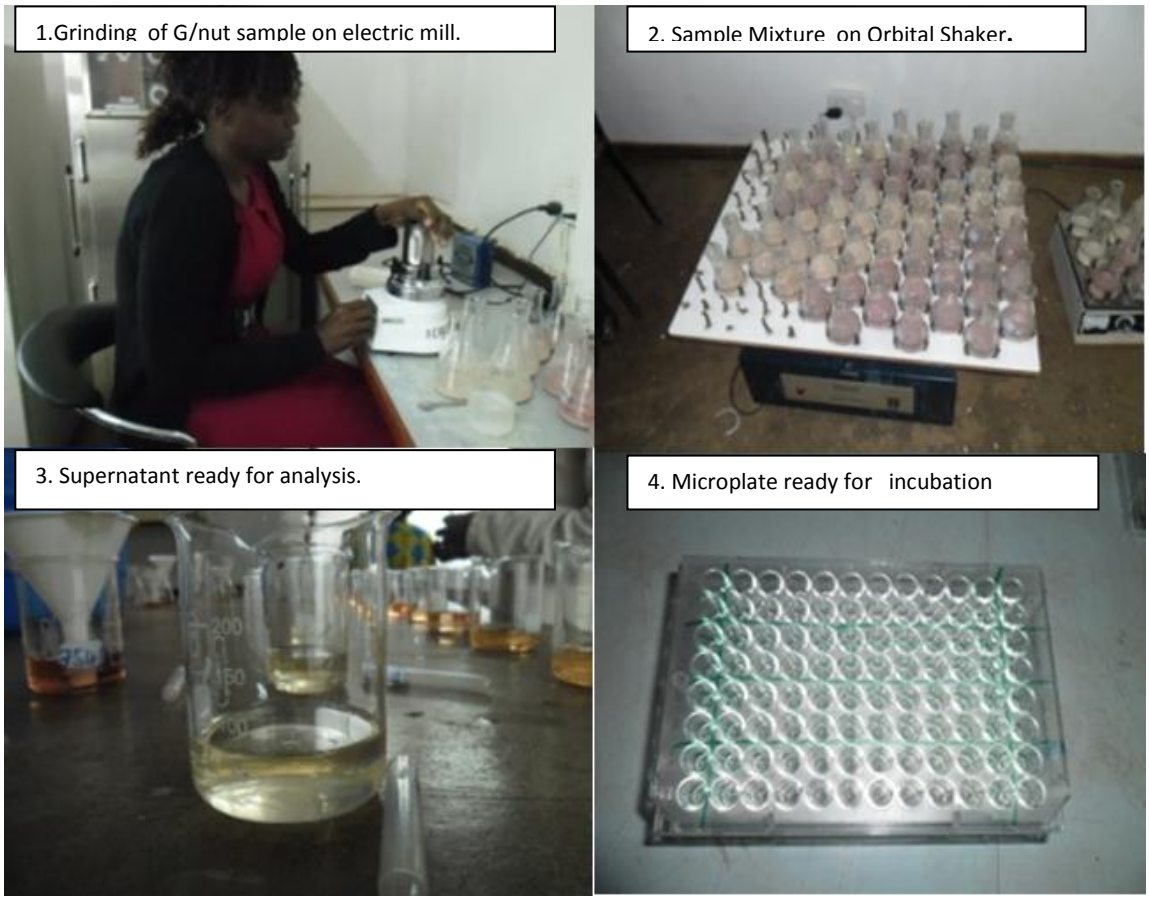

Figure 7: Determination of aflatoxin levels in the laboratory

3.8 Statistical analysis of data

The data was subjected to one way analyses of variance (ANOVA) using GENSTAT 14th version computer software package according to a completely randomized design (CRD). Mean squares were partitioned into difference due to male parents and female parents, which was attributed to general combining ability (GCA), and differences due to male x female interaction, which was attributed to specific combining ability (SCA). Fishers protected least significant difference (LSD) (Gomez and Gomez, 1984) test ($P<0.05$) was used to compare treatment means in case the *F*- statistic was significant. The genetic analyses of progenies from NCD II mating scheme was done according to Singh and Chaundhary (1985).

The following linear model was followed.

$$Y_{ijk} = \mu + M_i + F_j + MF_{ij} + R_k + \varepsilon_{ijk}$$

where,

Y_{ijk} = observed trait value, μ = mean effect, M_i = effect of the i^{th} male, F_j = effect of the j^{th} female, MF_{ij} = effect of interaction between i^{th} male and j^{th} female, R_k = effect of k^{th} replication and ε_{ijk} = experimental error

Bakers ratio was used to determine the additive and non-additive variances (Baker, 1978). Bakers ratio = (σ^2gca male + σ^2gca female)/ (σ^2gca male + σ^2gca female + σ^2sca).

The genetic variances were estimated by equating the variances to the respective mean squares from the ANOVA table shown below.

Table 3: ANOVA of NCD II (Comstock and Robinson, 1948) used in set A and B

Source of variation	df	MS	EMS Variance Components
Replications	r-1		
Males (M)	m-1	MS_m	$\sigma^2 + r\sigma^2_{f*m} + rf\sigma^2_m$
Females (F)	f-1	MS_f	$\sigma^2 + r\sigma^2_{f*m} + rm\sigma^2_f$
Males x Females (MxF)	(m-1)(f-1)	MS_{m*f}	$\sigma^2 + r\sigma^2_{f*m}$
Error	(r-1)(mf-1)	MS_E	σ^2_e
Total	mf (r-1)		

The variance components were calculated as follows

Males $\hat{\sigma}^2_m = \dfrac{MS_m - MS_{f*m}}{rf}$

Females $\hat{\sigma}^2_f = \dfrac{MS_f - MS_{f*m}}{rm}$

Male x female interaction $\hat{\sigma}^2_{m*f} = \dfrac{MS_{m*f} - MS_E}{r}$

Male additive variance $\hat{\sigma}^2_A = 4\hat{\sigma}^2_m$

Female additive variance

Dominance variance $\hat{\sigma}^2_D = 4\hat{\sigma}^2_{f*m}$

The heritability was calculated using variance ratios as follows

Narrow sense heritability

$$h^2 = \frac{\hat{\sigma}^2_A}{\hat{\sigma}^2 + \hat{\sigma}^2_A + \hat{\sigma}^2_D}$$

CHAPTER FOUR

4.0 RESULTS

4.1 Aflatoxin contamination levels and *Aspergillus flavus* colony forming units (CFU) for the F_1 crosses for Set A.

Mean squares and means for crosses for aflatoxin contamination levels and CFU are shown in Table 4 and Table 5 respectively.

4.1.1 Aflatoxin contamination levels (ppb) among the F_1 crosses

The analysis of variance revealed significant ($P<0.05$) differences for aflatoxin contamination levels among the crosses (Table 4). The mean differences in the levels of aflatoxin contamination among the crosses are shown in Table 5. The lowest mean was obtained from 55-437 x Katete (0.2ppb) while the highest was from J11 x N/common with aflatoxin levels of 7.3ppb. Most of the crosses registered aflatoxin levels lower than 2ppb and these included 55-437 x Katete (0.2ppb), J11 x Katete (1.3ppb) and J11 x Luena (1.83ppb), while those that registered 2ppb and above included 55-437 x Luena (2.70ppb), 55-437 x N/Common (2.90ppb) and J11 x N/Common (7.3ppb).

4.1.2 *Aspergillus flavus* colony forming units (CFU)

Significant ($P<0.01$) differences in propagule densities of *Aspergillus flavus* (Table 4) were observed. The means (Table 5) showed that the lowest densities were found in soils of J11 x Luena log(10) 4.11 cfu g^{-1} soil, followed by J11 x Katete log(10) 4.13 cfu g^{-1} soil, 55-437 x Luena had log(10) 4.58 cfu g^{-1} soil, J11 x N/common had log(10) 4.52 cfu g^{-1} soil and 55-437 x N/Common had log(10) 4.59 cfu g^{-1} soil, while the highest density log(10) 4.78 cfu g^{-1} soil was found in soils of 55-437 x Katete.

Table 4: Mean square for Aflatoxin contamination levels and Log CFU for the F_1 crosses for set A

Source of variation	d.f.	Aflatoxin	Log CFU
Crosses	5	18.08***	0.22**
Female (GCA_f)	2	29.23***	0.16*
Male (GCA_m)	1	10.71**	0.48**
Female.Male ($_{F*m}$)	2	10.62**	0.12
Error	8	0.80	0.03
CV%		**33.10**	**4.20**

Table 5: Means for Aflatoxins contamination levels and Log CFU for the F1 crosses for set A

Entry	Aflatoxin (ppb)	Log(10) cfu g^{-1} soil
55-437 x Katete	0.2	4.78
55-437 x Luena	2.7	4.36
55-437 x N/Common	2.9	4.59
J11 x Katete	1.3	4.13
J11 x Luena	1.83	4.11
J 11 x N/Common	7.3	4.52
Mean	**2.70**	**4.40**
CV%	**33.10**	**4.20**
$LSD_{0.05}$	**1.69**	**0.35**

4.1.3: General Combining Ability (GCA) and Specific Combining Ability (SCA) effects for resistance to Aflatoxin contamination by *Aspergillus flavus*

Analysis of variance for the male GCA, female GCA and SCA for aflatoxin contamination levels are presented in Table 4. Estimates for general combining ability (GCA) and specific combining ability (SCA) effects are presented in Table 6.

Significant ($P<0.05$) GCA males, GCA females and SCA were obtained (Table 4) for *A. flavus* resistance. Significant ($P<0.05$) positive and negative GCA effects for male and female parents were obtained in set A. Male parents 55-437 and J11 had (-0.77) and (0.77) respectively, while female parents Katete and N/Common registered (-1.95) and (2.39) respectively.

Significant ($P<0.05$) negative GCA effects for females were obtained for Katete (-1.95) while for N/Common significant ($P<0.01$) positive GCA effects (1.19) were recorded.

Significant ($P<0.05$) negative SCA effects were observed for crosses 55-437 x N/Common (-1.43) and J11 x Luena (-1.21), while positive and significant ($P<0.05$) SCA effects were obtained for 55-437 x Luena (1.21), 55-437 x Katete (2.92) and J11 x N/Common (1.43).

Table 6: Estimates of GCA and SCA effects for resistance to Aflatoxin contamination by *Aspergillus flavus* for set A

Males	**GCA**
55-437[A]	-0.77*
J11[A]	0.77*
Females	
Katete[B]	-1.95***
Luena[B]	-0.44
N/Common[B]	2.39***
F_1 crosses	**SCA**
55-437 x Katete	0.22
55-437 x Luena	1.21*
55-437 x N/Common	-1.43*
J11 x Katete	-0.22
J11 x Luena	-1.20*
J11 x N/Common	1.43*
SE_m	**0.36**
SE_f	**0.30**
SE_{mf}	**0.52**

4.1.4 Estimation of heritability for resistance to Aflatoxin contamination by *Aspergillus flavus*

Estimates revealed that the environmental variance contributed less than the genotypic variance to the total phenotypic variance for resistance to aflatoxin contamination by *A. Flavus* as shown in Table 7. The additive dominance value was found to be 2.0, while that of the dominance component was 13.1. The table also shows that the dominance components of genetic variation were seven times more than that of the additive components. The narrow sense heritability (h^2) was 0.1 and the Bakers ratio was 0.2.

Table 7: Estimates of components of genetic variance and narrow sense heritability (h^2) for resistance to Aflatoxin contamination by *Aspergillus flavus* for set A

Variance components	
$\hat{O}^2p$	15.9
$\hat{O}^2g$	15.2
$\hat{O}^2a$	2.0
$\hat{O}^2d$	13.1
$\hat{O}^2e$	0.8
$\hat{O}^2a$: $\hat{O}^2d$	1:7
h^2	0.1
Bakers ratio: $\hat{O}^2gca_m + \hat{O}^2gca_f / \hat{O}^2gca_m + \hat{O}^2gca_f + \hat{O}^2sca$	0.2

KEY: $\hat{O}^2p$ = total phenotypic variation, $\hat{O}^2g$ = genotypic variation, $\hat{O}^2a$ = additive variance , $\hat{O}^2d$ = dominance variance, $\hat{O}^2e$ = environmental variance, $\hat{O}^2a$: $\hat{O}^2d$ = additive to dominance ratio, h^2 = Narrow sense heritability, $\hat{O}^2gca_m$ =variance for gca_m, $\hat{O}^2gca_f$ = variance for gca_f , $\hat{O}^2sca$= variance for sca

4.1.5 Correlation analysis between aflatoxin contamination levels and *Aspergillus flavus* colony forming units (CFU).

The results of the simple correlation analysis done for set A revealed that the aflatoxin contamination levels were positively correlated (r=0.48) to the *A. flavus* colony forming units (CFU).

4.2 Aflatoxin contamination levels and *Aspergillus flavus* colony forming units (CFU) for the F_1 crosses for set B.

Mean squares for aflatoxin contamination levels and log CFU for crosses are shown in Table 8. The means for the aflatoxin contamination levels and log CFU are tabulated in Table 9.

4.2.1 Aflatoxin contamination levels (ppb) among the F_1 crosses

The analysis of variance revealed highly significant ($P<0.001$) differences for aflatoxin contamination levels among the crosses (Table 8). The mean differences (Table 9) revealed Ah 7223 x N/Common (0.77ppb) as having the lowest amount of aflatoxin, while the highest was from J11 x N/common with aflatoxin levels of 7.30ppb. The other two crosses J11 x Katete and Ah 7223 x Katete registered 1.30ppb and 2.00ppb aflatoxin levels respectively.

Table 8: Mean square for Aflatoxin contamination levels (ppb) and Log CFU for set B

Source of variation	d.f.	Aflatoxin	Log CFU
Crosses	3	27.27***	0.09
Female (GCAf)	1	17.04**	0.16
Male (GCAm)	1	25.52***	0.05
female.male (F*m)	1	39.24***	0.06
Error	8	0.81	0.04
CV%		**31.70**	**4.80**

Table 9: Means for Aflatoxin contamination levels (ppb) and Log CFU for set B

Entry	Aflatoxin (ppb)	Log(10) cfu g^{-1} soil
Ah 7223 x Katete	2.00	4.15
Ah 7223 x N/Common	0.77	4.25
J11 x Katete	1.30	4.14
J 11 x N/Common	7.30	4.51
Mean	**2.80**	**4.30**
CV%	**31.70**	**4.80**
$LSD_{0.05}$	**1.70**	**0.38**

4.2.2 General combining abilty (GCA) and Specific combining ability effects for resistance to Aflatoxin contamination by *Aspergillus flavus*.

Analysis of variance for the male GCA, female GCA and SCA (Table 8) revealed significant (P<0.05) differences. Estimates for general combining ability (GCA) and specific combining ability (SCA) effects are presented in Table 10. Significant (P<0.01) positive and negative GCA effects for male and female parents were obtained. Highly significant (P<0.01) positive and negative SCA effects in the F_1 crosses were recorded.

Significant (P<0.01) positive and negative GCA effects for male and female parents were obtained. Male parents Ah 7223 and J11 had (-1.46) and (1.46) respectively, while female parents Katete and N/Common registered (-1.19) and (1.19) respectively.

Significant (P<0.01) negative SCA effects for the crosses were observed with J11 x Katete (-1.81) and Ah 7223 x N/Common (-1.81). The following crosses Ah 7223 x Katete (1.81) and J11 x N/Common (1.81) all exhibited positive and significant SCA effects.

Table 10: Estimates of GCA and SCA effects for resistance to Aflatoxin contamination by *Aspergillus flavus* for set B

	Effects
Males	**GCA**
Ah 7223[A]	-1.46**
J11[A]	1.46**
Females	
Katete[A]	-1.19**
N/Common[B]	1.19**
F_1 crosses	**SCA**
Ah 7223 x katete	1.80***
Ah 7223 x N/common	-1.81***
J11 x Katete	-1.81***
J11 x N/Common	1.81***
SE_m	**0.37**
SE_f	**0.37**
SE_{mf}	**0.52**

4.2.3 Estimation of heritability for resistance to Aflatoxin contamination by *Aspergilus flavus.*

The estimates revealed that the environmental variance contributed less than the genotypic variance to the total phenotypic variance for resistance to aflatoxin contamination by *A. flavus* as shown in Table 11. Furthermore the value for the additive dominance components was found to be 9.6, while that of the dominance component was positive (51.2). The table also shows that the dominance components of genetic

variation exceeded the additive components by 5 times. The narrow sense heritability was found to be 0.2 and the Bakers ratio was 0.3.

Table 11: Estimates of components of generic variation and narrow sense heritability (h^2) for resistance to aflatoxin contamination by *Aspergillus flavus* for set B

Variance components	
$\hat{O}^2p$	61.6
$\hat{O}^2g$	60.8
$\hat{O}^2a$	9.6
$\hat{O}^2d$	51.2
$\hat{O}^2e$	0.81
$\hat{O}^2a$: $\hat{O}^2d$	1:5
h^2	0.2
Bakers ratio	
$\hat{O}^2gca_m + \hat{O}^2gca_f / \hat{O}^2gca_m + \hat{O}^2gca_f + \hat{O}^2sca$	0.3

KEY: $\hat{O}^2p$ = total phenotypic variation, $\hat{O}^2g$ = genotypic variation, $\hat{O}^2a$ = additive variance ,$\hat{O}^2d$ = dominance variance, $\hat{O}^2e$ = environmental variance, $\hat{O}^2a$: $\hat{O}^2d$ = additive to dominance ratio,

h^2 = Narrow sense heritability, $\hat{O}^2gca_m$ =variance for gca_m, $\hat{O}^2gca_f$ = variance for gca_f, $\hat{O}^2sca$= variance for sca

4.2.4 Correlation analysis between aflatoxin contamination levels and *Aspergillus flavus* colony forming units (CFU).

The results of the simple correlation analysis done for set B revealed that the aflatoxin accumulation levels were positively correlated (r=0.27) to the *A. flavus* colony forming units (cfu).

CHAPTER 5

5.0 DISCUSSION

5.1 Relationship between aflatoxin contamination levels in the groundnuts and Aspergillus flavus population densities in the soil.

In this study the relationship between *A. flavus* population densities in the soil and aflatoxin contamination in the seed was examined. The population densities for *A. flavus* enumerated from the soil samples ranged from 4.11 to 4.78 cfu g^{-1} soil. The densities obtained were compared to those reported by Zablotowicz *el at* (2007) in two seasons in 2001 and 2007. These researchers reported *A. flavus* densities from 12 sites ranging from 2.0 to 3.8 cfu g^{-1} soil in 2001 and 2.3 to 4.3 cfu g^{-1} soil in 2007 from 15 sites. The population densities obtained from the study are in agreement with those obtained by Zablotowicz *el at* (2007) and they further agree with those reported by Horn and Dorner (1998) for soils from warm temperate to semi tropical climates used for peanut, corn and cotton.

The aflatoxin contamination levels obtained from the study had 90% B_1 contamination levels less than 4ppb ranging from 0.2ppb to 2.90ppb with only one registering above 4ppb. The results obtained are however not in agreement for those obtained by Monyo *et al.*, (2012). Their results revealed 46% and 23% of the total samples, from 2008 to 2009, respectively, had B_1 contamination levels greater than 4 ppb, and those above 20 ppb were 21% for 2008 and 8% for 2009, respectively.

The total aflatoxin concentration levels obtained from 90% of the samples are lower than those permitted by international agencies. The maximum allowable levels in the United States is 20ppb and the European Union has an allowable level of 4ppb total aflatoxins and under 2ppb aflatoxin B_1 (Holbrook *et al.*, 2008). These regulations are directed at minimizing human exposure to aflatoxins (Cotty and Garcia, 2007). In Zambia there is no set minimum level of aflotoxins for groundnuts however a standard exists for maize. The requirements for the Food Reserve Agency (FRA) when purchasing maize from farmers and other traders include maximum aflatoxin content of 10ppb although the Zambian standard (ZS 186:2004) for cereals and cereal products is instructive only

stating that mycotoxins should not be harmful to human health (Kankolongo *et al*., 2008).

No significant positive correlation between aflatoxin B_1 contamination in the groundnuts and the quantity of *A. flavus* colonies in the soil was detected in both sets of population in the current study. The lowest aflatoxin contamination levels were found in the cross with the highest *A. flavus* densities. This is contrary to what Monyo *et al.,* (2012) working on groundnuts reported as they found significant positive correlation between aflatoxin contamination and the quantity of *Aspergilli* in the soil. Egal *et al.,* (2005) working on maize also reported a significant positive correlation between *A.flavus* densities and aflatoxin contamination levels.

The results from this study therefore imply that seed contamination by *A. flavus* depends on many factors beyond the densities of the fungi and these factors may include the seeds innate susceptibility, environmental factors, fungal community structure in the soil and the ability of the fungus to reach and penetrate as well as the amount of organic matter contained in the soil (Horn, 2003, Zablotowich *et al*., 2007). 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. It has also being argued that fungi in *Aspergillus* section Flavi exist in complex communities composed of species that vary widely in aflatoxin producing ability (Cotty, 2006). The species that do not produce aflatoxins, called atoxigenic, are common in *A. flavus* (Garber and Cotty, 1997; Lisker *et al*., 1993) and hence may not be related to the aflatoxin produced in the groundnuts.

5.2 Inheritance of resistance to aflatoxin contamination by *Aspergillus flavus.*

The F_1 groundnut crosses were evaluated for resistance to aflatoxin contamination by *Aspergillus flavus* in an effort to establish the gene action controlling this trait.

The observed general combining ability (GCA) and the specific combining ability (SCA) mean squares in both Set A and Set B for the aflatoxin contamination trait under study were significant. The significant GCA and SCA mean squares demonstrated the variability in the GCA of the parents and the contribution of both additive and non-additive gene-action in the inheritance of resistance to aflatoxin contamination by

Aspergillus flavus. 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 *Aspergillus flavus* aflatoxin contamination resistance in groundnuts. Literature on groundnuts resistance studies is limited in referencing GCA and SCA means squares in explaining the implications but a study in maize by Asea *et al*., (2012) pointed to the same implications as those of Yoopum *et al*., (2005) that significant GCA and SCA mean squares represent presence of variability among materials used in the study for possibility of isolating resistant genotypes to aflatoxin contamination by *Aspergillus flavus.*

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. These findings are in agreement with those reported by Utomo *et al*, (1990), who found heritability estimates ranging from 21 to 23% for resistance to aflatoxin production. Arunyanark *et al.,* (2010) also noted low to moderate (0.30 to 0.65) heritability estimates for resistance to aflatoxin contamination. . 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). This calls for employment of selection methods that allow for fixation of a trait in the early generation and postponing the selection to later generations (Baker, 1968). The selection would be for superior hybrid combinations rather than the performance of the parents involved (Habaninema *et al*., 2012).

The current study presented significant positive and negative combining ability effects. In Set A, the GCA effects of two males, 55-437 and Ah 7223, had significant negative values of -0.77 and -1.46, respectively, while male parent, J11, had significant positive

GCA effects of 1.46. Female parent Katete had significant negative GCA effects in both sets of -1.95 and N/Common had significant positive GCA effects in both set A and B of 2.39 and 1.95, respectively. These results are in agreement with Rao *et al*., (1989) who working on similar materials, found that Ah 7223 had significant negative GCA effects and, therefore, had good combining ability for resistance to *A.flavus* via seed-coat resistance. In contrast to the results regarding J11, Upadhyaya *et al*., (2000) and Rao *et al.*, (1989) found that the parent had nonsignificant GCA effects for resistance to *In vitro* seed colonization of *A. flavus* (IVSCAF) and seed coat resistance.

Negative effects are desirable in resistance studies because they indicate contribution of the genotype towards resistance, while positive combining ability effects indicates contribution of susceptibility in the crosses (Marinkovic, 1982; Kenga *et al*., 2004). In a study done on maize by Mukanga *et al*., (2010) and on cassava by Lokko *et al*, (2004) they noted that negative effects obtained for the GCAs and SCAs were desirable for the contribution of the genotypes towards resistance to ear rot and cassava mosaic disease respectively.

The significance of negative GCA for both male and female 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, 2004). The positive GCA values suggest that it is pointless to utilize these genotypes in the development of crosses for resistance to aflatoxin contamination by *A. flavus* as they would contribute to the susceptibility of the progeny.

The analysis of genetic effects, therefore, showed that the two parental male genotypes 55-437 and Ah 7223 and the female genotype Katete were good combiners, while male parent J11 and female parent N/Common were poor combiners for the Aflatoxin contamination trait. The three good combiners are good candidates for use in enhancement of the average performance of *A. flavus* aflatoxin contamination resistance in the progeny.

The significant negative SCA effects implied that these crosses were good specific combiners and that non-additive gene action could play a vital role in the improvement of the trait of interest. The positive SCA effects implied the crosses were poor specific

combiners and were therefore susceptible to aflatoxin contamination. The non additive significant negative SCA effects are important in breeding, as breeders at times tend to make selections based on SCA values with the view of increasing the efficiency of hybrid improvement. 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, (2008) while working on beans, a self-pollinated crop.

Cross J11 x N/Common had significant negative SCA effects, in both sets, though derived from parents that were poor general combiners based on the GCA effects while cross 55-437 x Katete had significant positive SCA effects, in set A, though derived from parents that were good general combiners based on the GCA effects. This manifestation of progenies having reactions not related to the parents' 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 a similar phenomenon in groundnuts and cowpeas respectively and attributed it to genetic interaction between favourable alleles and between unfavourable alleles contributed by both pa\rents. This suggests that inheritance of this trait is not simple and that inter-allellic interactions due to epistasis could be responsible.

CHAPTER 6

6.0 CONCLUSIONS

It is concluded from this study that sufficient genetic variability exists among groundnut materials used to isolate superior ones for aflatoxin contamination resistance and that this trait is predominantly conditioned by non-additive gene action. The predominance of non additive gene effects indicates that non-fixable dominance deviation and epistatic effects will result in difficulties with directly improving the aflatoxin contamination resistant trait as they confound the selection of superior genotypes using simple selection procedures. The selection of genotypes with desirable traits in the early segregating generations is going to be difficult because the genes could not yet be fixed in individual genotypes. Thus procedures like pure line selection, pedigree selection, and their modification will not be useful. The development of alternative selection strategies such as the recurrent selection procedure accomplished by selection of crosses having high SCA effects and advancing progenies to later filial generations will be required.

The study identified 55-437, Ah 7223 and Katete as good general combiners and Ah 7223 x N/Common, J11 x Katete, 55-437 x N/Common and J11 x Luena as good specific combiners. The identification of these genotypes with desired GCAs and SCAs as sources of resistance to aflatoxin contamination by *Aspergillus flavus* is important. The performance of the genotypes could be exploited for developing improved resistant lines in Zambian breeding programs in future. Furthermore an investigation into the presence of epistasis in future studies will be required to help assist designing an appropriate improvement strategy to be used.

The development of *Aspergillus flavus* resistant cultivars for Zambia will be very beneficial in helping to manage the aflatoxin contamination challenge in the country. Zambia lies in the semiarid tropics where pre-harvest aflatoxin contamination is a serious problem. It is therefore imperative to aim for cultivars that confer high resistance to *Aspergillus flavus* to help reduce aflatoxin contamination and its associated health hazards. It would therefore be imperative to use modern techniques such as marker assisted selection to speed up the identification and development of more resistant cultivars to help mitigate the problem.

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8.0 APPENDICES

Appendix 1: Preparation of Modified Rose Bengal Agar (MRBA)

The Media is prepared by combining the following per litre of distilled water;

Inorganic salts

$NaNO_3$ 3.0g
KH_2PO_4 0.3g
K_2HPO_4 0.7g
$MgSO_4.7\ H_2O$ 0.5g
KCl. 0.5g
NaCl 10.0g B
acto agar 20.0g

Antibiotics

Chloramphenical 50.0mg
Dicholran 10.0mg
Streptomycin 0.3g
Rose Bengal Stock 5ml Sucrose 3.0g

Appendix 2: Data transformation of CFU to Log CFU for set A

Cross	Male	Female	Rep	CFU	Log CFU
1	55-437	Luena	1	43030.303	4.634
1	55-437	Luena	2	27070.606	4.432
1	55-437	Luena	3	10100.99	4.004
2	55-437	Natal common	1	38686.97	4.588
2	55-437	Natal common	2	*	*
2	55-437	Natal common	3	*	*
3	55-437	Katete	1	61010	4.785
3	55-437	Katete	2	*	*
3	55-437	Katete	3	*	*
4	J11	Luena	1	10706.97	4.03
4	J11	Luena	2	14949.394	4.175
4	J11	Luena	3	12323.333	4.091
5	J11	Natal common	1	33535.455	4.526
5	J11	Natal common	2	21818.182	4.339
5	J11	Natal common	3	46060.606	4.663
6	J11	Katete	1	13939.394	4.144
6	J11	Katete	2	11717.273	4.069
6	J11	Katete	3	15959.697	4.203

Appendix 3: ANOVA for Aflatoxin accumulation levels for A

Source of variation	d.f.	s.s.	m.s.	F-value	F pr.
Cross	5	90.4275	18.0855	22.58	<.001
Female	2	58.4603	29.2302	36.5	<.001
Male	1	10.716	10.716	13.38	0.006
female.male	2	21.2512	10.6256	13.27	0.003
Residual	8	6.4067	0.8008		
Total	**13**	**82.7321**			

Appendix 4: ANOVA for Log CFU for set A

Source of variation	d.f.	s.s.	m.s.	F-value	F pr.
Cross	5	1.06492	0.21298	6.10	0.013
Female	2	0.33039	0.16519	4.73	0.044
Male	1	0.48224	0.48224	13.8	0.006
female.male	2	0.25229	0.12615	3.61	0.076
Residual	8	0.27949	0.03494		
Total	**13**	**0.92178**			

Appendix 5: Data transformation of CFU to Log CFU for set A

Cross	Male	Female	Rep	CFU	Log CFU
1	Ah 7223	Katete	1	15050.606	4.178
1	Ah 7223	Katete	2	22424.242	4.351
1	Ah 7223	Katete	3	8484.848	3.929
2	Ah 7223	Natalcommon	1	12020.303	4.08
2	Ah 7223	Natalcommon	2	11717.273	4.069
2	Ah 7223	Natalcommon	3	39293.03	4.594
3	J11	Katete	1	13939.394	4.144
3	J11	Katete	2	11717.273	4.069
3	J11	Katete	3	15959.697	4.203
4	J11	Natalcommon	1	33535.455	4.526
4	J11	Natalcommon	2	21818.182	4.339
4	J11	Natalcommon	3	46060.606	4.663

Appendix 6: ANOVA for Aflatoxin accumulation levels

Source of variation	d.f.	s.s.	m.s.	F.value	F pr.
Cross	3	81.8025	27.2675	33.53	<.001
Female	1	17.0408	17.0408	20.95	0.002
Male	1	25.5208	25.5208	31.38	<.001
female.male	1	39.2408	39.2408	48.25	<.001
Residual	8	6.5067	0.8133		
Total	**11**	**88.3092**			

Appendix 7: ANOVA for Log CFU for set B

Source of variation	d.f.	s.s.	m.s.	F.value	F pr.
Cross	3	0.26563	0.08854	2.13	0.174
Female	1	0.16263	0.16263	3.92	0.083
Male	1	0.046	0.046	1.11	0.323
female.male	1	0.05699	0.05699	1.37	0.275
Residual	8	0.33191	0.04149		
Total	**11**	**0.59754**			