ASSESSMENT OF GROUNDNUT (*Arachis hypogaea* L.) FOR GENETIC DIVERSITY USING AGRO-MORPHOLOGICAL TRAITS AND SSR MARKERS

BY

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GENERAL ABSTRACT

Groundnut *(Arachis hypogaea L.)* offers one of the cheapest sources of proteins and economic empowerment to smallholder farmers in Africa, contributing significantly to world production and trade. Thus, improved groundnut seed with high quality attributes is needed. Therefore, pre-breeding activities involving agro-morphological attributes such as yield, disease tolerance/resistance, plant architecture among others are important in order to develop superior genotypes with the needed quality attributes. This study focused on assessing the performance and level of phenotypic variability and genetic diversity of groundnut genotypes using agronomic and morphological attributes, and simple sequence repeat (SSR) markers. Twenty-seven groundnut genotypes collected from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT-Malawi) and Chitedze Agricultural Research Station (Malawi) showed highly significant differences in relation to number of branches, days to flowering, leaf color, seed yield and shelling percentage except for aflatoxin content and groundnut rosette disease. Moderate to high broad-sense heritability (0.56-0.71) was observed for number of branches, days to flowering and leafspot disease. The genotypes grouped into three main distinct clusters with those bred for low aflatoxin accumulation falling in the same cluster. Principal component analysis (PCA) had two PCs explaining 57.7% of the total variation with number of branches, flowering and aflatoxin contributing the most to the first PCA. Five genotypes; MP-68, ICGV-94379, ICGV-93305, CDI-1314 and CDI-0009 were identified as high yielding with low aflatoxin concentration hence are recommended for further pre-breeding activities such as increasing yield and resistance to diseases and aflatoxin. Using 20 SSR markers, 39 groundnut genotypes of diverse origin maintained at Agricultural Research Council – Grain Crops Institute in South Africa (ARC-GCI were assessed for genetic diversity. Results showed polymorphic information content (PIC) averaging 0.71, indicating the markers were very informative. A wide genotypic diversity with highest dissimilarity index of 6.4 between genotype pair RG562 and RG288, and smallest dissimilarity index of 0.9 between RG512 and RG562 was observed. Allelic diversity analysis showed high diversity among genotypes from southern Africa and southern America as indicated by the Shannon information index, mean number of observed alleles (Na) and mean number of effective alleles (Ne) which were relatively higher than in other groups. Analysis of molecular variation (AMOVA) results indicated that variation between and within individuals was more significant than between populations. Discrimination of the genotypes was not dependant on the geographical origin as genotypes belonging to different origins clustered in the same groups. Thus, genotypes with wide diversity can be used in breeding programmes as parents.

DECLARATION

I, Olivia Chipeta, declare that,

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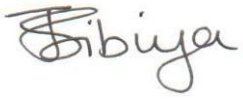

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Dr Julia Sibiya

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LIST OF ABBREVIATIONS

%GA	=	Percentage of genotypes amplified
%P	=	Percentage polymorphic loci
AF	=	Aflatoxin concentration
AFB1-BSA	=	Aflatoxin B1-Bovine serum albumin
AFLP	=	Amplified fragment length polymorphism
AMOVA	=	Analysis of molecular variance
ANOVA	=	Analysis of variance
ARC-GCI	=	Agricultural Research Council – Grain Crops Institute
BHS	=	Branch habit
CARS	=	Chitedze Agricultural Research Station
CGIAR	=	Consultative Group on International Agricultural Research
CTAB	=	Cetyl trimethylammonium bromide
CV	=	Coefficient of Variation
DF	=	Degrees of freedom
DNA	=	Deoxyribonucleic acid
DTF	=	Days to seventy-five percent flowering
ELISA	=	Enzyme-linked immunosorbent assay
EU	=	European Union
F	=	Inbreeding coefficient
FIS	=	Fixation index or inbreeding coefficient
FIT	=	Overall fixation index
FPS	=	Final plant stand
FST	=	Genetic differentiation
GA	=	Genetic advance
GAM	=	Genetic advance mean
GCV	=	Genetic coefficient variation
GD	=	Genetic distance
GHS	=	Growth habit
GI	=	Genetic identity
GRI	=	Groundnut rosette incidence
GS	=	Genomic selection
GXE	=	Genotype by environment interactions
He	=	Expected heterozygosity
Ho	=	Observed heterozygosity

HPLC	=	High-performance liquid chromatography
I	=	Shannon’s information index
ICRISAT	=	International Crops Research Institute for the Semi-Arid ropics
INIFAT	=	Instituto de investigaciones fundamentals en agricultura tropica
IP	=	Initial plant stand
IVSC	=	In-vitro seed colonisation
LC	=	Leaf colour
LOS	=	Leafspot ordinary scale
LTS	=	Leaf spot transformed scale
MA	=	Major allele
MAF	=	Major allele frequency per locus
MS	=	Mean squares
N	=	Number of observations
Na	=	Number of alleles per locus
NB	=	Number of branches
Ne	=	Number of effective alleles per locus
PA	=	Private allele per population
PBST	=	Phosphate buffer saline in Tween 20
PCA	=	Principal component analysis
PCR	=	Polymerase chain reaction
PCV	=	Phenotypic coefficient variation
PIC	=	Polymorphic information content
PPB	=	Parts per billion
QTL	=	Quantitative trait locus
RAPD	=	Random amplification of polymorphic DNA
RFLP	=	Restriction fragment length polymorphism
RUTF	=	Ready-to-Use Therapeutic Food
SCAR	=	Sequence characterized amplified regions
SE	=	Standard error
SH	=	Shelling percent
SNP	=	Single nucleotide polymorphisms
SS	=	Sum of squares
SSA	=	Sub-Saharan Africa
SSR	=	Simple sequence repeats/Microsatellites
SY	=	Seed yield
SY	=	Seed yield
TLC	=	Thin-layer chromatography

UWPGMA	=	Un-weighted pair group method
VAR	=	Variance

CHAPTER 1
INTRODUCTION

Groundnut (*Arachis hypogaea* L) is one of the major legumes that is grown around the world. It is a huge source of income for households and national economies in Africa because of trade and exports. Globally, groundnut is a source of oil, food and feed for livestock through its by-products such as groundnut cake (Arya et al, 2016; Nautiyal, 2002). It is mainly composed of 44-56% oil, 22-30% dietary protein, 10-25% carbohydrates with vitamins E, K and B complex, minerals and fibre (Nigam, 2014). Comparably, its nutrition composition such as vitamins, potassium, phosphorus and other elements are higher than those of other nuts such as cashew nuts, almonds, pistachios, pecans and walnuts among others, hence making it important (Settaluri et al, 2012). As of 2014/2015 season, groundnut was ranked as the world's fifth oil crop contributing 5.1% of world production (ITC, 2015). Its area of production was over 14 million hectares of land with production over 14 million tonnes more than that of dry beans, pigeon peas, potatoes and sweet potatoes. Southern Africa contributed 77,541 tonnes of this production (FAOSTAT, 2018).

In Malawi, groundnut is particularly grown by smallholder farmers who account for 93% of all production. Yield levels have increased from 18,000 tonnes in 1990 to 280,000 tonnes in 2010 (Derlagen and Phiri, 2012) compared to South Africa the same period (FAOSTAT, 2018). About 40% of the production from Malawi is marketed, of which 10% is exported to countries like South Africa, Kenya and Tanzania (Derlagen and Phiri, 2012). The National Smallholder Farmers Association of Malawi (NASFAM) dominates this export market. Thus, the crop is a source of income and food for smallholder farmers in Malawi. About 35% is processed into oil, peanut butter and groundnut cake, whilst 37% is used for domestic consumption and the remainder is used as seed (Derlagen and Phiri, 2012).

The groundnut seed industry is faced with several challenges including diseases such as groundnut rosette disease (GRD) which is spread by the aphid vector *Aphis craccivora,* early and late leaf spot caused by *Cercospora arachidicola* and *Phaesariopsis personata,* respectively, rust (*Puccinia arachidis*), and mycotoxins such as aflatoxins. These result into poor food quality and quantity of the produce. In addition, there is poor productivity as a result of poor management practices and the use of unimproved varieties (Simtowe et al, 2012). This is a challenge that occurs despite several efforts from scientists and other government initiatives to promote the use of improved varieties that are high yielding with better tolerance to diseases (Derlagen and Phiri, 2012). Most cultivars grown by farmers in Malawi are highly

susceptible to GRD and are subject to significant yield losses. In addition, farmers lack awareness of new varieties whose seed is also not readily available (Simtowe et al, 2012).

Moreover, aflatoxin contamination has negative implications on trade (Rios et al, 2013), thus affecting the economic status of the countries due to stringent measures on maximum allowable amounts of aflatoxin in groundnuts (Guchi, 2015; Babu et al, 1994). Exposure to aflatoxin contamination (aflatoxicosis) has serious health challenges including liver cancer, immunosuppression, stunting in kids, and in extreme cases, death, as has been reported in several countries (Unnevehr and Grace, 2013; CAST, 2003). One of the first cases of death due to aflatoxicosis was reported in 1974 in India where 108 people died and other death incidences have been reported in Kenya, Malaysia, India and Thailand (Unnevehr and Grace, 2013; Krishnamachari et al, 1975). In addition, operations to reduce post-harvest losses do not practically eliminate the toxin; hence, prevention needs to start from the field. Furthermore, incidences of climate change as reported will increase the effect of aflatoxins due to rise in temperatures, in turn, leading to frequent droughts. More so, incidences of pests are going to increase hence pest attack will also predispose aflatoxin contamination (Medina et al, 2014). There is, therefore, a need to find long-term solutions because none of the released varieties in Malawi are aflatoxin tolerant. This can be achieved through screening of accessions for tolerance and further breeding.

Genetically, groundnut has been reported to have a narrow genetic base (Upadhyaya et al, 2003); however, morphological diversity has been reported to be broader (Garba et al, 2015). Genetic improvement of any crop relies on genetic variation and hence groundnut is not an exception. Pre-breeding activities like the assessment of genetic diversity are highly recommended prior to initiating a crop breeding programme (Shimelis and Laing, 2012). This involves identifying potential sources of genetic material that have a wider gene pool. Morphological characterisation is thus an important pre-breeding component of a large crop breeding programme. In addition, marker assisted selection (MAS); together with genomic assisted selection (Meuwissen, 2003) are robust methods of improving genetic gains for quantitatively inherited traits across stress and non-stress environments.

1.1 Problem statement

Groundnut plays a vital role nutritionally as a source of proteins, and economically when the produce is sold locally and exported. It is also an important oil crop thus contributes to the oil industry. However, aflatoxins are a growing threat to groundnut production in Malawi as they reduce crop quality and quantity, which in the end affects the marketability of the groundnuts. Although the use of host plant resistance has been identified to be an economical and

sustainable way of reducing aflatoxin contamination in groundnut, none of the released varieties in Malawi are resistant to aflatoxins. The major drawback is that there is lack of populations that have good levels of resistance to aflatoxins. In addition, breeding for aflatoxin resistance is a complex process because of the suitability of climates in sub-Saharan Africa (SSA). Breeding for aflatoxin resistance has been ongoing in Malawi since the 1970s without much success. This is due to the trait being complex and highly interactive with the environments, thus a successful buildup of sources of resistance has remained elusive. As a result, there is a need to identify new sources of resistance and or tolerance and evaluate the identified genotypes across several environments to establish their utility in the groundnut breeding projects. To achieve this, pre-breeding activities like phenotypic characterisation and genetic diversity analysis to identify heterotic patterns and possible ways of improving yields are necessary. However, groundnuts have been reported to have a narrow genetic base, therefore, exploiting new sources of variation at molecular level is essential. Simple sequence repeat (SSR) markers were, therefore used in this study for the genotyping due to their effectiveness in detecting variation.

1.2 Research justification

Genetic and phenotypic diversity analysis allows identification of suitable genotypes for advancement across multiple environments. The use of molecular markers in African breeding programmes is still booming and offers the potential for advancing the breeding system through shortening the breeding cycle hence reducing overall costs in years, but also improves selection as it is trait specific. As a result, markers are an upgrade and complementary tool for conventional breeding. SSR markers have the potential of identifying diversity, even from low genetic pool groundnut populations, and can greatly improve yields hence the purpose of this study. In addition, trait specific breeding is of paramount importance as it reflects the economic need for a particular region. Pre-breeding activities help in selection of promising genotypes for further advancement in the breeding programme. ICRISAT Malawi is a regional African hub for groundnut breeding and there is need to build a gene pool that has sufficient levels of aflatoxin resistance for Malawian niche market. As a result, this would enable genotypes to be selected based on their heterotic grouping, resistance to aflatoxin contamination and stability across selected environments. In addition, the genetic information generated would help future breeding work in targeting specific traits to fit within specific product niches.

1.3 Research objectives

1.3.1 Main objective

- The overall objective of the study was to evaluate groundnut genotypes for reaction to aflatoxin and assess their level of diversity at molecular (SSR markers) and phenotypic levels

1.3.2 Specific objectives

- To evaluate reaction of groundnut genotypes to aflatoxin contamination in the field and in the glass house under natural conditions
- To determine the phenotypic diversity of groundnut genotypes using genetic parameters.
- To evaluate genetic diversity of groundnut genotypes using simple sequence repeat (SSR) markers

1.4 Research hypotheses

The study was based on the following hypotheses:

- There are significant differences in reaction of groundnut genotypes to aflatoxin contamination.
- There is significant phenotypic diversity amongst groundnut genotypes using various genetic parameters
- There is useful genetic diversity among the selected groundnut genotypes based on SSR markers

Thesis presentation

The specific objectives mentioned were achieved and are addressed in the various chapters, which constitute this thesis. Each chapter is an independent, potential manuscript for journal publication and therefore there may be some overlaps of content and references with other chapters. The chapters are divided as follows:

Chapter 1: Thesis introduction

Chapter 2: Literature review

Chapter 3: Phenotypic diversity of Malawian groundnut genotypes using agro-morphological traits and reaction to aflatoxin

Chapter 4: Genetic diversity of groundnut genotype collection from the South African gene bank based on SSR marker

Chapter 5: Research conclusions and recommendations

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

This chapter discusses the origin and centres of diversity for groundnuts. It also elucidates the botany and significance of groundnuts in the world, ideal conditions for groundnut production and current challenges in the industry. The research involves a number of traits, however, the review covers mainly about aflatoxin, which includes but not limited to the status of aflatoxin contamination and what the breeding sector has done. It also deliberates the ideal conditions for aflatoxin contamination. The chapter concludes with the thematic study of phenotypic and genetic diversity in relation to groundnut production.

2.2 Origin and taxonomy of groundnuts

Groundnut (*Arachis hypogaea* L.) also known as peanut originated from north-western Argentina on the eastern slopes of the Andes and southern Bolivia (Rao, 1987).The genus *Arachis* is naturally limited to Bolivia, Argentina, Brazil, Uruguay and Paraguay in South America. Other literature suggests that it is not known exactly where cultivated groundnut originated. However, the most possible place is a region in the eastern foothill of the Andes (north-western Argentina and southern Bolivia) (Nigam, 2014). Taxonomically, the genus *Arachis* has 80 species and nine sections (Koppolu et al, 2010). The nine sections include Caulorrhizae, Trierectoides, Erectoides, Triseminatae, Rhizomatosae, Extranervosae, Procumbentes, Heteranthae, and *Arachis* (Nigam, 2014). *A. hypogaea* is an annual herb of indeterminate growth pattern (IBPGR, 1992) divided into two subspecies: *A. hypogaea* subsp. *hypogaea* and *A. hypogaea* subsp. *fastigiata*. These are characterised by their differences in the port, and leaf colour among others (Garba et al, 2015).

The centres of highest genetic diversity in *Arachis* occur in South America. As a result, six gene centres have been recognised and they include the Goias and Minas Gerais in Brazil, the Guarani region, the Rondonia and northwest Mato Grosso in Brazil, north-eastern Brazil, the eastern foothills of the Andes in Bolivia and Peru. Africa has been identified as a secondary centre of diversity (Nigam, 2014).

2.3 Groundnuts botany

Groundnut is an allotetraploid 2n=4x=40 (AABB genomes) (Nigam, 2014) with sets of chromosomes four times of a haploid that came about naturally from hybridisation of diploid

wild species, *A. duranesis* (AA) and *A. ipaensis* (BB) (Liang et al, 2017). Botanically, its leaves are tetra-foliate, which have leaflets on the main stem, which differ in shape and size compared to those on the side branches. Its growth habit is commonly grouped into three classes; bunch/erect, semi-runner/semi-spreading, and runner/spreading types.

Branching habit consist of two major groups, which are either alternate or sequential although a third type of irregular also exists. Presence or absence of flowers on the main stem and branching pattern on side branches occur independently although they are closely linked. For example, alternate branching has absence of reproductive axes on the main stem whilst sequential branching has them on the main axes (Nigam, 2014).

The flowers abide in inflorescences which are found in the axils of leaves; preceding opening of flowers. Generally, a flower is made up of five petals, ten stamens and a pistil. One flower opens up at each node during sunrise. After fertilisation, pegs form, which go down the ground due to gravitation force, as a result, pod growth is underground. The pods differ in size, shape and texture and may have up to five seeds. Seed development occurs when the pods have reached their full size (Nigam, 2014).

The groundnut has a tap root system with many lateral roots. The roots can grow as deep as 135 cm but are commonly restricted to 5-35 cm zone within a radius of 12-14 cm. Groundnut roots have no typical root hairs, but instead have bunch of hairs produced in the axil of developing side roots to provide place for nodulation (Nigam, 2014).

2.4 Importance of groundnuts

Groundnut (*Arachis hypogea* L) is an annual legume that is mainly used as a source of food, edible oil and protein (18-25%) (Upadhyaya et al, 2005). Globally, it ranks 6th among oil seed crops such as soybean, rape seed and sunflower (Nigam, 2014). It is mainly used directly as food or in confectionaries and industries. As such, it can be consumed directly raw, roasted, boiled or processed. Groundnut is a source of nutritional security as it supplements maize with vitamins, micronutrients, proteins and oil in many rural economies. In addition, the groundnut crop is also used as a source of nitrogen (100-152 kg/ha N) for the soil through its ability to fix atmospheric nitrogen (Nigam, 2014).

Utilization of groundnut is region-specific. In most parts of Africa, groundnuts are a source of food, followed by oil, while in southern America and countries like India; it is primarily used as an oil crop (Nigam, 2014; Reddy and Bantilan, 2012). As a high protein value crop, its use is highly biased towards supplementation for the malnourished and people living with HIV/AIDS in Malawi (AICC, 2014). Malawian Companies like Rab Processors and Valid Nutrition

produce Ready-to-Use Therapeutic Food (RUTF) supplements made from groundnut providing the essential nutrients to the malnourished. It is also an important soil nutrition ameliorator, which forms an advantage to millions of people who cannot afford mineral fertilization of their crops.

Groundnut is an essential cash crop for both domestic and export markets as some of the production in Malawi is exported (AICC, 2014). Thus, it provides income at both domestic household and national levels. The vegetative parts, after harvest, can also be used as manure or animal fodder while shells provide fuel (form briquettes) and feed for livestock (Nigam, 2014; Babu et al, 1994).

2.5 Growing conditions for groundnuts

Groundnuts grow in well-drained loamy-sand, sandy-clay-loam or sandy-loam soils with enough calcium and organic matter and pH between 5.0-6.2. The ideal soil temperatures range between 18 - 30°C. Groundnut is a low temperature-sensitive crop not suitable for colder regions. During sowing, low temperatures may delay germination whilst extreme temperatures and low relative humidity affect flowering and pegging. Tropical and subtropical regions that characterize Malawian regions are suitable for groundnut production. These suitable regions have a characteristic 4-5 month growing season with stable, relatively high to moderate temperature and rainfall that is uniformly distributed for adequate soil moisture (AICC, 2014).

2.6 Challenges in groundnut production

Groundnut production constraints are similar at global and regional level only that they differ in their magnitude and specifications. Some of the major challenges in Africa include lack of adoption of new varieties and technology for improving cropping systems, low yields and fluctuations and access to inputs. Other challenges are pests such as white flies, aphids and diseases including rosette, peanut stripe virus and root-knot nematodes among others (Yao, 2004). In addition, Africa is experiencing challenges due to climate change. Intermittent droughts, which have been rampant in the last few years have been a major constraint in groundnut production affecting yield quality and quantity. In Ethiopia, major groundnut problems include poor soil fertility, limited land for production, diseases and aflatoxins (Chala et al, 2014). While lack of credit facilities, fertilizer, high cost of improved seed and technical expertise are some of the challenges faced by women farmers in Nigeria. In the sub-Saharan Africa, diseases such as rosette are a major challenge in groundnut production (Naidu et al, 1999). In China, the world's largest groundnut producer, challenges are similar to those

experienced in Africa, while in the United States of America they are more to do with policy issues resulting in a decline in area of production although yields are still high (Schnepf, 2016).

As production constraints vary geographically, from region to region; the International Crops Research Institute for the Semi-Arid Crops (ICRISAT), revealed that lack of financial input, unfavourable climate and lack of seed are the most limiting groundnut production constraints in Malawi (ICRISAT, 2007). Climate change has affected groundnut production because groundnut yields are higher when the crop is planted early at the start of the season. On the other hand, dry spells, towards the end of the rainy season; affect yield and quality as pods fail to develop and mature well hence end up with shrivelled nuts and pops (Arunachalam and Kannan, 2013). Seed has also been a problem because groundnut is a self-pollinated crop and therefore farmers can use recycled seed with minimal yield penalty. Thus, most companies are not willing to venture into seed business because it is hard to know the demand for certified seed and only get involved in production and marketing of the seed through government's farm input subsidy programme. As a result, accessibility of improved seed is still a problem hence yield is affected (ICRISAT, 2018).

Other challenges include low soil fertility, poor insect pest and disease control, weed control and lack of labour saving technologies. Moreover, there are increased incidences of contamination due to aflatoxins in groundnuts, which have recently reduced trade between Malawi and other countries including the European Union (Diaz Rios and Jaffee, 2008). In order to sustain the groundnut industry and value chain, improve household and national level income, the control of fungi causing aflatoxins becomes a priority and a sustainable goal. Control can be achieved by the use of host plant resistance, and the goals are achievable and realistic, and will have more advantages to the smallholder farmers' communities who are sustained by groundnuts.

2.7 Aflatoxin contamination in groundnut

Aflatoxins are highly toxic and carcinogenic chemical substances (to plants, humans and livestock) produced by fungi species *Aspergillus* that cause liver and other cancers, immune-suppression, compromised growth, synergisms and death (Waliyar et al, 2007). There are two species, which are most responsible for producing aflatoxin; *Aspergillus flavus* and *Aspergillus parasiticus,* which are both found in the soil and can spread via the air (Nigam, 2014). These mycotoxins are more enhanced in arid and semi-arid tropics where the environments are conducive (Monyo, 2010). They are classified based on whether they affect plants or animals, based on the colour of inflorescence of the fungus under ultra-violet light and their relative

mobility by thin-layer chromatography on silica gel, resulting in Aflatoxins B1, B2, G1, and G2 (AFB1, AFB2, AFG1, and AFG2).

Aflatoxins were discovered in 1960 after a turkey disease called 'Turkey X' killed many birds. Since then, aflatoxin adverse health effects on both livestock and humans has been documented, with one of the worst recorded case being that of Kenya where 125 people died after eating contaminated maize (Unnevehr and Grace, 2013; Lewis et al, 2005). Aflatoxins are most prevalent on crops such as cassava, maize and groundnuts where they cause significant yield losses (Guchi, 2015).

Contamination of these mycotoxins occur both at pre- and post-harvest stages of the crop cycle. Drought towards the end of the rainy season is the main predisposing factor of aflatoxin in the field and is attributed to the high temperatures, which facilitate the growth of the fungi (Guo et al, 2005). In addition, drought enhances pod damage through cracks in the pod wall, which exposes the seed to mould growth and subsequent entry of the fungus. Moreover, nutrient deficiency (Bhatnagar-Mathur et al, 2015) and insect damage also provide entry routes for the fungus (Monyo, 2010) and increase severity.

The occurrences of aflatoxin contamination on groundnuts has greatly affected production and trade in Malawi. The country used to be a major exporter of groundnuts until the 1980s when the market collapsed due to the aflatoxin problem. The aflatoxin contamination led to rejection of a huge volume (42%) of groundnuts by the EU market in 2005 (Diaz Rios and Jaffee, 2008). The EU regulates aflatoxin contamination levels on any crops entering the region (Otsuki et al, 2001). Losses estimated around US$11 million by the year 2017 were incurred in Malawi as reported by Diaz Rios and Jaffee (2008).

Several strategies have been used to control aflatoxins which include improved postharvest handling (Upadhyaya et al, 2003), biological control using non-toxic strains of *Aspergillus* (Dorner, 2008) and breeding for host plant resistance (Nigam et al, 2009). However, using a single approach to reduce aflatoxin incidence has not been completely successful. Integrated aflatoxin management is the best mitigatory approach to reduce yield losses due to aflatoxins. This includes the use of resistant varieties, which offers a sustainable and more economical strategy to deal with aflatoxin contamination. Research has indicated that resistant cultivars are less affected by the fungi even when environments are highly conducive, resulting in yields that are better than susceptible varieties (Guchi, 2015). The majority of smallholder farmers cannot afford the high costs of fungicides and thus using resistant cultivars is a cost-advantage strategy in the management of aflatoxin contamination (Monyo, 2010). Research on identifying potential sources of aflatoxin resistance in groundnuts is still scarce in Malawi, and currently

most known cultivars adapted to Malawian environments are susceptible to aflatoxins. There is, therefore, a need for identifying potential sources of aflatoxin resistance.

2.8 Breeding for aflatoxin resistance

Progress in breeding for aflatoxin resistance is underpinned by genetic variance for resistance, accessibility and use of dependable and effective screening methods in order to find plants with resistant genes (Holbrook et al, 2009). Aflatoxin resistance in plants is polygenically controlled and several QTLs have been successfully mapped on maize (Busboom and White, 2004). Therefore, the trait is greatly affected by the environments and genotype by environment (G x E) interactions. Even though this is the case, research indicates that there are three forms of resistance to aflatoxin contamination, which include pod infection (pod wall), seed invasion and colonisation (seed coat) and resistance to aflatoxin production (cotyledons) (Upadhyaya et al, 2002).

Although the forms of resistance differ, genotypes respond differently to aflatoxin production depending on the form of resistance. In addition, genotypes may do well in one form of resistance and fail in another hence; genotypes differ in their ability to support aflatoxin production. Studies have shown, for example, that genotypes such as U 4-7-5 and V R R 245 support only low levels of aflatoxin production, but were susceptible to seed colonization by *A. flavus* (Mehan, 1989). In other few cases, other accessions such as ICGs 1326, 3263, 3700, 4749, 4888, 7633, and 9407 have been reported to have resistance to both seed infection and seed colonization (Nigam et al, 2009). Rahmianna et al (2015) evaluated ten Indonesian groundnut genotypes for aflatoxin accumulation in the genotypes whereby one had the lowest concentration of aflatoxin <5 ppb, indicating resistance.

Efforts to eradicate the aflatoxin problem through genetic manipulation have been put in place since the late 1970s and is on-going (Nigam et al, 2009). Mixon and Rogers (1973) were the first to suggest the use of resistant genotypes to control aflatoxin (Nigam et al, 2009).They found that genotypes P.I. 337394 and P.I. 337409 had low 5-9%% seed infection indicating high resistance for aflatoxin contamination. At ICRISAT, about 2000 accessions were screened for *A. flavus* seed infection resistance in a sick plot under drought stressed conditions and 21 genotypes were identified as being resistant with < = 2% seed infection (Singh et al, 1997). In 2005 rainy season, 24 resistant lines were re-assessed under field and *in-vitro* conditions (Nigam et al, 2009) and out of these, 10 genotypes; ICG's 1859,1994,1326,3267, 10094, 3241,1422, 3251, 9820, and 4160 did not show pre-harvest seed infection or aflatoxin production (Nigam et al, 2009). In as much as efforts for breeding of aflatoxin resistance have been on going, the main challenge in breeding for aflatoxin

resistance has been lack of resistance genes which show stability and high levels of resistance (Torres et al, 2014; Mehan and Gowda, 1997).

High genotype by environment interactions have also been reported indicating that selection of resistant genotypes has to be environment specific (Anderson et al, 1995). In addition, to these challenges, the inheritance pattern for In-vitro Seed Colonisation (IVSC), pre-harvest seed infection or aflatoxin production has not been studied much. Few studies have been conducted on broad sense heritability and combining ability for aflatoxin resistance as reported by Mehan and Gowda (1997). Several measures to minimise this problem such as drying, curing and storage can help if followed appropriately. However, since infection is pre-harvest, meaning that once contamination occurs it cannot be reversed, breeding for resistance remains an important aspect as it can cater for the post-harvest aflatoxin production (Nigam et al, 2009).

2.9 Aflatoxin and drought

Drought and high temperatures are a common feature in tropical and subtropical regions. Studies have implicated that drought and heat play a pivotal role on aflatoxin incidences on crops (Guo et al., 2005). Cole et al (1985) reported that temperatures of 29.6°C and above under drought stress conditions cause increased aflatoxin contamination. The findings were also confirmed by Bhatnagar-Mathur et al (2015) who demonstrated that drought and temperatures between 29-31°C increase aflatoxin contamination in the last three-six weeks of the groundnut life cycle. Aflatoxin contamination increases with drought and increased temperatures. Holbrook et al (2000) observed that drought tolerant genotypes had less pre-harvest aflatoxin contamination than drought susceptible types, implying a positive relationship between drought tolerance and aflatoxin contamination. However, research by Hamidou et al (2014) showed that drought tolerance does not mean low aflatoxin contamination.

2.10 Phenotypic diversity

According to Araus et al (2018), crop phenotyping means assessing genotypic differences of a crop in respect of its traits such as yield, stress resilience, and quality among others. This is done in order to measure the amount of genetic gain a crop may have over time due to selection intensity, selection accuracy, genetic variation and time taken to complete a breeding cycle. Phenotypic traits evaluation is important as it helps in breeding for complex traits, which are difficult to breed as it requires use of secondary traits that are highly correlated to the trait of interest. This is because complex traits are usually influenced by high GXE interactions

hence improvements and/or genetic gains are low. Therefore, improvement of correlated traits is possible.

Studies have revealed that there is huge agro-morphological diversity in the cultivated peanut (Garba et al, 2015; Mace et al, 2006). As a result, selection based on phenotypic diversity cannot be a problem because there is a wide pool of variation. However, genetically, this is different as groundnut stands on a narrow genetic base. Since selection is based on both genetic and phenotypic attributes, phenotypic diversity cannot be ignored. Some studies based on this have been conducted precisely using many accessions for phenotypic characterisation. For example, Swamy et al (2003) evaluated a core collection for agronomic traits in India to estimate phenotypic diversity and define the significance of diverse descriptor traits. All characteristics excluding leaflet length and width, seed length and width pod length and width showed genotype by location interactions. There were also some significant phenotypic correlations between the different traits. For this type of characterisation, it becomes easier to select for good traits for consequent breeding, utilisation or conservation of the genotypes.

Further, a groundnut core collection was assessed at ICRISAT, India for phenotypic diversity to categorise 21 early maturing landraces including three known sources of early maturity (Gangapuri, Chico and JL24).The landraces had similar maturity to Chico and Gangapuri (80–90 DAS) thus earlier than JL 24 (90–95 DAS). The landraces differed in eight of the 14 morphological traits studied and showed a wide range of agronomic traits in clusters 2 and 3 indicating their usefulness in breeding programmes for early maturity and high yield (Upadhyaya et al, 2006).

2.11 SSR markers and genetic diversity

The use of marker-assisted selection in plant breeding experiments has greatly improved genetic gains in yield and agronomic components. Such breeding technologies have been useful on major plants like maize, wheat, rice among others. They have also been useful on groundnut improvement. Molecular markers have been used to complement conventional breeding mostly because they reduce efforts, time and costs for developing improved cultivars thereby shortening the breeding cycle.

Therefore, the use of phenotypic traits in groundnuts must be complimented by marker-assisted selection as genetic variability is very low. This is done at molecular level using DNA markers of different characteristics. Examples of such markers include random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), sequence

characterized amplified regions (SCARs), single nucleotide polymorphisms (SNPs) and simple sequence repeats or microsatellites (SSRs). Molecular markers are used in plant breeding as well as in DNA fingerprinting, genome mapping and study of genetic diversity (Gupta and Varshney, 2000).

Several studies based on different molecular markers have been carried out in relation to genetic diversity in groundnut and other crops. For example, using random amplified polymorphic DNA (RAPD) assay, 26 accessions of groundnuts were studied for molecular diversity to select genotypes with distinctive DNA profiles for mapping and genetic enhancement. The genetic similarity ranged from 59% to 98.9% with an average of 86.2% (Dwivedi et al, 2001). However, these markers come with limitations compared to SSR markers. Consequently, a lot more studies have used SSR or microsatellite markers for genetic diversity in groundnuts. This is due to their codominance nature, abundance, huge extent of allelic diversity, high reproducibility among others. SSR markers provide new prospects for molecular diversity analysis of groundnut (Mace et al., 2006).

In a study by Kanyika et al (2015), 799 SSR markers were screened to identify markers that were associated with resistance to important groundnut diseases like groundnut rosette disease, rust, early leaf spot and aflatoxin contamination. Results indicated that 376 of these markers were polymorphic and were used to show the relatedness of the genotypes to assist in selection of diverse genotypes for improvement. Two hundred and thirty-seven of these markers were identified and recommended for use in introgression of resistance of multiple biotic constraints to farmer-preferred varieties in the sub Saharan Africa (Kanyika et al, 2015).

In a different study, Upadhyaya et al (2005) studied the Asian groundnut core collection of 508 accessions and found that the 60 genotypes were diverse. Oteng-Frimpong et al (2015) also assessed 48 genotypes for diversity using SSR markers. In both studies, SSR markers were discriminatory of the genotypes even where the differences were low. Further studies were conducted by Mace et al (2006) to ascertain diverse groundnut germplasm that is disease resistant in order to develop mapping populations and for their introduction in breeding programmes. The genotypes used comprised 22 genotypes with varying levels of disease resistance to rust and LLS and were assessed using 23 SSR markers. The results detected 135 alleles of 23 loci and 12 of the 23 SSRs exhibited a high level of polymorphism as their polymorphism information content (PIC) values were ≥ 0.5. In addition, Li et al. (2011) used 709 SSR markers obtained from the public database of which 556 were used to characterize groundnut genotypes. Polymorphism information content scores and heterozygosity indices were calculated to estimate genetic diversity of the groundnuts. Results indicated that 410 of these markers had one allele confirming that the diversity of the cultivated groundnuts is

indeed limited. The genetic relationships established through cladogram, which symbolizes hypothetic relationships also confirmed that SSR markers are a useful tool to study the diversity of groundnuts as the pedigrees and origins of these tested groundnuts were in agreement with the cladogram (Li et al, 2011)

Naito et al (2008) also assessed the diversity and genetic relationships of Japan groundnut germplasm through using allelic variation of selected set of 13 SSR markers. Two-hundred and one accessions of *A. hypogaea* and 13 accessions of *Arachis* wild species were analyzed. Results indicated that there were 108 polymorphic alleles in *A. hypogaea* using 13 primer pairs. The alleles ranged from 3-15 with an average of 8.3 per marker. A phenogram divided *A. hypogaea* and *A. monticola* into a different group from diploid species and these were in turn divided further into two groups; one group consisted mainly of species of *fastigiata* accessions whilst the second group had mainly species of tetraploid wild peanut *A. Monticola* and *hypogaea* accessions.

Ren et al. (2014) also used 146 highly polymorphic SSR markers to evaluate the genetic diversity and population structure of 196 groundnut cultivars in different regions in China. Results indicated high genetic variations between cultivars from the north and the south, which provided molecular foundation for understanding the genetic diversity of Chinese cultivars (Ren et al, 2014). Twenty-four accessions each from the four botanical varieties of the cultivated groundnuts were evaluated for their genetic diversity using 34 SSR markers. Of the tested accessions, ten to sixteen pairs of SSR primers showed polymorphisms (Tang et al, 2007).

Although, the use of single nucleotide polymorphism (SNP) markers is becoming more popular due to their low cost per data point, high genomic abundance, co-dominance, potential for high throughput analysis and lower genotyping error, there are still limited platforms for studying genetic diversity in groundnuts using SNP markers. However, Kakeeto (2018) using the diversity array technology (DART) studied genetic diversity of 104 Ugandan groundnut accessions using 2896 single nucleotide polymorphisms (SNPs). He found that 50% of these SNPs were polymorphic with a mean polymorphic information content (PIC) of 0.15.

In addition, genomic selection (GS), is one of the latest developments in marker-assisted selection where the whole genome markers; major or minor, are used in predicting breeding values within a short period time shortening the breeding cycle (Crossa et al, 2017; Newell and Jannink, 2014; Heslot et al, 2012; Jannink et al, 2010). However, Goddard and Hayes (2007) indicated that genomic selection also comes with its limitations in that it requires a large amount of markers for it to be possible and it is expensive to conduct. As a result, marker

assisted selection methods using SSRs are still important as they use fewer markers and the cost of production is not as high as genomic selection.

2.12 Genetic variability

Day (1973) described genetic variability as the genetic differences within a population that make up a plant. This variation is between crop species and within individual crops. It is depicted by the variation in performance for the different traits of the crops. Means are used to calculate the phenotypic, genotypic and environmental variances which are used to find coefficient of variation phenotypically and genotypically (Ogunniyan and Olakojo, 2014). Usually, a higher phenotypic coefficient of variation (PCV) and genetic coefficient of variation (GCV) value difference indicates that the trait was more under environmental influence; as such, its genetic improvement would be difficult compared to a case where GCV was higher. This is so because a lower GCV and PCV difference means that additive genes are more influential than the environment, hence selection of the observed trait is easier (Meena et al, 2017; Meles et al, 2017; Singh et al, 2015; Ogunbayo et al, 2014; Sidhya et al, 2014; Wolie et al, 2013).

2.13 Heritability

Selection of a good trait depends on its ability to be passed on to its offspring. As a result, genetic variability cannot be acted independently without regards to heritability. This is so because if a trait is largely influenced by the environment, it means its heritability could be low as such it cannot be inherited by the offspring. Heritability is in two forms; narrow sense and broad sense. Narrow sense heritability is described as the ratio of phenotypic variance that is due to genetic effects whilst broad sense heritability describes the phenotypic effects that are due to both to environmental and genotypic influence (Falconer and Mackay, 1996; Nyquist and Baker, 1991). Heritability is used mostly as a predictor for gain after selection, as high heritability means the trait under observation is heritable whilst low heritability means that it is under the influence of the environment hence less heritable (Holland et al, 2003). As a result, heritability has been used in several studies as a means of selection (Sengwayo et al, 2018; Bahmankar et al, 2014; Jogloy et al, 2011; Xu et al, 2009; Condon and Richards, 1992).

2.14 Conclusion

This review has revealed that groundnut is an important crop for both feed and income in Malawi and South Africa. A lot has been done for this crop in assessing phenotypic and genetic diversity. However, not much has been done in Malawi and South Africa in this field, hence, the current challenges facing the industry. New improved varieties of groundnuts need to be

developed from the accessions that the gene banks hold thus, the need to assess the level of phenotypic diversity using various attributes for phenotypic selection. Molecular markers will assist in selection of desirable parents and shortening the breeding cycle hence the need to assess genetic diversity in the groundnut accessions.

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CHAPTER 3
ASSESSMENT OF PHENOTYPIC DIVERSITY OF GROUNDNUT GENOTYPES USING AGRO-MORPHOLOGICAL TRAITS AND REACTION TO AFLATOXIN

Abstract

Phenotypic variation plays a significant role in crop improvement especially during pre-breeding activities. Thus, phenotypic characterization is imperative for any breeding programme if meaningful gains are to be realised. The objective of this study was, therefore, to assess phenotypic diversity of 27 groundnut genotypes collected from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and Chitedze Agricultural Research Station (CARS) in Malawi in relation to their reaction to aflatoxins, agronomic and morphological traits. Analysis of variance showed that the genotypes were significant in relation to number of branches, days to flowering, leaf color, seed yield and shelling percentage except for aflatoxin content and groundnut rosette disease. Furthermore, moderate to high broad-sense heritability (0.56-0.71) was observed for number of branches, days to flowering and leafspot disease. There was a negative correlation between aflatoxin and leafspot suggesting that as leafspot attack increases, there would be less aflatoxin accumulated suggesting that the two pathogens could be antagonistic. Cluster analysis divided the genotypes into four main distinct clusters with most genotypes bred for low aflatoxin accumulation belonging to the same clusters. Principal components analysis had two PCA's explaining 57.7% of the total variation with number of branches, flowering and aflatoxin contributing the most to the variation for the first PCA. From this study, five genotypes namely; MP-68, ICGV-94379, ICGV-93305, CDI-1314 and CDI-0009 were identified with high yields and low aflatoxin concentration hence would possibly be used further for pre-breeding activities.

Key words: Groundnuts, phenotypic diversity, PCA, cluster analysis, genetic variability

3.1 Introduction

Groundnut is an annual legume that is grown throughout the world for its nuts, protein and oil. It mainly consists of 48-50% oil and 25-28% protein (Pasupuleti et al, 2013). Thus, it is the second oil-producing legume after soybean (Khan et al, 2004). In Africa, it is one of the most important food and cash crop (Guchi, 2015).

Botanically, groundnuts are mainly grouped into three types depending on their differences. These include Virginia, Valencia and Spanish, which are mostly large seeded, medium sized and small two seeded, respectively (Kotzamanidis et al, 2006). However, there are many differences as recorded by Nigam (2014) owing to its morphological diversity. This diversity helps in improving the genetic pool of the already limited genetic pool of groundnuts since genetic potential of any plant depends upon its phenotypic variation (Ru et al, 2016). Furthermore, it helps in increasing the use of groundnuts in different agro-ecological zones of the world as morphological diversity means widening adaptability. A good example is that of CG7, a popular Virginia groundnut variety in Malawi, which is preferred mostly for its wide adaptability, whilst some varieties like the Spanish types were bred for the low lying areas (Monyo, 2015).

A study of groundnuts morphological and agronomic traits using vegetative and reproductive structures has been conducted in order to establish a representative mini core collection at the INIFAT gene bank in Cuba (Mayor et al, 2004). Furthermore, at ICRISAT, characterisation of several mini core collections from the ICRISAT gene bank has been conducted based on the agronomic and morphological traits (Swamy et al, 2003; Upadhyaya, 2003; Upadhyaya et al, 2003). Additionally, phenotypic diversity is also very important as it directly reflects the significance of traits to each other and their end use by plant breeders or consumers. For example, in Greece, large seeded varieties are the most preferred by consumers and those with small pod constriction are not preferred since they easily break affecting yield quality and quantity (Kotzamanidis et al, 2006). Moreover, by-products from vegetative parts are most preferred for feeding animals.

In Malawi, the trend has revealed that confectionary, oil and disease resistance are some of the important traits that are preferred by the market. Most of the genotypes used in this study were not primarily bred in Malawi, but rather have been released in other countries as varieties for low aflatoxin contamination among other traits. Aflatoxins are one of the major drawback factors, which contributed to loss of the European export market in Malawi in the 1980s and still remains an issue towards production, export and trade (Rios et al, 2013; Monyo, 2010; Babu et al, 1994). Breeding for important traits such as diseases, aflatoxin is still an on-going

process at ICRISAT with pre-breeding activities necessary for consequent use in future breeding programmes. Thus, this study was undertaken to assess the phenotypic diversity of groundnut genotypes using agro-morphological traits and reaction to aflatoxin contamination.

3.2 Materials and methods

3.2.1 Field experiment geographical location

Twenty-six genotypes and one local check (Table 3.1) sourced from ICRISAT Malawi and Chitedze Agricultural Research station breeding programmes from which 18 of these were reported and recommended to be of low aflatoxin content when used in other regions by Partnership for aflatoxin control in Africa (PACA) whilst the remainder were mapped for different traits such as oleic acid content. The local check used was selected based on its aflatoxin susceptibility state. These were planted at Masenjere Research Station in Chikhwawa district, southern region of Malawi during the 2016/2017 winter season. During the 2017/2018 rainy season, the genotypes were planted in the central region on station at ICRISAT greenhouses and Chitedze Research station field. Geographical information for these sites is described in Table 3.2

Table 3.1 List of the evaluated genotypes and their source

NO.	GENOTYPE	SOURCE
1	CDI_0009	CARS
2	CDI_1314	CARS
3	CDI_2189	CARS
4	ICG_13603	ICRISAT
5	ICG_1415	ICRISAT
6	ICG_14630	ICRISAT
7	ICG_3584	ICRISAT
8	ICG_5195	ICRISAT
9	ICG_6703	ICRISAT
10	ICG_6888	ICRISAT
11	ICGV_91278	ICRISAT
12	ICGV_91324	ICRISAT
13	ICGV_93305	ICRISAT
14	ICGV_94114	ICRISAT
15	ICGV_94379	ICRISAT
16	ICGV_SM08528	ICRISAT
17	ICGV_SM08533	ICRISAT

NO.	GENOTYPE	SOURCE
18	ICGV_SM08540	ICRISAT
19	ICGV_SM08547	ICRISAT
20	ICGV_SM08556	ICRISAT
21	ICGV_SM08586	ICRISAT
22	JL_24 (local check)	ICRISAT
23	MP_F2	CARS
24	MP_F28	CARS
25	MP_F68	CARS
26	MP_F82	CARS
27	MP_F87	CARS

CARS= Chitedze Agricultural Research Station and ICRISAT= International Crops Research Institute of the Semi-Arid Tropics

Table 3.2 Trial geographical locations and their information

Location	Altitude (m)	Latitude (^{0}S)	Longitude (^{o}E)	Min temp ^{o}C	Max temp ^{o}C
Chitedze	1146	13°85	33°38	18	29
Masenjere	73.42	16°20	35°5	15	30

Min temp= Minimum temperature and max temp= maximum temperature

3.2.2 Field experimental design and agronomic management practices

The field experiments were laid out in a 4 x 9 alpha lattice design with two replicates. Field plot size measured 1.5 m length with 0.75 m between ridges. Whole plot had two ridges at Chikhwawa whilst at Chitedze it had four ridges. Management practices involved weeding as the weeds appeared and banding was done after every weeding. Fungicides (Chlorathalonil) and pesticides (Cypermenthrin) were applied every two weeks from 50 days after sowing. Harvesting was done on whole plots using hand hoes and stripping was done soon after harvesting. Sun drying was done until the nuts reached 5-7% moisture content.

3.2.3 Greenhouse experimental design and management practices

Seeds were planted in wooden boxes measuring 60 cm by 40 cm by 40 cm in length, width and breadth, respectively. The wooden boxes were designed in such a way that each had five compartments measuring as above. Three types of soil composed of red, wetland and sand were used to make the media in a ratio of 40:40:20. The soil was sieved then sterilised in a

Wagtech soil steriliser for 1 hr 30 min at 121˚C to kill all pathogens. After sterilisation, the soil was cooled, and placed into the wooden boxes. The wooden boxes were then covered with a black perforated plastic sheet to ease flow of water.

Figure 3.1 Lay-out of the genotypes in the screenhouse

Seeds were sown at 15 cm apart. Plants were watered daily and rainfall supplemented as the screen house was made of small wire net. Weeding and other crop husbandry practices such as spraying for prevention of fungal and viral diseases were done as needed every two weeks after 50 days of sowing. Harvesting was done manually by lifting the plants from the boxes. Stripping and drying was done as above.

3.3 Data collection

Table 3.3 shows the description of the qualitative and quantitative data collected for the evaluated genotypes.

Table 3.3 Description of qualitative and quantitative traits

Trait	Abbreviation	Description
Initial plant stand	IP	Counting of number of plants after emergence
Days to 75% flowering	DTF	Days to flower of 75% genotype
Growth habit	GHS	A qualitative description of the growth habit of the plant
Branch habit	BHS	A qualitative description of the branch habit of the plant
Number of branches	NB	Number of branches per plant
Leaf colour	LC	A Qualitative expression of leaf colour
Final plant stand	FPS	Number of plants in a plot after maturity
Seed yield	SY	Weight of pods per unit area, plot & plant
Aflatoxin concentration	AF	Quantity of aflatoxin per genotype in parts per billion
Groundnut Rosette incidence	GRI	Counting number of diseased plants
Leaf spot	LTS	Visual estimate of diseased plants

Rosette disease was scored using percent disease incidence (PDI) where by total number of plants infected in a plot were expressed as a percentage over total number of plants in a plot according to Waliyar et al (2007). The formula is given as below

$$\frac{Total\ number\ of\ infected\ plants}{Total\ number\ of\ plants\ in\ a\ plot} \times 100 \quad \text{.....Equation 1}$$

Leaf spot disease was scored using a 1-9 scale where 1 represented a clean plot and 9 represented a major severe attack.

3.3.1 Aflatoxin quantitative method

Aflatoxin content was quantified using indirect competitive enzyme linked immune-sorbent assay as explained by Waliyar and Sudini (2012). First, the aflatoxin was extracted by grinding 20 g of seeds per genotype into fine powder using a blender. Then the sample powder was triturated in a blender with 100 ml of 70% methanol (v/v 70 ml absolute methanol in 30 ml distilled water) which contained 0.5% potassium chloride. The extract was transferred into a

conical flask. Then it was shaken on a Gallenkamp orbital shaker for thirty minutes at 300 revolutions per minute. Then the mixture was filtrated using Whatman filter paper. Finally, it was diluted in 1:10 phosphate buffer saline in Tween 20 (PBST) that is, 1 ml of extract to 9 ml of buffer.

Aflatoxin analysis followed by coating the 96 microtiter ELISA plate with AFB_1-BSA antigen. Then blocking followed washing three times with PBST and incubating at 37^0C for one hour. Samples were added in sample wells while specific antibodies were added in both standard and sample wells in order to compete with the bound AFB_1-BSA antigen with the aid of immunoglobulins. An enzyme conjugate, anti-rabbit-lgG-ALP, was added and incubation followed for one hour at 37^0C. The substrate, 4-nitrophenyl phosphate disodium salt hexahydrate, was added for colour development. Finally, the colour reaction was read using an ELISA plate reader. The optical density values obtained were used to produce a regression curve and a standard curve as extrapolated with a known correlation coefficient, which gave the AFB_1 concentrations in parts per billion.

3.4 Data analysis

Combined data analysis was done unless specified otherwise as follows; quantitative traits were subjected to analysis of variance (ANOVA) and means were separated through Duncan's multiple range tests in Genstat 18 while for qualitative-data, mode and kurtosis were calculated in Excel. Before this, leafspot diseases data was transformed through square-root method as outlined below

Sqrt (leaf spot score +0.05)=Transformed scale……...Equation 2

Principal components analysis (PCA) was done in Genstat 18 as well as, hierarchical clustering using complete linkage method. Genetic parameters such as genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), genetic advance, broad sense heritability were also calculated using variance components derived from SAS 9.4 with formulas as below.

The genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) components were calculated as given by Burton and Devane (1953);

$$\mathbf{GCV = (\sigma_g / \bar{x}) \times 100}$$

$$\mathbf{PCV = (\sigma_p / \bar{x}) \times 100}$$

Where:

σ_g is genotypic standard deviation

σ_p is phenotypic standard deviation

$\bar{x}$ is the mean for a specific trait

Broad sense heritability was calculated using the formula as given by Abraha et al (2017);

$$H^2 = \sigma^2_g / \sigma^2_p$$

Where, H^2 is heritability in the broad sense

σ^2_p is the phenotypic variance for a particular trait $= \sigma^2_p = \sigma^2_g + \sigma^2_{gs}/s + \sigma^2_e/sr$

σ^2_g is the genotypic variance for a particular trait

Genetic advance (GA) and genetic advance as percent of mean (GAM) were found using the formulae given by Johnson et al (1955);

$$GA = k\, H^2\, \sigma_p$$

Where:

GA = Genetic advance

k is the coefficient of selection intensity

H^2 is heritability in the broad sense for that particular trait

σ_p is the phenotypic standard deviation of that particular trait

Genetic advance as percentage of mean (GAM) was calculated as given by Abraha et al (2017)

$$GAM = (GA / \bar{x}) \times 100$$

Where **GA** is Genetic advance

$\bar{x}$ is the mean for a particular trait

3.5 Results

3.5.1 Combined analysis of variance for seven quantitative traits

Combined ANOVA results for both greenhouse and field experiments are presented in Table 3.4 below. Five quantitative traits namely; days to flowering, leafspot disease, number of branches, shelling percentage and seed yield were highly significant at p<0.001, 0.01 and 0.05 for genotypes, environment, genotypes by environment except for groundnut rosette disease and aflatoxin concentration.

Table 3.4 Combined Analysis of Variance for seven quantitative traits

Source	DF	AF	DTF	GRI	LTS	NB	SH	SY
Reps	1	747	24.89	2240.3	0.01859	44.08	417.4	0.06624
Genotypes	26	1709ns	25.74***	1146.8ns	0.07***	5.46***	276.6*	2.35***
Environment	2	20055***	453.38***	978.5ns	5.73***	4.50ns	5116.5***	1.08***
Genotypes *Environment	52	1960ns	8.16*	745.62ns	0.03*	1.34ns	179.2ns	1.63***
Error	80	1959	5.34	514.5	0.02	2.14	120.0	0.08

*** Significant at 0.001, ** significant at 0.01,* significant at 0.05, ns=non-significant, DF= Degrees of freedom, AF= Aflatoxin concentration, DTF=Days to 75% flowering, GRI=Groundnut Rosette incidence, LTS=Leafspot transformed scale, NB=Number of branches, SH=Shelling percentage, SY= Seed yield

3.5.2 Mean comparisons for the seven quantitative traits

Results for mean comparisons for agronomic performance of the evaluated quantitative traits are presented in Table 3.5.The results indicated that number of branches ranged from 3 to 13 with a mean of 6 branches. Days to 75% flowering ranged from 26 to 49 with a mean of 36. Original data for leaf spot score was from 1 to 5 with a mean of 2 while the transformed scale was from 1 to 2 with a mean of 1.3. Groundnut rosette disease incidence ranged from 0% to 94% with a mean of 40%. Seed yield ranged from 0 to 335 grams per plant with a mean of 29.2. Shelling percent was between 4% and 98% a mean of 65%. Aflatoxin concentration ranged from 8 ppb to 80 ppb with a mean 27.4 ppb.

Table 3.5 Combined mean comparisons of quantitative traits of the groundnut genotypes

Genotypes	NB	DTF	LOS	LTS	GRI	SY	SH	AF
CDI_0009	7.67ab	38.42abc	1.64de	1.13h	47.22ab	23.34cd	66.64b-e	16.97b
CDI_1314	9.17a	38.92a	1.94bcde	1.24e-h	33.33ab	17.76bcd	53.66e	18.85ab
CDI_2189	5.67b-f	38.83ab	1.5e	1.23fgh	57.84ab	56.53bcd	65.21b-e	13.69b
ICG_13603	6.92b-e	38.17a-d	1.89bcde	1.37c-g	34.26ab	22.92bcd	71.07bc	30.15ab
ICG_1415	5.50c-f	36.67a-g	1.97bcde	1.34d-g	48.49ab	11.56d	64.04b-e	19.45ab
ICG_14630	6.00b-f	34.08e-h	2.42abc	1.56ab	57.63ab	19.16d	59.73b-e	9.06b
ICG_3584	6.00b-f	39.17a	2.06a-e	1.44a-d	46.99ab	22.04d	65.8b-e	28.06ab
ICG_5195	5.42c-f	33.5gh	2.03a-e	1.53abc	47.02ab	47.22bcd	72.89ab	25.88ab
ICG_6703	6.33b-f	38.42abc	2.61a	1.61a	35.66ab	16.67cd	70.33bcd	48.71ab
ICG_6888	5.25def	33.5gh	1.72de	1.412b-f	36.69ab	11.8d	62.76b-e	16.12b
ICGV_91278	6.17b-f	38.17a-d	1.86bcde	1.41b-f	35.08ab	30.82bcd	64.58b-e	16.99b
ICGV_91324	5.33def	35.67b-g	2.14a-e	1.41b-f	58.21ab	26.16d	57.4b-e	28.13ab
ICGV_93305	6b-f	33.92e-h	1.89bcde	1.50a-d	32.83ab	29.38bcd	64.97b-e	8.24b
ICGV_94114	5.83b-f	36.58a-g	1.75de	1.36c-g	25.66ab	37.83cd	70.8bc	22.87ab
ICGV_94379	5ef	32h	1.86bcde	1.35c-g	35.31ab	73.11bcd	68.7b-e	12.54b
ICGV_SM08528	6.5b-f	38.33abc	1.56de	1.32d-g	45.04ab	34.22d	68.23b-e	35.93ab
ICGV_SM08533	7b-e	34.5e-h	1.97a-e	1.38b-g	11.7ab	24.9bcd	69.83bcd	32.5ab
ICGV_SM08540	6.58b-f	36a-g	1.92bcde	1.35c-g	32.69ab	24.89bcd	70.74bc	16.13b
ICGV_SM08547	6.42b-f	37ab-e	2.03a-e	1.224gh	4.8b	18.15ab	84.74a	20.1ab
ICGV_SM08556	7.17bcd	36.75a-f	2.06a-e	1.33d-g	27.27ab	36.45a	59.74b-e	52.79ab
ICGV_SM08586	5.17def	38.67ab	1.81cde	1.23fgh	24.82ab	67.26cd	62.95b-e	80.49a
JL_24	6.08b-f	35.67b-g	2a-e	1.37 c-g	50.83ab	17.36bcd	57.13cde	12.46b

Genotypes	NB	DTF	LOS	LTS	GRI	SY	SH	AF
MP_F2	4.75f	34.92e-h	1.86bcde	1.44a-d	46.23ab	13.76d	61.49b-e	32.13ab
MP_F28	5.58c-f	35.25c-g	1.97a-e	1.44a-d	44.23ab	21.73bcd	68.37b-e	59.14ab
MP_F68	7.42abc	35.08d-h	2.17abcd	1.42b-e	67.45a	18.39d	60.8b-e	18.45ab
MP_F82	5.75b-f	33.58fgh	2.47ab	1.53abc	41.33ab	12.71d	64.05b-e	42.49ab
MP_F87	5.58c-f	34.5e-h	2a-e	1.43a-d	44.47ab	51.98abc	54.8de	22.76ab
Mean	6.16	36.16	1.966	1.38	39.7	29.2	65.24	27.4
SEM	0.28	0.45	0.09	0.03	4.37	1.91	2.19	25.57
Minimum	3	26	1	1.03	0	0	3.83	0
Maximum	13	49	5.33	2.32	94.4	334.8	98	403.8
P. Value	<.001	<.001	0.028	<.001	0.760	<0.028	0.06	0.64
CV %	26.73	10.79	45.48	22.96	53.80	24.49	23.16	168.4

NB=Number of branches, DTF= Days to 75% flowering, LOS= Leafspot original scale, LTS= Leafspot transformed scale, GRI= Groundnut Rosette incidence (%), SY= Seed yield (g/plant), SH=Shelling percent and AF= Aflatoxin quantity (µg/Kg). Means followed by the same letter means that the genotypes are not differing in Duncan's multiple range test.

3.5.3 Qualitative traits mode and kurtosis

The mode for branching habit and growth habit was erect whilst leaf colour was green. Kurtosis level was high (54) in both branching habit and growth habit whilst in leaf colour it was -0.61747 as can be seen in Table 3.6.

Table 3.6 Kurtosis and mode for qualitative traits

Genotypes	BHS	GHS	LC
CDI_0009	1	1	2
CDI_1314	1	1	2
CDI_2189	3	3	2 & 3
ICG_13603	1	1	2
ICG_1415	1	1	3
ICG_14630	1	1	3
ICG_3584	1	1	3
ICG_5195	1	1	2
ICG_6703	1	1	3
ICG_6888	1	1	2
ICGV_91278	1	1	2
ICGV_91324	1	1	2 & 3
ICGV_93305	1	1	2
ICGV_94114	1	1	2
ICGV_94379	1	1	3
ICGV_SM08528	1	1	2
ICGV_SM08533	1	1	1 & 2
ICGV_SM08540	1	1	1 & 2
ICGV_SM08547	1	1	2 & 3
ICGV_SM08556	1	1	2
ICGV_SM08586	1	1	2 & 3
JL_24	1	1	2 & 3
MP_F2	1	1	1& 3
MP_F28	1	1	1
MP_F68	1	1	3 & 3
MP_F82	1	1	2 & 3
MP_F87	1	1	2
Mode	1	1	2
Kurtosis	54	54	-0.61747

BHS=Branch habit score, GHS=Growth habit score and LC=Leaf colour

BHS and GHS 1=erect, 2=prostrate, 3= semi-spreading, 4= semi erect

LC 1= light green, 2= green and 3=yellow green

3.5.4 Principal components analysis

Principal component analysis grouped the variation into seven components, which accounted for 97.55% variation and six eigen values had a value more than one. The first component had an eigen value of 13.227 and had number of branches, days to 75% flowering and leafspot contributing much to the first PCA whilst number of branches, aflatoxin and days to 75% flowering contributed most of variation to the second PCA. This PCA had an eigen value of 5.804. Principal component analysis eigen vectors and values are listed in Table 3.7. Number of branches had the highest contribution of eigen vectors in both PCA 1 (0.70) and 2 (0.61). Days to flowering, leafspot and aflatoxin contributed much to PCA 1 whilst aflatoxin, days to flowering and seed yield contributed significantly to PCA 2.

Table 3.7 Eigen vectors and values showing their contribution to individual and cumulative percentages of the variation to the PCAs

	Eigen vectors contribution	
TRAITS	PCA 1	PCA 2
AF	0.04	-0.50
DTF	0.60	-0.43
LTS	-0.40	0.26
NB	0.70	0.61
GRI	-0.06	0.08
SY	-0.00	-0.29
SH	-0.17	0.21
Eigen value	13.23	5.80
Individual %	40.1	17.6
Cumulative %	40.1	57.7

AF=Aflatoxin concentration (µg/Kg), DTF=Days to 75% flowering, LTS=Leafspot transformed scale, NB=Number of branches, GRI=Groundnut Rosette incidence (%), SY=Seed yield (g/plant), SH=Shelling percent

3.5.5 Principal component biplot

Principal component one and two formed the PCA biplot as Figure 3.2 indicates. The smaller angles of the vectors going in the same direction showed a strong correlation as in the case of seed yield and aflatoxin. Genotypes that were close to the vector line but further away from

the convex hull vertices indicated that they were good in that particular trait. As a result, six genotypes with genotype ID's number 21, 15, 13, 25, 2 and 21 representing genotypes ICGV-SM08586, ICGV-94379, ICGV-93305, MP-68, CDI-1314 and CDI-0009 were selected for high seed yield and low aflatoxin concentration except for genotype ICGV-SM08586.

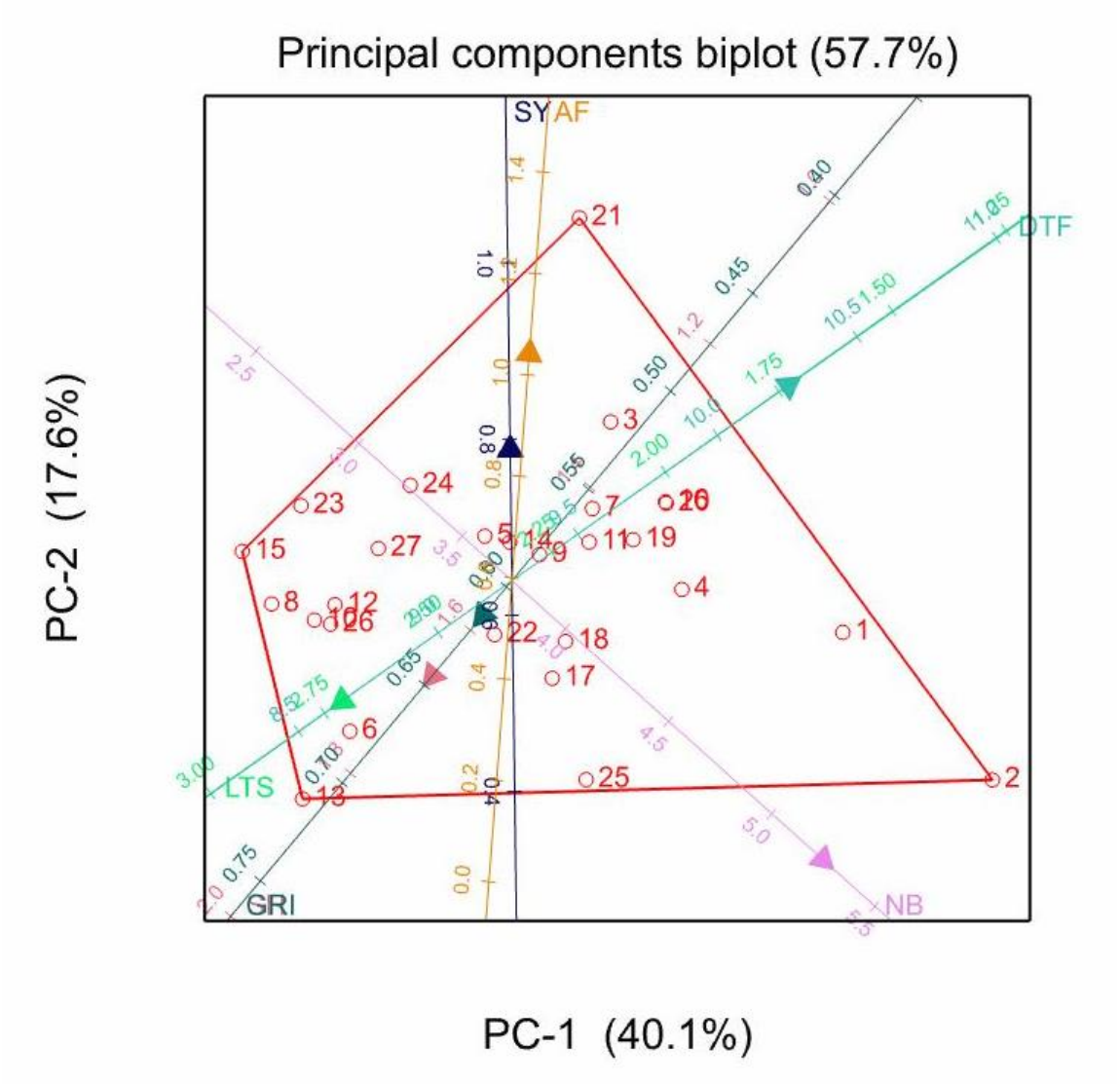

Figure 3.2 Principal Component Analysis biplot showing individual and cumulative percentage of variation for the quantitative traits

3.5.6 Cluster analysis of the evaluated genotypes

Hierarchical cluster analysis was performed in order to discriminate genotypes according to their similarities or differences. In this study, a dendrogram (Figure 3.3) based on complete linkage method was constructed to show phenotypic relationships among the genotypes. Three clusters resulted from such and genotypes belonging to the same breeding station but different origin were mostly grouped together. Since most (18) of the genotypes used in this

study were previously reported to have low aflatoxin contamination, the genotypes were spread across the clusters with most genotypes belonging to cluster 1 alongside five of the eight genotypes belonging to Chitedze Agriculture Research station mapped for other traits like oleic acid. This means that cluster one had the highest number of similar genotypes than the others.

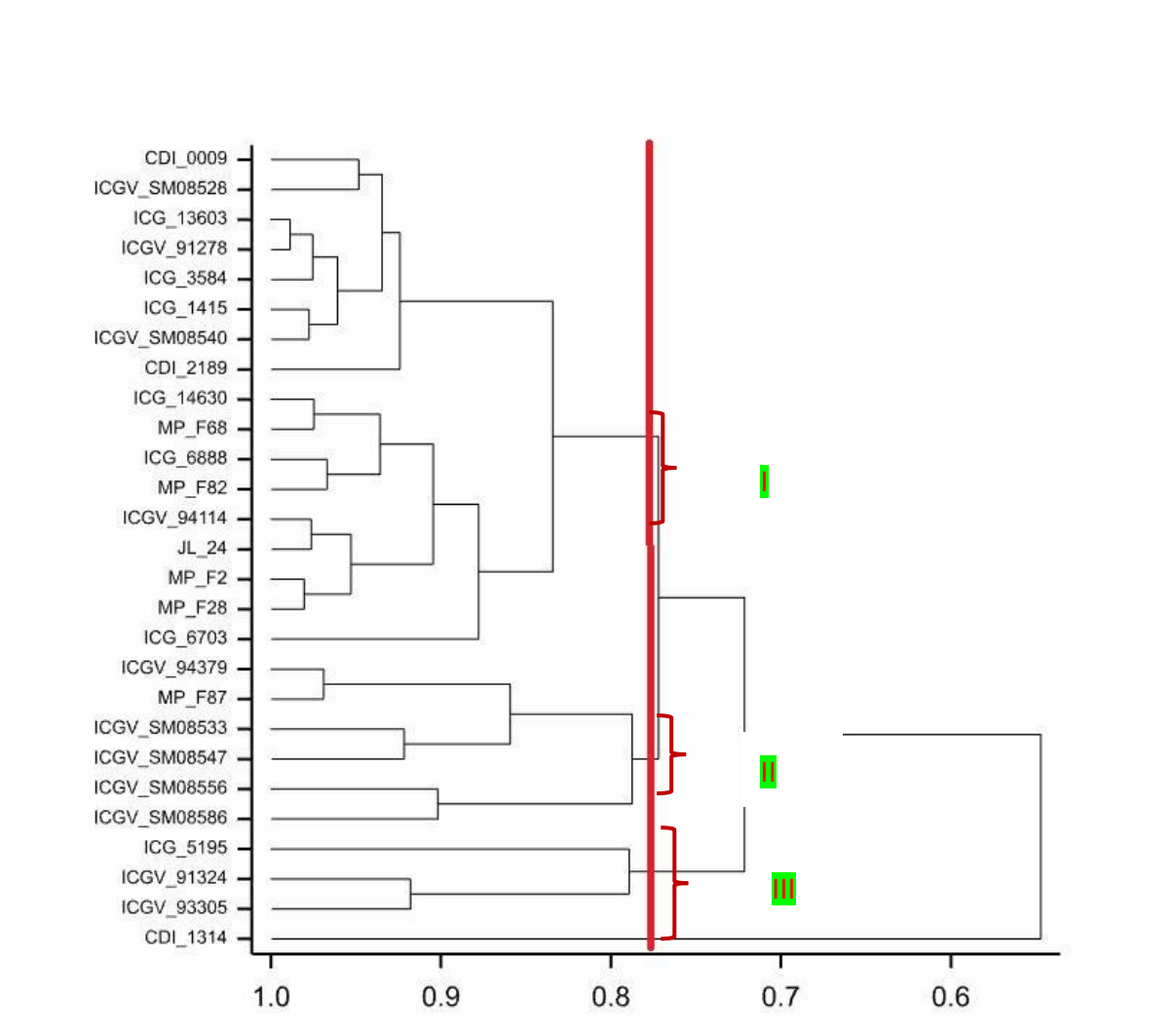

Figure 3.3 Dendrogram based on complete linkage method showing the phenotypic relationships among the groundnut genotypes

3.5.7 Phenotypic and genotypic coefficient of variation

The phenotypic and genotypic coefficient of variations for six quantitative traits are presented in Table 3.8. Results indicated that GCV and PCV were both low (<10%) for days to 75% flowering, seed yield, leafspot transformed scale and shelling percent while number of branches had moderate (16.22) PCV and (13.67) GCV. Aflatoxin had a very high (64.72) PCV with a low GCV (0).

Table 3.8 Genetic variability components of some quantitave traits

TRAIT	var(gen)	var(env)	Var(error)	Mean	σ^2p	σ^2g	GCV%	PCV%
NB	0.71	0.00	1.74	6.16	1.00	0.71	13.67	16.22
DTF	2.90	7.96	5.02	36.16	4.27	2.90	4.71	5.72
LTS	0.00	0.11	0.017	1.38	0.01	0.01	5.89	7.85
SH	9.76	89.75	120.43	65.24	41.85	9.76	4.79	9.92
AF	0.00	313.25	1886.80	27.40	314.47	0.00	0.00	64.72
SY	0.10	0.00	0.11	29.2	0.37	0.10	1.09	2.09

Var (gen)=Variance genetic, Var (env)= Variance environment, Var (error)= Variance Error, Mean=Grand mean, σ^2p=phenotypic variance,σ^2g=genotypic variance, GCV%=Genotypic coefficient variance percent and PCV%=Phenotypic coefficient variance percent.NB=Number of branches, DTF=Days to 75% flowering, LTS=Leafspot transformed scale, SH=Shelling percent, AF=Aflatoxin concentration and SY=Seed yield

3.5.8 Heritability of the quantitative traits

Heritability, genetic advance and genetic advance mean estimates are presented in Table 3.9. Results indicated that the broad sense heritability ranged from 0.00 to 0.70 expressed as percentage as 0% and 70%, respectively. Robinson et al (1949) described broad sense heritability percentages in 3 categories, where heritability values greater than 60% are high, 30-60% - moderate, and 0-30% - low. In this study, number of branches and days to flowering had high heritability estimates of 71% and 68% respectively. Leafspot had moderate heritability (56%) while shelling percent; aflatoxin and seed yield had the lowest heritability ranging from 0-27%. Genetic advance ranged from 0-2.66 whilst genetic advance as % of the mean ranged from 0-20.27%.

Table 3.9 Broad sense heritability,genetic advance and genetic advance mean values for the quantitative traits

Trait	Heritability	Heritability %	Genetic advance	GAM% (% of mean)
NB	0.71	71	1.25	20.27
DTF	0.68	68	2.47	6.84
LTS	0.56	56	0.11	7.78
SH	0.23	23	2.66	4.07
AF	0.00	0	0.00	0.00
SY	0.27	27	0.29	1.00

NB=Number of branches, DTF=Days to 75% flowering, LTS=Leafspot transformed scale, SH=Shelling percent, AF=Aflatoxin concentration and SY=Seed yield

3.6 Discussion

Genetic improvement of a crop depends on its variable traits. The means for quantitative traits of the groundnut genotypes were significant for five traits namely days to flowering, number of branches, leafspot, seed yield and shelling percentage. These results affirm what Zongo et al (2017) found. Flowering is an important aspect as far as podding and maturity are concerned as indicated by Upadhyaya and Nigam (1994). This is because early flowering depicts that early harvest may occur hence the genotypes may be able to escape biotic and abiotic stresses that occur in the late season (Yol et al, 2018). As a result, genotypes ICGV-94379, ICG-5195, ICG-6888, MP-F82 and ICGV-93305 would be ideal for early maturity trait with genotype ICGV-94379 being the best as it also had the highest seed yield per plant. This trait also contributed significantly to the first principal component vector loading along with number of branches similar to what Yol et al (2018) established. High heritability values were also found for these traits as confirmed by Zongo et al (2017).

Number of branches are important as they have a direct bearing on yield. Often times, more number of branches means higher yield from the plant. In this study, number of branches, yield and shelling percentage were similar and higher than those reported by other scientists

(Yol et al, 2018; Upadhyaya et al, 2003; Upadhyaya et al, 2002). Examples of such genotypes were CDI-1314, CDI-0009 and MP-F68. These genotypes' rosette score was higher as such yield was low compared to CDI-2189, MP-F87 and ICGV-SM08586, which had significant rosette attack. This could mean that the latter genotypes are resistant. Worth noting is the fact that, genotypes MP-F87 and ICGV-SM08586 were also grouped in the same cluster whilst CDI-1314 was clustered alone.

Low leafspot scores were observed for all genotypes indicating that the genotypes could be resistant even though they were not inoculated but the disease was readily available (Subrahmanyam et al 1995). Leafspot disease is caused by the fungi *Cercospora arachidicola.* It is one of the major diseases in Africa as it contributes 50-70% yield losses as such breeding for this trait is significant (Zongo et al, 2017). These genotypes showed a comparably good resistance result as they contributed significantly to the first and second PCAs with negative and positive loadings, respectively. As a result, these genotypes could be sources of early leafspot resistance as they showed a moderate broad sense heritability in line with other studies (Anderson et al, 1991; Green and Wynne, 1987; Anderson et al, 1986). This assumes that genetic selection of this trait could be possible.

Shelling percentage is a trait that shows how good the seed yield will be based on the number of shelled nuts (Horrocks and Zuber, 1970). In this study, mean shelling percentage was higher than other findings in genotypes ICGV-SM08547, ICG-5195, ICG-13603, ICGV-94114, ICGV-SM08540, ICG-6703, ICGV-SM08533, ICGV-94379, MP-F28, ICGV-SM08528 and CDI-0009 (Yol et al, 2018; Oteng-Frimpong et al, 2017; Zongo et al, 2017). Seed yield is one of the important traits that breeders place much weight on when selecting parents for making crosses depending on the main objective. The present investigation reported high seed yield in genotypes compared to other results (Olayinka and Etejere, 2015; Yambhatnal et al, 2012; Songsri et al, 2009). This could be possibly because of the high yield values from the two sites whose groundnuts were under irrigation.

One of the main objective of this study was to select desirable genotypes with aflatoxin tolerance apart from other traits. Results showed that the genotypes did not react differently to aflatoxin concentration even though aflatoxin concentration was lower than what Korani et al (2017) recently established. The phenotypic coefficient of variation was high and broad sense heritability was zero suggesting that the trait was largely influenced by the environment. These findings on broad sense heritability agree with the results of other studies (Arunyanark et al, 2010; Girdthai et al, 2010). Furthermore, Nigam et al (2009) reported that high genotype and environmental interactions and low to moderate heritability are often associated with the

trait. As a result, breeding for this trait has been difficult suggesting that multi-location testing for these genotypes would be essential.

Skewness is a measure of asymmetry of the data set. Kurtosis measures how skewed the data is. Data set that has a positive kurtosis reveals that it is heavier on one tail whilst a negative kurtosis means that the data is flat i.e. the mean and standard deviation are equal (Kim, 2013; Groeneveld & Meeden, 1984). In this study, the kurtosis for branching habit and growth habit was positive showing that the data was one tailed. This is because most of the genotypes used were of the same branching and growth habit. The mode depicts the most frequent result and for this study; green colour, erect growth and branching habit were the most frequent traits observed. Green colour is an expression of relative content of chlorophyll. Plants that have green colour compared to other colours tend to have more photosynthesis hence more food output, thus affecting yield (Ferguson et al, 1972). The results of the yield revealed that the genotypes had higher yields than those reported as stated above. Leaf colour could be one of the attributes.

Growth and branching habit have an effect on determining yield as Giayetto et al (2013) established when they subjected two different groundnut genotypes to different temperatures and sowing date. It was revealed that secondary branches determine yield. This study revealed higher number of main branches than mostly reported as stated above signifying the relationship between the higher yields reported.

Cluster analysis revealed three distinct clusters in a dendrogram truncated at 77% similarity threshold. Genotypes bred for aflatoxin were spread regardless of their breeding source showing their level of dissimilarity similar to Xiong et al (2011). Furthermore, the genotypes belonging to drought tolerant traits were grouped with other genotypes of different traits such as aflatoxin, similar to what Dwivedi et al (2001) found. This means that, although the genotypes were not different in their reaction to aflatoxin, differences still exist as genotypes were clustered differently due to combination of other traits. Therefore, this suggests that the trait can be selected using other traits.

Genetic variability showed that number of branches had a moderate GCV and PCV whilst Aflatoxin had a higher PCV. The rest of the traits namely leaf spot, shelling percentage, flowering and seed yield had low GCV and PCV. Similar results were obtained by other experiments where phenotypic coefficient variation was slightly higher than genotypic coefficient variation insinuating that environmental conditions had an upper hand in influencing the genotypes (Narasimhulu et al, 2012; Nath and Alam, 2002). This means that these traits coupled with low heritability will make it difficult to improve as additive genes were not

influential hence making it difficult to inherit. Genetic coefficient variation measures the extent of variation whilst heritability describes how heritable a trait is. Some of the traits such as number of branches, days to flowering and leafspot had moderate to high heritability. However, except for number of branches, their GCV and GAM was low as a result genetic improvement for these traits would be difficult. Aflatoxin had a high PCV but no genetic parameters making it belong to the same category. This could be as a result of environment interactions as the genotypes were planted in different environments and their reactions were significantly different since aflatoxin accumulation is highly dependent on genotype by environment interactions. In addition, genetic inheritance levels are usually low (Girdthai et al, 2010). It should also be noted that as a result of prolonged dry spells during the growing season, the genotypes were predisposed to high accumulation of the toxin than those that were supplemented with irrigation hence the high CV as combined analysis involved extreme variations. Dry spells expose the risk of aflatoxins accumulation.

Principal component analysis measures variation of traits by grouping similar components into possible parallel components thus reduces the similarity into distinct differences. This is achieved through the construction of PCA biplot, which gives the highest possible cumulative variation. Traits that are close to each other in a biplot imply that they are positively correlated whilst those further from each other at 180^0 angle are negatively correlated and those traits at 90^0 angle are not correlated. The results showed five genotypes namely; MP-68, ICGV-94379, ICGV-93305, CDI-1314 and CDI-0009 that had low aflatoxin and high yield compared to results of other studies (Olayinka and Etejere, 2015; Yambhatnal et al, 2012). Codex (1995) is an international body that sets the limits for food and feed contaminants and toxins such as aflatoxin. The current maximum level of aflatoxin is 15 parts per billion (ppb). Genotype ICGV-93305 had aflatoxin less than 10 ppb, meeting the Codex requirement. However, the maximum levels differ from country to country as others have less than the standard like European market (4 ppb), South Africa, east Africa (10 ppb) and China (20 ppb). Therefore, breeding for this trait should also consider market variations in terms of maximum allowable levels, hence the need to screen these genotypes under various environments for a stable result.

3.7 Conclusion

This study aimed at assessing the level of phenotypic diversity in the groundnut accessions for pre-breeding activities in Malawi. Results indicated that the genotypes were variable for five quantitative traits namely; seed yield, number of branches, leafspot, shelling percentage and days to flowering. Cluster analysis also showed wide diversity as depicted by the number of clusters that were formed. Genetic parameters revealed that the environment played a

significant role in most traits even though most of these had moderate to high broad-sense heritability. PCA revealed five genotypes with higher yields than other studies with low aflatoxin concentration. These are MP-68, ICGV-94379, ICGV-93305, CDI-1314 and CDI-0009. As a result, selection of these genotypes would largely depend on multi-environment testing of the genotypes for aflatoxin tolerance.

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CHAPTER 4
GENETIC DIVERSITY STUDY OF GROUNDNUT GENOTYPES BASED ON SSR MARKERS

Abstract

Genetic diversity is an important aspect as far as improvement of a crop is concerned. A total of 53 groundnut (*Arachis hypogaea* L.) genotypes of diverse origin maintained at Agricultural Research Council – Grain Crops Institute in South Africa (ARC-GCI) were assessed for level of diversity using 20 SSR markers. Results showed that the markers were very informative as revealed by their high polymorphic information content ranging from 0.31 to 0.89 and averaging 0.71. Genotypic diversity analysis through computation of Jaccard dissimilarity indices and clustering indicated that there was wide genotypic diversity with highest dissimilarity index (6.4) between genotype pair RG562 and RG288, and smallest dissimilarity index (0.9) between RG512 and RG562. Allelic diversity analysis showed that there was high diversity among genotypes from southern Africa and southern America as indicated by the Shannon information index, mean number of observed alleles (Na) and mean number of effective alleles (Ne) which were relatively higher than in other groups. Analysis of molecular variation (AMOVA) results indicated that larger variability (59%) was due to variation within individuals whilst the remaining variation was accounted for by variation among individuals within population. This indicated that variation between and within individuals is more significant than between population. Cluster analysis revealed that the discrimination of the genotypes was not dependant on the origin as genotypes belonging to different geographical origins clustered together. It was concluded that the SSR markers were able to detect wide diversity among genotypes and since geographical location did not imply diversity, we conclude that morphological selection between and within individuals would be important in selecting the genotypes for further breeding.

Key Words: Genetic diversity, genetic improvement, groundnut, simple sequence repeats, South Africa

4.1 Introduction

Groundnut, *Arachis hypogaea,* is native to South America (Hammons et al, 2016) specifically southern Bolivia and northwestern Argentina on the eastern slopes of the Andes (Rao, 1987). Ranked as 6th oil seed crop in the world (Nigam, 2014), groundnut production in Africa is at an escalating rate as it contributes over 37% to the world production (FAOSTAT, 2018).

Groundnut is used as food for both humans and farm animals (Asibuo et al, 2008). It is also commonly grown for its edible oil, which makes 40-60% (Singh et al, 2013; Upadhyaya et al, 2005). In South Africa, it is grown by smallholder farmers and used mainly for consumption. Groundnut in South Africa has shown variable production trends over time due to several constraints faced by the sector (FAOSTAT, 2018).

Groundnut production is constrained by several biotic and abiotic factors such as diseases, pests, aflatoxin contamination, nematodes and drought (Singh et al., 2013; Nigam, 2014). Several efforts have been made to improve groundnut production and productivity. However, groundnut has been reported to have a narrow genetic base (Bhad et al, 2016), thus enhancing the genetic base of the crop is one of the strategies that has been put in place in various breeding programmes for successful selection of genotypes. Genetic diversity studies with the aim of assessing the morphological, biochemical and genetic variability present in a given population are crucial for groundnut improvement (Bhad et al, 2016; Sai et al, 2016), hence, knowledge of genetic diversity within or among genotypes is important for the crop's improvement.

Molecular markers have proven to be an efficient tool to assess variation within and among groundnut populations (Kanyika et al, 2015) and to isolate genes linked to desirable traits (Zongo et al, 2017; Bhad et al, 2016; Asibuo et al, 2008; Mace et al, 2006). Simple sequence repeats (SSRs) are one of the PCR- based markers, which have been extensively used for genetic diversity analysis (Moretzsohn et al, 2004). The SSR markers are codominant markers that are relatively abundant, highly polymorphic, and show simplicity of genotyping (Matus and Hayes, 2002). However, little or no information is found on the genetic diversity among groundnut accessions maintained at the Agricultural Research Council – Grain Crops Institute in South Africa.The aim of the present study was, thus, to evaluate the present genetic diversity among the accessions based on simple sequence repeat (SSR) markers, for subsequent breeding and conservation.

Past studies indicate that there is genetic diversity in groundnuts regardless of its narrow genetic status. Krishna et al (2004) observed considerable diversity amongst 48 Valencia groundnut genotypes that were studied in United States of America. More so, 41 genotypes

of diverse backgrounds revealed that Virginia genotypes were more diverse than the other genotypes as reported by Knauft and Gorbet (1989). Several other studies confirmed large genetic diversity (Garba et al, 2015; Zaman et al, 2011; Moretzsohn et al, 2004; Dwivedi et al, 2001; Alam et al, 1985). Nonetheless, low variability has also been reported (Herselman, 2003; Halward et al, 1991).

4.2 Materials and methods

4.2.1 Plant materials

Fifty-three genotypes (Table 4.1) of various origins maintained at Agricultural Research Council-Grain Crops Institute were planted during the 2016/2017 growing season at ARC Potchefstroom, South Africa for leaf sampling and SSR genotyping. Plants were planted at 10 cm apart on ridges measuring 1.0 m in length spaced at 0.75 m apart. Ten leaf samples were collected from each genotype after four weeks of planting for genotyping.

Table 4.1 The geographic origins of the 53 groundnut accessions used in this study

No.	Accession	Country of origin	Geographic origin	No	Accession	Country of origin	Geographic origin
1	RG 46	USA	North America	28	RG 479	Zimbabwe	Southern Africa
2	RG 288	USA	North America	29	RG 355	Zimbabwe	Southern Africa
3	RG 321	USA	North America	30	RG 483	South Africa	Southern Africa
4	RG 394	USA	North America	31	RG 307	South Africa	Southern Africa
5	RG 422	USA	North America	32	RG 313	South Africa	Southern Africa
6	RG 423	USA	North America	33	RG 726	South Africa	Southern Africa
7	RG 489	USA	North America	34	RG 893	South Africa	Southern Africa
8	RG 521	USA	North America	35	RG 1033	South Africa	Southern Africa
9	RG 562	USA	North America	36	RG 1062	South Africa	Southern Africa
10	RG 1042	USA	North America	37	AS-ARC-Oleic	South Africa	Southern Africa
11	RG 1037	USA	North America	38	BS-ARC-Opal	South Africa	Southern Africa
12	RG 327	Bolivia	South America	39	C. TAFA	South Africa	Southern Africa
13	RG 346	Bolivia	South America	40	RG 267	Malawi	Southern Africa
14	RG 347	Bolivia	South America	41	RG 512	Malawi	Southern Africa
15	RG 353	Bolivia	South America	42	RG 863	ICRISAT Malawi	Southern Africa
16	RG 357	Bolivia	South America	43	RG 387	Madagascar	Southern Africa
17	RG 333	Brazil	South America	44	RG 255	Kenya	Others
18	RG 335	Brazil	South America	45	RG 256	Australia	Others
19	RG 337	Brazil	South America	46	RG 410	Senegal	Others
20	RG 418	Brazil	South America	47	RG 416	Senegal	Others
21	RG 414	Argentina	South America	48	RG 532	ICRISAT	Others
22	RG 329	Paraguay	South America	49	RG 536	ICRISAT	Others
23	RG 260	Zimbabwe	Southern Africa	50	RG 571	Taiwan	Others
24	RG 261	Zimbabwe	Southern Africa	51	RG 1056	Semi-runner	Others
25	RG 451	Zimbabwe	Southern Africa	52	RG 1057	Runner	Others
26	RG 452	Zimbabwe	Southern Africa	53	RG 1061	Runner	Others
27	RG 472	Zimbabwe	Southern Africa				

4.2.2 DNA extraction

Fresh leaf samples from 53 groundnut accessions were collected as required by the standard protocol given by SciCorp Laboratories (Pty) Ltd, Pietermaritzburg, South Africa where genotyping was done. Young fresh leaves were harvested from 10 plants of each genotype. The leaf samples were bulked per genotype and placed in a 1.5 ml eppendorf tube and freeze-dried for three days. The dried leaf samples were sealed in a clean small box and posted to SciCorp Laboratories for genotyping. DNA extraction was performed using the standard CTAB extraction protocol. A 100 mg of ground plant tissue was added to 500 µl of CTAB buffer and incubated for an hour at 65°C. After centrifugation, the supernatant was mixed with phenol: chloroform: iso-amyl alcohol (25:24:1). After a second centrifugation, the DNA was precipitated from the aqueous layer by the addition of a salt and ethanol. The upper aqueous phase only (contains DNA) was transferred to a clean microfuge tube. The resulting pellet was dried and re-suspended in TE buffer.

4.2.3 PCR and SSR analysis

Twenty selected polymorphic SSR markers were used to genotype the 53 groundnut accessions (Table 4.2). The genomic DNA samples of all the accessions were amplified through polymerase chain reaction (PCR) using SSR primers. However, due to the poor quality of DNA extracted from 11 genotypes, only 42 genotypes were included in the analysis. PCR products were fluorescently labelled and separated by capillary electrophoresis on ABI 3130 automatic sequencer (Applied Bio systems,). The fragment size of the amplified products was measured. Two approaches were adopted to investigate the genetic structure and diversity among the groundnut accessions. In the first approach, the amplified products were scored for the presence (1) or absence (0) of alleles. The binary data were then used to obtain a dissimilarity matrix using the Jaccard index. The matrix was used to run a cluster analysis based on unweighted neighbor-joining algorithm employing the software DARwin 5.0 (Perrier and Jacquemoud-Collet, 2006). However, to assess the genetic structure within and among genotypes, a second approach based on the co-dominant nature of the marker was adopted and analysis was done using GENALEX version 6.5 (Peakall and Smouse, 2012; Peakall and Smouse, 2006).

Genetic diversity parameters, such as number of alleles per locus (N_a), number of effective alleles per locus (N_e), observed (H_o) and expected (H_e) heterozygosity, and Shannon's Information Index (I) were calculated using GenAlex version 6.5 (Peakall and Smouse, 2012) according to the protocol described by (Nei and Li, 1979). The number of polymorphic loci was

estimated for each predetermined group, based on geographic origin. Further, an indirect estimate of the level of gene flow (Ilesanmi and Ilesanmi, 2011) was calculated using the formula: N_m = 0.25 (1 – FST/FST) using GenAlex. The F-statistics such as genetic differentiation (F_{ST}), fixation index or inbreeding coefficient (F_{IS}), and overall fixation index (F_{IT}) were calculated according to Wright's original derivation (Wright, 1949). Polymorphic information content (PIC) was calculated using the formula: PIC =1 - ΣP_{ij}^2, where P_{ij} is the frequency of j^{th} allele of the i^{th} locus. Nei's unbiased genetic distance was also estimated to determine the degree of population differentiation among the study material. Nei's unbiased genetic distance and identity were estimated according to (Nei, 1978) using GenAlex.

Table 4.2 Description of the simple sequence repeats (SSR) primers used for groundnut genetic diversity analysis

No	Marker	Forward primer	Reverse primer	Repeat Type
1	PM375	CGGCAACAGTTTTGATGGTT	GAAAAATATGCCGCCGTTG	(CT)10
2	PM3	GAAAGAAATTATACACTCCAATTATGC	CGGCATGACAGCTCTATGTT	(GA)14
3	AC2H11	TCCTTTACTTGTGCAGTTGTGC	AAAACGCCATGTGGTGGAT	(CT)18 + (CA)17
4	AC2A04	GATCACTCCAGATAATCAC	AAGGTTATCACTCACGTC	(TG)15
5	TC9B08	GGTTGGGTTGAGAACAAGG	ACCCTCACCACTAACTCCATTA	(GA)22
6	pPGPseq2e6	TACAGCATTGCCTTCTGGTG	CCTGGGCTGGGGTATTATTT	(GA)
7	TC2C07	CACCACACTCCCAAGGTTTT	TCAAGAACGGCTCCAGAGTT	CT)23
8	PMc297	ATG CAC CTG CAA GTG AAG AG	TCA AGG ATG CAG CAA GAC AC	(AAT)4(CAT)(AAT)2
9	PM137	AACCAATTCAACAAACCCAGT	GAAGATGGATGAAAACGGATG	(GA)20
10	AH-10	ATCACCATCAGAACGATCCC	TTTGTAGCCTTCTGGCGAGT	
11	PM183	TTCTAATGAAAACCGACAAGTTT	CGTGCCAATAGAGTTTTATACGG	(CT)24
12	PM50	CAATTCATGATAGTATTTTATTGGACA	CTTTCTCCTCCCCAATTTGA	(TAA)4, (GA)19
13	TC3A12	GCCCATATCAAGCTCCAAAA	TAGCCAGCGAAGGACTCAAT	(TC)27
14	AH-8	ATCATTGTGCTGAGGGAAGG	CACCATTTTTCTTTTTCACCG	
15	TC2D06	AGGGGGAGTCAAAGGAAAGA	TCACGATCCCTTCTCCTTCA	(AG)30
16	PM036	ACTCGCCATAGCCAACAAAC	CATTCCCACAACTCCCACAT	(GA)18
17	IPAHM103	GCATTCACCACCATAGTCCA	TCCTCTGACTTTCCTCCATCA	
18	PM35	TGTGAAACCAAATCACTTTCATTC	TGGTGAAAAGAAAGGGGAAA	(GA)18(GAA)2
19	TC11C06	TCCAACAAACCCTCTCTCTCT	GAACAAGGAAGCGAAAAGAA	(CT)5 + (TC))13
20	SEQ3A05	CATTCTCATTCTCTCATTCATTCA	CGAACCCTCTGATTTGTGAT	(TC)11 + (CA)7

Source:www.biomedcentral.com/content/supplementary/1471-2229-10-17-s1.xls and www.biomedcentral.com/content/supplementary/s12863-016-0337-x-s1.xlsx

4.3 Results

4.3.1 Allelic diversity of SSR markers

Fifty-three (53) genotypes were collected for genotyping, however, 17 genotypes were omitted from the analysis. For these 17 genotypes, the SSR primers either failed to amplify any band or less than 2% of the markers were amplified. The 20 SSR primer pairs used in this study amplified 162 putative alleles (different fragment sizes). Of which more than 59% (96) were effective in discriminating the genotypes. The genotypes showed a wide range of allelic diversity from 3 to 15, with a mean of 8.10 alleles per locus. The highest allele number was observed from marker pPGPseq2e6 and the lowest was from SEQ3A05. The PIC value ranged from 0.31 to 0.89, with a mean of 0.71. Most of the markers were polymorphic with PIC values of > 0.50 except two markers (AH-10 and SEQ3A05). Markers AC2A04 and AH-10 had the same number of alleles, however, the PIC values were 0.72 and 0.32, respectively. This was observed due to the differences in allele frequencies in that the major allele frequency in AC2A04 was 0.30 while the major allele frequency in AH-10 was 0.82. All the alleles amplified by the SSR primers in this study showed an allele frequency of less than 0.50 except for two markers (AH-10 and SEQ3A05) suggesting even distribution among the genotypes.

The mean observed heterozygosity per locus was 0.57 and with the highest (1.00) and lowest (0.03) values detected from PM35 TC11C06 and TC9B08, and TC3A12, respectively. About 45% of the markers showed H_0 value of > 0.80 and a negative inbreeding coefficient (F_{IS}) value. F_{IS} values represent the average deviation of the population's genotypic proportions from Hardy-Weinberg equilibrium and the values ranged from 0 to 1. A negative F_{IS} value represents an excess of heterozygotes. For example, for loci TC3A12, PM35 and TC11C06, 73%, 87% and 81% of the genotypes are expected to be heterozygous at the specific loci under random mating conditions, respectively. However, 100% of the genotypes at these loci were heterozygotes. It may be due to high outcrossing or mutation at the specific loci. Gene diversity (H_e) ranged from 0.32 (AH-10) to 0.9 (IPAHM103 and PM3) with a mean of 0.75 was detected. Table 4.3 summarises these results.

Table 4.3 Genetic diversity parameters generated by 20 SSR markers among groundnut accessions

Loci	%GA	Na	Ne	Ho	He	FIS	PIC	MA	MAF
PM375	92.31	11	7.30	0.67	0.88	0.23	0.86	121	0.25
PM3	94.87	11	8.78	0.97	0.90	-0.10	0.88	221	0.16
AC2H11	61.54	4	2.55	0.46	0.62	0.25	0.54	158/241	0.44
AC2A04	89.74	4	3.86	0.97	0.75	-0.31	0.72	191	0.30
TC9B08	82.05	7	4.52	0.03	0.79	0.96	0.76	113	0.34
pPGPseq2e6	89.74	15	7.40	0.60	0.88	0.31	0.86	267	0.26
TC2C07	84.62	7	2.61	0.82	0.63	-0.33	0.51	223	0.56
PMc297	61.54	4	3.73	0.58	0.75	0.20	0.71	240	0.33
PM137	76.92	6	3.96	0.03	0.76	0.96	0.73	164	0.33
AH-10	48.72	4	1.46	0.37	0.32	-0.17	0.32	254	0.82
PM183	51.28	8	3.72	0.45	0.75	0.38	0.70	147	0.38
PM50	71.79	12	7.54	0.32	0.88	0.63	0.86	121	0.25
TC3A12	71.79	7	3.50	1.00	0.73	-0.40	0.67	182	0.45
AH-8	58.97	7	4.62	0.26	0.80	0.67	0.77	252	0.30
TC2D06	56.41	6	3.18	0.00	0.70	1.00	0.64	215	0.41
PM036	92.31	13	5.34	0.86	0.82	-0.06	0.80	221	0.28
IPAHM103	94.87	16	9.22	0.95	0.90	-0.06	0.89	147	0.18
PM35	87.18	11	7.16	1.00	0.87	-0.16	0.86	110/112/125/158	0.16
TC11C06	97.44	6	5.09	1.00	0.81	-0.24	0.80	186/199	0.22
SEQ3A05	92.31	3	1.95	0.06	0.49	0.89	0.31	232	0.64
Mean	77.82	8.10	4.87	0.57	0.75	0.23	0.71	-	0.35
SE	3.54	0.87	0.51	0.08	0.03	0.11	0.04	-	0.04

%GA= percentage of genotypes amplified; N_a= Number of alleles per locus; N_e = number of effective alleles per locus; H_o= observed heterozygosity, H_e = expected heterozygosity; F = Inbreeding coefficient; PIC = polymorphic information content, MA= major allele; MAF major allele frequency per locus, SE= Standard error

4.3.2 Population divergence

Genetic parameter estimates of groundnut populations stratified based on geographic origin are presented in Table 4.4. Genotypes that originated from southern Africa revealed the highest variation for most of the genetic parameters. The mean observed (Na) and effective (Ne) number of alleles was higher for genotypes from southern Africa and South America, respectively. Shannon information index was higher for genotypes from southern Africa followed by genotypes from South America with mean values of 1.51, and 1.42, respectively. The highest mean observed heterozygosity (0.60) was observed from genotypes originated from North America and the lowest H_0 (0.54) was detected from South America genotypes. On the contrary, the highest expected mean gene diversity (0.76) was detected from genotypes driven from southern Africa followed by South America genotypes (0.74). The mean fixation index was relatively higher for South America and Southern Africa genotypes. Highest number of private alleles (19) per population was detected from Southern Africa collections followed genotypes collected from diverse origin (10). All genotypes originating from South and North America, and Southern Africa showed the highest percentage of polymorphic loci (Table 4.4).

Table 4.4 Genetic diversity parameter estimates of groundnut populations based on geographic origin

Population	N	Na	Ne	I	Ho	He	F_{IS}	PA	%P
North America	7	4.75	3.52	1.31	0.60	0.73	0.11	9.00	100.0%
South America	10	5.45	4.25	1.42	0.54	0.74	0.24	8.00	100.0%
Southern Africa	14	6.10	4.31	1.51	0.58	0.76	0.20	19.00	100.0%
Others	8	4.75	3.82	1.30	0.56	0.72	0.19	10.00	95.0%
Mean	7.59	5.26	3.97	1.39	0.57	0.74	0.18	-	98.8%
SE	0.30	0.27	0.21	0.05	0.04	0.02	0.06	-	1.25%

N= Number of observations; N_a= number of alleles per locus; N_e= number of effective alleles per locus; I= Shannon's information index; H_o= observed heterozygosity; H_e= expected heterozygosity; F_{IS}= Inbreeding coefficient; PA = Private allele per population; %P = Percentage polymorphic loci

Genetic differentiation (FST) among the geographic origin ranged from 0.041 between South America and southern Africa and 0.059 between North America and others, suggesting there was little to moderate differentiation among the four populations (Wright, 1978). The relatively

low values of FST imply that there is high frequency of identical alleles among population. Gene flow among the groundnut population within geographic origin ranged from 3.99 between North America and genotypes collected from diverse sources to 5.90 between Southern Africa and others (Table 4.5). The populations maintained higher genetic identity and low genetic distances.

Table 4.5 Pair-wise estimates of gene flow (above diagonal, within the brackets), genetic differentiation (F_{ST}) (above diagonal off brackets); genetic distance (G D) (lower diagonal off brackets) and genetic identity (GI) (lower diagonal within the brackets).

Population	North America	South America	Southern Africa	Others
North America		0.043 (5.59)	0.052 (4.55)	0.059 (3.99)
South America	0.021 (0.98)		0.041 (5.90)	0.052 (4.55)
Southern Africa	0.111 (0.89)	0.057 (0.95)		0.043 (5.60)
Others	0.078 (0.93)	0.067 (0.94)	0.021 (0.98)	

4.3.3 Analysis of molecular variance (AMOVA)

Analysis of molecular variance among groundnut populations stratified based on geographic origin are shown in Table 4.6. No significant genetic differentiation was observed among the four populations (P =0.955). However, a highly significant difference (P < 0.001) of molecular variation was observed among individuals within the population. Similarly, highly significant (P < 0.001) variation was detected within individual in all the 39 groundnut genotypes collected from diverse geographic locations. Larger genetic variability (59%) was attributed to variation within individuals, and the remaining variation was explained by variation among individuals within population (Table 4.6). This signifies that in groundnut the between and within individual variation is more crucial that the between population variation.

Table 4.6 Analysis of molecular (AMOVA) among 53 groundnut accessions classified based on geographic origin using 20 SSR markers

Source of variation	df	SS	MS	Est. Var.	Perc. Var.	F-statistics
Among populations	3	28.511	9.504	0.000	0%	0.955
Among individuals	35	393.796	11.251	3.267	41%	0.001
Within individuals	39	184.000	4.718	4.718	59%	0.001
Total	77	606.308		7.985	100%	

Df = degrees of freedom; SS = sum of squares; MS = mean squares, Est. var.= Estimated variance, Perc. Var = Percentage variance

4.3.4 Cluster analysis

The genetic relationship among the 39 groundnut genotypes was assessed using neighbour-joining algorithm using the unweighted pair group method. The analyses indicated the presence of two distinct subpopulation (Figure 4.1). The clustering patterns of the genotypes did not match with the geographical origin probably due to high gene flow (Table 4.5). Cluster I contained the highest proportion of the genotypes (62%) and dominated by the southern Africa collections. This cluster further sub-divided into four sub-clusters. Cluster II had three sub-clusters comprising of 15 genotypes. This cluster was represented by relatively equal proposition of genotypes from each subpopulation.

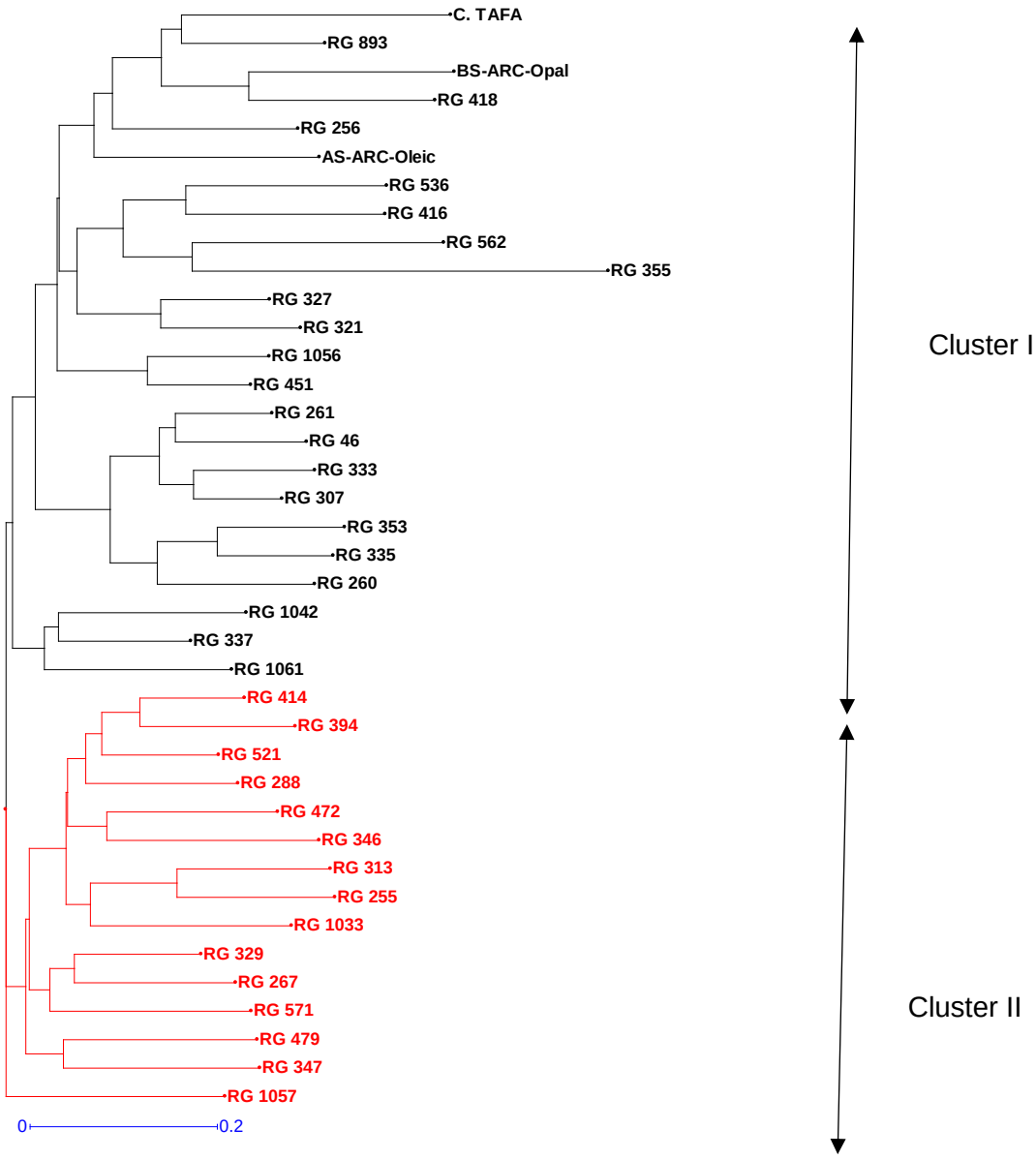

Figure 4.1 Un-weighted pair group method (UWPGMA) dendogram showing genetic relationship of the 39 groundnut genotypes determined using 20 selected SSR markers

4.4 Discussion

The current study examined genetic diversity and population structure of groundnut accessions collected from diverse geographic origins and maintained at ARC-GCI using SSR markers. The SSR markers produced number of alleles ranging from 4 to 11. Hildebrand et al (1994) and He et al (2003) reported similar results. However, Koppolu et al (2010) reported much higher number of alleles ranging 4 – 28. All the SSR markers used in this study were highly polymorphic with a mean number of 8 alleles per locus. This result is much higher than in previous findings reported by various researchers (He et al, 2005; He et al, 2003; He and Prakash, 1997). Of the total 162 putative alleles detected, 59% of the alleles were effective in discriminating the genotypes suggesting the alleles were evenly distributed among the genotypes. The major allele frequency ranged from 0.16 to 0.82 with a mean of 0.35. Goddard et al (2000) suggested that markers with major allele frequency between 0.5 and 0.8 can be useful in QTL mapping. Therefore, markers PMc297, AH-10 and SEQ3A05 can be useful in providing information about linkage disequilibrium and QTL mapping in groundnut.

The PIC range observed was from 0.31 to 0.86 in this study was similar to that reported by (Moretzsohn et al, 2004; Matus and Hayes, 2002). However, the mean PIC value obtained in the current study was 0.71 and values with > 0.50 were observed in 90% of the loci analysed. This result was much higher than the findings of Cuc et al (2008) where only 34% and 44% of SSR markers showed PIC values > 0.50, respectively. This suggested that the loci used in this study were highly polymorphic and the observed alleles were evenly distributed within the genotypes. This, in turn, indicates that these markers had a high discriminatory power and were found to be highly suitable for genetic diversity analysis (Tang et al, 2007). In case agro-morphological traits failed to detect variability due to the similarity in growing environments, SSR markers can be a useful tool in discriminating differences of genotypes at molecular level. Tang et al (2007) in their genetic diversity analysis of groundnut genotypes that belong to var. *hirsuta* in southern China using agro-morphological traits found that all the genotypes were similar. However, using SSR markers they were able to discriminate the variation present among the genotypes (Tang et al, 2007).

The mean observed heterozygosity (H_o) of 0.57 and fixation index (F_{IS}) value of 0.23 was detected in this study. The high H_0 and the low F_{IS} values suggested that these genotypes are highly heterozygous and this is not the case with self-pollinated crops such as groundnut (Sharma et al. 2016). This might have resulted from mutation or high natural outcrossing rate. However, it was reported that groundnuts exhibit low natural outcrossing rates ranging from 0 to 8% (Reddy et al, 1993; Knauft et al, 1992). The other more logical reason might be that genotypes were sampled from breeding population at early stages of the breeding cycle.

Similarly, high mean expected heterozygosity (He) value of 0.75 was observed among the genotypes, indicating the possibility of two randomly sampled alleles in a given genotype to be diverse was greater than 75%. This, is due to the fact that SSR markers are co-dominant in nature hence able to discriminate the differences (Pandey et al, 2014). As a result, it suggested that this collection of genotypes was highly genetically diverse and this is a good foundation for genetic improvement of the crops considering that the genetic base of groundnuts genetically stands at a lower level. As a result, these individuals could be potentially used as parents for future breeding.

Analysis of molecular variance among groundnut populations revealed 41 and 51% of the variation was attributable to among individuals and within individuals respectively. The geographic origin had no impact on the genetic diversity of the crop as it was revealed by low to moderate genetic differentiation observed among the regions. According to standard guidelines for the interpretation of genetic differentiation (Wright, 1978), the range 0.0 - 0.005 indicates little, 0.05 - 0.15 moderate, 0.15 - 0.25 great, and above 0.25 very large genetic differentiations. The results indicate that genetic differentiation was relatively low (0.041) between South America and Southern Africa and moderate (0.059) between North America and others. This might have resulted from the high gene flow (3.99 – 5.90) observed among the regions. According to Slatkin (1989) and Morjan and Rieseberg (2004), gene flow <1 is considered to be low, while $N_m = 1$ is considered to be moderate and $N_m > 1$ is considered to be high. Moderate or relatively low levels of gene flow can significantly alleviate the loss of genetic diversity by preventing the effect of genetic drift (Aguilar et al, 2008). The high level of gene flow observed may be attributed to an exchange of genetic materials. Cluster analysis revealed two main distinct genetic groups among the studied groundnut genotypes revealing wide genetic diversity for breeding and strategic conservation. The clustering of genotypes was independent of geographical origin in that genotypes from different geographic origin were clustered in the same group. This is common that groundnut genotypes of the same botanical group were clustered in different groups, as was reported by Xiong et al (2011).

4.5 Conclusion

In conclusion, this study was undertaken to assess the genetic diversity present among the 39 groundnut accessions using a set of 20 SSR markers. The results of this study highlight that the germplasm used in this study were genetically diverse and they can be used as a good foundation to select potential genotypes for further genetic improvement and broadening of the genetic base of the crop. However, selection may be more meaningful, if the information obtained in the current study can be supplemented by morphological data. A set of 20 SSR markers were able to detect considerable level of genetic diversity among the groundnut

genotypes collected from diverse geographic origins. The SSR markers proved to be more reliable and efficient in discriminating the genotypes into two distinct subpopulations. However, stratification based on geographic origin had no influence on the genetic diversity of the crop. This suggested that future germplasm collection programmes should not be based on geographical background rather should be dictated by the prevailing morphological variation.

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CHAPTER 5
STUDY OVERVIEW

5.1 Introduction

Groundnut is an important crop worldwide including Malawi and South Africa. Its nutrition composition and food benefits make it significant for its production to be boosted with limited resources available such as use of improved seed. However, breeding of improved seed is limited by the state of the crop genetically as it has a narrow genetic base hence the need for continuous deliberate exploitation of other sources of diversity. Genetic diversity is the foundation of all variation. However, it cannot be a stand-alone attribute as phenotypic diversity completes the whole process of selection. As a result, the need to know the status of these two is relevant for any breeding program. In addition, aflatoxins are also a bottleneck to production and post-harvest management hence the need to identify sources of resistance coupled with good agronomic attributes. Therefore, the objectives of this study were 1) to identify phenotypic diversity of groundnuts obtained from ICRISAT Malawi and Chitedze Agricultural Research Station (CARS-Malawi) using agro-morphological traits such as aflatoxins and yield and 2) to assess the level of genetic diversity of groundnut accessions maintained at ARC-GCI in South Africa using SSR markers.

5.2 Summary of results

5.2.1 Phenotypic diversity of Malawian groundnuts

Twenty-six groundnut genotypes with one local check were used to assess the level of phenotypic diversity during the 2016/2017 winter period and 2017/2018 rainy season in three locations. Data was analysed using Genstat 18 and SAS 9.4 to obtain analysis of variance, principal components analysis and genetic variability parameters.

- Analysis of variance revealed that the genotypes' seed yield, number of branches, shelling percent, leaf spot disease and days to flowering were variable except for aflatoxin concentration and groundnut rosette disease.
- Hierarchical clustering further divided the genotypes into three clusters showing variability between them. Most genotypes (16) were grouped into the first cluster, which was made up of 11 genotypes from ICRISAT Malawi alongside five of the eight genotypes from Chitedze Agriculture Research station.

- Moderate to high heritability (0.56-0.71) was estimated for leafspot disease, days to flowering and number of branches indicating that genetic improvement for these traits is possible through genetic inheritance.
- Low GCV and PCV (<10%) values were observed for flowering, seed yield, leafspot and shelling percent whilst number of branches had moderate (16.22%) GCV and (13.67) PCV. Aflatoxin had high PCV (64.72) and zero GCV indicating that the environment had a higher influence on this trait making breeding complex.
- Five genotypes namely; ICGV-94379, ICGV-93305, MP-68, CDI-1314 and CDI-0009 were identified as the best genotypes with high yields and low aflatoxin content. These could be potentially used in breeding activities at ICRISAT.

5.2.2 Assessment of genetic diversity using SSR markers

- Twenty SSR markers were used to produce the molecular data of 39 groundnut genotypes
- High polymorphic information content was reported that ranged from 0.31 to 0.89 and averaged 0.71.
- Highest genetic distance (6.4) was between genotype pair RG562 and RG288 indicating that they are diverse hence could be used as potential parents whilst the smallest dissimilarity index (0.9) was between RG512 and RG562 showing that they are close.
- Southern Africa and southern America accessions showed that there was wide diversity among them as indicated by the Shannon information index, mean number of observed alleles (Na) and mean number of effective alleles (Ne) which were relatively higher than in other groups.
- AMOVA results showed that larger variability (59%) was due to variation within individuals whilst the remaining variation was accounted for by variation among individuals within population.
- Cluster analysis revealed two main clusters that were discriminated regardless of geographical origin.
- SSR markers were able to detect wide diversity in the accessions giving the potential that these accessions can be used as parents in breeding programmes.

5.3 General recommendations

The following recommendations and implications were realised from this study;

Most traits in this study were identified as variable except aflatoxin and rosette disease giving the potential to be used in breeding programmes. Genotypes that were identified as having high yield and low aflatoxin content can be used for further breeding activities in breeding programmes. However, there will be a need to do multi-location testing of these genotypes for stability analysis of traits like yield and aflatoxin that tend to be variable in response to environment.

SSR markers are a tool that can be used in assessing genetic diversity in groundnuts as they are able to reveal high polymorphism even in narrow based genetic diversity. The diversity revealed in this study can be used to pair accessions such as RG562 and RG288 using genetic distances for diverse sources of various traits. Therefore, identifying potential sources of resistances for various traits in these genotypes will be essential for introgressing resistant genes.