ASSESSING THE MORPHOLOGICAL VARIATION AND CHARACTERISING THE PROTEINS OF BAMBARA GROUNDNUT (*VIGNA SUBTERRANEA* L. VERDC).

BY

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DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in any candidature for any degree.

Signed …….. Date ……...

STATEMENT 1

This dissertation is being submitted in partial fulfilment of the requirements for the degree of Magister Technologiae Biotechnology.

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This dissertation is the result of my own independent investigation, except where otherwise stated. Other sources are acknowledged by giving explicit references. A bibliography is appended.

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ABSTRACT

Bambara groundnut (*Vigna subterranea* L. Verdc) is an underutilized crop in the African continent. It is a drought tolerant crop and fixes atmospheric nitrogen. Bambara groundnut is primarily grown for the protein content of its seeds and is mainly produced by small scale farmers at the subsistence level. However, despite its importance as a subsistence crop in many African countries, only local landraces of bambara groundnut are still cultivated. Mass selection of a few local varieties for the main agronomic characteristics has been carried out. All the bambara groundnut germplasm in South Africa has not been morphologically characterized. Although the protein of bambara groundnut is of good quality and is rich in lysine, there is no information on the characterisation of these proteins. The presence of antinutritional factors in the crop has also received little attention. This study focused on three major objectives including: (I) to assess the extent of morphological variations among thirty selected landraces of bambara groundnut, (II) to characterize the major seed proteins in these accessions using one dimensional gel electrophoresis, and (III) to determine the presence of any anti-nutritional factors in the seeds of the selected bambara groundnut landraces.

30 accessions of bambara groundnut were evaluated for their variability in agronomic and morphological traits. The field experiment was conducted at ARC-VOPI in Roodeplaat research farm during the 2014/2015 summer cropping season. The field trial was arranged as a complete randomized block design with 3 replications. 18 quantitative traits were recorded to estimate the level of genetic variability among accessions. 4 different methods were employed to extract seed proteins from 30 bambara groundnut accessions in order to ascertain the best method for protein extraction. These methods included: 10%-80% isopropanol, 10% trichloroacetic acid (TCA) in acetone solution, sonication and 2x Lammeli buffer extraction methods. The quick start Qubit® fluorometer protein kit was used to determine the protein concentration in each sample. The samples were then subjected to one dimensional gel electrophoresis. For antinutritional analysis, 5 factors (condensed tannins, free and phytic acid phosphate, polyphenol and trypsin contents) were used to determine the amount of antinutrient in 30 bambara seeds that were ground to a fine powdery flour. 3 replicates of all the samples were ground for each assay evaluated. The flour was then immediately extracted and used for the different assays.

The analysis of variance revealed significant differences only in 10 of the 18 phenotypic traits that were evaluated. The UPGMA cluster analysis based on the quantitative traits produced

four distinct groups of genotypes and a singleton. Genotypes SB11-1A, SB19-1A, SB12-3B and Bambara-12 were found to possess good vegetative characters and are recommended for use as suitable parents when breeding cultivars for fodder production. Desirable yield and yield-related traits were identified in B7-1, SB4-4C, SB19-1A, Bambara-12 and SB16-5A and are recommended as suitable parental lines for bambara groundnut grain production improvement. The quantitative characters therefore provided a useful measure of genetic variability among bambara genotypes and will enable the identification of potential parental materials for future breeding programmes in South Africa.

Out of the 4 different seed protein extraction methods exploited for this study, the 2x Laemmli buffer extraction method produced the best result with clear protein bands. A unique feature from all extraction methods was the presence of a common protein band at ~ 75 kDa. All extraction methods except 10 % TCA-Acetone resolved common banding patterns in all the bambara groundnut samples. This data suggests that there is very little or no intraspecific genetic diversity among the seed proteins of bambara groundnut accessions studied.

There was wide variation in the content of the five antinutritional compounds among the thirty bambara groundnut accessions. The mean values for condensed tannin content ranged between 0.20 - 6.20 mg/g. Free phosphate recorded an overall mean of 1.71 mg/g while a range of 1.35 - 4.93 mg/g was observed by phytic acid phosphate (PAP). The polyphenol content had an overall mean of 0.39 mg/g and trypsin inhibitor (TIA) was quite variable among the bambara groundnut accessions ranging from 5.30 - 73.40 TIA/mg. Generally, higher levels of antinutrients were observed in this study compared to the other studies. The results obtained in this study led to a conclusion that although variations exits among the accessions studied, further research is required to verify the extent of morphological variations, the efficiency of protein extractions methods evaluated and the effects of these antinutrients in human and animal feeds.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 Background

Bambara groundnut (*Vigna subterranea* L. Verdc) is a legume indigenous to Africa and primarily grown for its seeds. It is becoming increasingly popular as a food crop in the rural areas of many countries in Africa (Vurayai *et al.* 2011). Bambara groundnut has been ranked as the third most important grain legume after groundnut *(Arachis hypogaea* L.*)* and cowpea *(Vigna unguiculata)* (Howell *et al.* 1994) in Africa. The crop has been cultivated in the tropical regions of sub-Saharan Africa and in Madagascar for many centuries (Godwin & Moses 2013). Bambara groundnut is essentially grown for human consumption and has been described as a complete balanced diet due to the high carbohydrate (65%) and protein (18%) content of its seed (Ouedraogo *et al*. 2008).

The immature seeds of the crop can be boiled or grilled before being eaten while the mature seeds can be roasted in oil or ground into flour and then mixed with oil or butter to form a porridge. In South Africa and Swaziland, bambara groundnut is used to add variety to the daily diets and the boiled seeds can also be pounded and mixed with samp or used to make a soup (Masindeni 2006). The leaves can also be used to feed livestock. Traditionally, it is used to cure nausea especially in pregnant women by chewing and swallowing the raw bean (Department of Agriculture, Forestry and Fisheries 2011).

Bambara groundnut is a drought tolerant crop and is readily adaptable to different environmental conditions and has the ability to be intercropped making it an important economic crop in many developing countries (Rungnoi *et al.* 2012). This legume has a symbiotic relationship with bacteria (rhizobia) that form root nodules. Rhizobia can fix free nitrogen from the air and incorporates it in the root tissue of its host plant (Masindeni 2006). This increases the amount of nitrogen in the soil which is directly beneficial to agriculture. Consequently, farmers may require less or no fertilizer during cultivation thus saving on much needed and scarce resources.

In Africa, bambara groundnut is confined to the dry regions, between the desert and the savanna (Southern fringe of the Sahara) and adapted to growing in areas of relatively higher temperatures for many leguminous crops (Tindall 1997). Bambara groundnut is not attacked by diseases and pests in any of its production regions. However, in damp conditions, it may be

susceptible to various fungal diseases (Baudoin & Mergeai 2001). It has a very low insect pest and disease susceptibility (Tweneboah 2000).

The breeding system of bambara groundnut is not well understood and landraces of the crop are still been cultivated. There is a need to develop improved varieties for particular agro-ecological conditions or production systems. Many researchers have used several morphological traits to characterize bambara groundnut accessions. Goli *et al.* (1997) characterized and evaluated the collection of bambara groundnut at the International Institute of Tropical Agriculture. The variability between local and exotic bambara groundnut landraces in Botswana was reported by Karikari (2000). Jonah *et al.* (2012) evaluated the seasonal variation and the correlation between yield and yield components in bambara groundnut accessions in Nigeria. Another study in Nigeria used multivariate analysis and character association for the growth and yield of bambara groundnut (Jonah *et al*. 2014). Mohammed (2014) studied pre-breeding of bambara groundnut accessions in Kano State of Nigeria. Shegro *et al.* (2013) reported variation in bambara groundnut using morphological traits in South Africa. A large number of bambara groundnut landraces are planted in South Africa and it is important to further assess the variation in these germplasms for use in breeding programmes.

The continuous increase in population and inadequate supply of protein has inadvertently increased the occurrence of malnutrition in developing countries (Siddhuraju *et al.* 1996). Adebowale *et al.* (2005) reported that plant protein products have gained increased interest as ingredients in food systems throughout many parts of the world and the success of utilizing plant proteins as additives depends greatly upon the favourable characteristics that they impart to foods. The partial replacement of animal foods with legumes has been shown to improve overall nutritional status (Guillion & Champ 1996) due to lower cholesterol level in plant foods. Polyacrylamide gel electrophoresis (PAGE) is considered as a useful tool for study in population genetics (Ghafoor *et al.* 2002; Nisar *et al.* 2007). There are no reports on an efficient extraction protocol for bambara groundnut seed protein. Hence this study will also assess four different protein extraction methods for bambara groundnut using one dimensional gel electrophoresis.

According to Holloway & Bradbury (1999), various legumes such as bambara groundnut also contain certain nutritional inhibitors and toxic substances. These nutritional inhibitors are called antinutritional factors. These factors modify the nutritional value of some staple foods and the effect can be detrimental on the health of consumers (Offor *et al.* 2011).

The digestion and bioavailability of the nutrients in the seeds of bambara for animal and human nutrition is limited by antinutrients such as trypsin inhibitors, condensed tannins and phytate (Hefnawy 2011). Other antinutrients such as phenols may play beneficial roles in human diets by acting as anti-carcinogens or by promoting health in other ways such as decreasing the risk of heart disease or diabetes (Holloway & Bradbury 1999). Recent studies have shown that malnutrition among children in developing countries is mainly due to the consumption of cereal based porridge which is bulky, low in energy and density and high in antinutrients (Michaelsen & Henrik 1998). In order to improve the protein quality of most staple foods, it is important to minimize or eliminate their toxins and antinutritional factors. Osagie (1998) reported that simple boiling, cooking and soaking can reduce the concentration of antinutrients in food.

Due to the high nutritional variability of bambara groundnut, it is mainly grown for its edible seeds for protein content and shows complementary advantage when consumed with cereals crops (Doku 1996; Ntundu *et al.* 2006; Olukolu *et al.* 2012). The crop is rich in iron and protein with a high lysine and methionine content (Adu-Dapaah & Sangwan 2004; Massawe *et al.* 2005). Despite these advantages, it is unfortunately, one of the neglected and under-utilized crops in sub Saharan Africa (Okpuzor *et al.* 2010). Bambara groundnut has a high potential for food security especially in unpredictable drought prone regions (Baryeh 2001). The presence of anti-nutritional factors in the crop has received little attention and the types of proteins in bambara groundnut are not known.

1.2 Research Aim

The aim of this study was to assess the extent of morphological variation and identify the major proteins and antinutritional factors in bambara groundnut accessions in South Africa.

1.3 Research Objectives

i. To assess the extent of morphological variation among 30 selected landraces of bambara groundnut.

ii. To characterize the major seed proteins in bambara groundnut accessions using one dimensional gel electrophoresis.

iii. To determine the presence of any antinutritional factors in the seeds of the selected bambara groundnut landraces.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Botanical origin and distribution

Bambara groundnut, (*Vigna subterranea (L) Verdc.*), is a leguminous crop that originated in West Africa. It has been widely cultivated in the tropical regions of Africa since the seventeenth century (Yamaguchi 1983). Bambara groundnut was first mentioned in the 17th-century literature (Marcgrav de Liebstad 1648), where it was referred to as 'mandubi d'Angola'. In 1806, Du Petit-Thouars found the crop in Madagascar, under the vernacular name 'voanjo', subsequently written as 'voandzou' in French. He then proposed the name *Voandzeia subterranea* (L.) Thouars, a name that was widely used by subsequent researchers for over a century. In addition to being present in sub-Saharan Africa, it is now found in many parts of South America, Asia and Oceania (Baudoin & Mergeai 2001). Purseglove (1992) believed that bambara groundnut was taken at an early date to Madagascar, probably by Arabs and it reached Brazil and Surinam in the early seventeenth century. It was later taken to the Philippines, India, Malaysia, Thailand and Indonesia by slaves. Investigators interested in the origin of bambara groundnut (Dalziel 1937; Jacques-Felix 1946; Rassel 1960; Hepper 1963; Begemann 1988a) all agreed that the crop originated from the African continent. The common name actually appears to be derived from a tribe, 'the bambara' who now lives mainly in Mali (Goli 1997).

Figure 1: Bambara groundnut field at 50% maturity (Source: ARC-VOPI Research farm Roodeplaat Pretoria, 2015).

The exact area of origin of bambara groundnut in Africa has been a matter of debate. In 1937, bambara groundnut was found by Dalziel in its genuinely wild state, in the North Yola province

of Nigeria. The author reported that Ledermann also found the wild plant the same year, near Garoua in northern Cameroon. Dalziel's finding was confirmed by Hepper in 1970. The distribution of wild bambara groundnut is known to extend from the Jos Plateau and Yola in Nigeria to Garoua in Cameroon and probably beyond (Goli 1997). As further confirmation, Begemann (1988a) carried out detailed analyses of the seed-pattern diversity within the large collection of bambara groundnut at IITA. The author found that samples collected less than 200 km from the putative center of origin, between Yola and Garoua, consistently showed a greater seed-pattern diversity. Diversity indices for the number of days to maturity, pod length, number of stems per plant and internode length, were comparatively higher for accessions from Nigeria and Cameroon. His conclusion confirmed the hypothesis that the center of origin of bambara groundnut is in the region of northeastern Nigeria and northern Cameroon.

There are different opinions on how bambara groundnut reached South Africa. According to Swanevelder (1998), the people of Bolobedu claimed they brought the crop when they first arrived in the south, while on the other hand the Venda people claimed they are the ones who first came with it from Central Africa. The latter case is supported by some proof: the name 'Ndluhu-mvenda', which means groundnut of Vendaland, is still used today. Locally it is called various names such as Phonda (Venda), jugo beans (Xhosa), Ditloo-marapo (Sepedi) and Tindhluwa (Tsonga) (Holm & Marloth 1940). In some other African countries, the nuts are also known as ntoyo ciBemba (Republic of Zambia), Gurjiya or Kwaruru (Hausa, Nigeria), Okpa (Ibo, Nigeria), Epa-Roro (Yoruba, Nigeria) and Nyimo beans (Zimbabwe) (Bamshaiye *et al*. 2011).

2.2 Bambara groundnut taxonomy

Bambara groundnut belongs to the family *Leguminosae*, subfamily Papilionoideae and genus *Vigna* (Jonah *et al*. 2012). It was described in *Species Plantarum* in 1763 by Linnaeus and named *Glycine subterranea*, in accordance with his system of nomenclature. Recently, detailed botanical studies were undertaken by Maréchal *et al*. (1978) who found great similarities between bambara groundnut and plant species of the genus *Vigna*. This confirmed studies done by Verdcourt, who seized the opportunity in 1980 to propose the current name *Vigna subterranea* (L.) Verdc. *Vigna* consists of approximately 80 species that are grouped into six subgenera: *Vigna, Ceratotropis, Plectotropis, Sigmoidotropis, Lasiosporon* and *Haydonia* (Table 1) (Molosiwa 2012).

Table 1: Summary of *Vigna* classifications based on 6 sub-genera, and some examples from each section. Adapted from (African *Vigna*, Available from www.bioversityinternational.org).

Subgenus	**Section**	**Species**
Vigna	Vigna	*V. subterranea*
	Comosae	*V. comosa, V. haumaniana*
	Macrodontae	
	Reticulatae	*V. reticulata*
	Liebrechtsia	*V. frutescens*
	Catiang	*V. anguiculata*
Haydonia	Haydonia	*V. monophylla*
	Microspermae	*V. microsperma*
	Glossostylus	*V. nigritia*
Plectotropis	Plectotropis	*V. verixllata*
	Pseudoliebrechtista	*V. nuda*
Ceratotropis	Ceratotropis	*V. mungo, V. radiata*
	Aconitifoliae	*V. aconitifolia*
	Angulares	*V. angularis*
Lasiospron	Lasiospron	*V. longifolia*
Sigmoidotropis	Sigmoidotropis	*V. elegans*
		V. peduncularis
		V. caracalla
		V. venusta
		V. adenantha

Subgenus *Vigna* (Fig. 2) comprises 39 species, and the important agricultural crops in this genus includes: azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi], bambara groundnut [*Vigna subterranean* (L.) Verdc.], blackgram [*Vigna mungo* (L.) Hepper], cowpea [*Vigna unguiculata* (L.) Walps], mothbean [*Vigna aconitifolia* (Jaqc.) Maréchal] mungbean [*Vigna radiata* (L.) Wilczek] and rice bean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi]. Bambara groundnut and cowpea originated from Africa, while the other five species are of Asian origin (Rungnoi *et al*. 2012). The species *subterranea* is further divided into two groups: *var. spontanea*, comprising the wild forms found in a small area around northern Cameroon and Nigeria, and *var. subterranea* comprising the cultivated forms in parts of the tropics, mostly in sub-Saharan Africa (Pasquet *et al.*1999; Basu *et al.* 2007). The chromosome number in both wild and cultivated plants is 2n = 2x = 22 (Forni-Martins 1986).

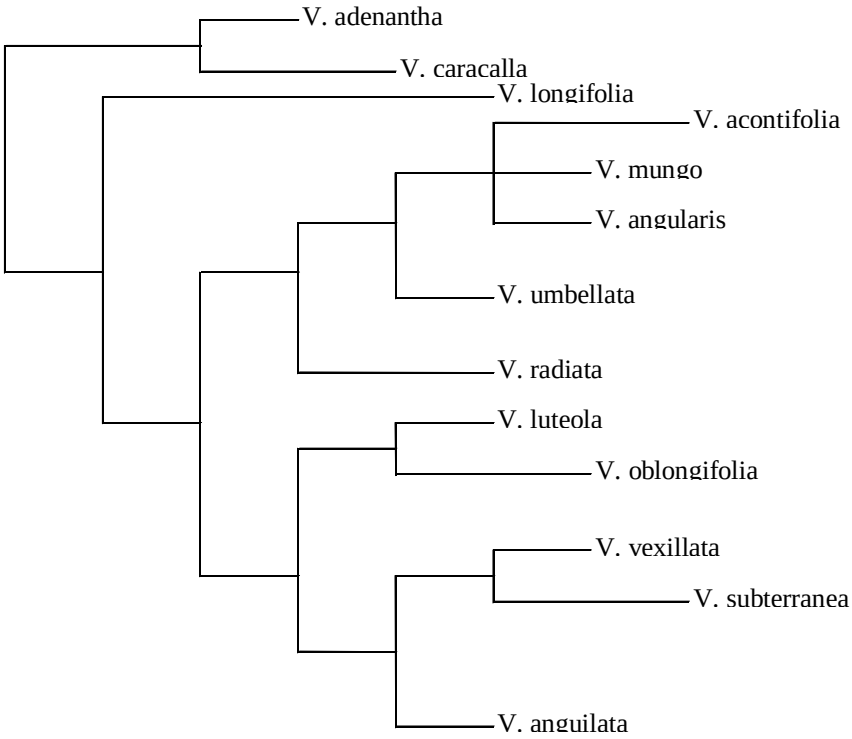

Figure 2: Phylogenetic tree showing the relationship between *Vigna* species from various *Vigna subgenera* and sections. Source (Wang *et al.* 2008).

2.3 Morphological characteristics of the crop

The morphological features of bambara groundnut is similar to that of groundnut (*Arachis hypogea* L.). Bamishaiye *et al.* (2011), reported that the crop is an intermediate herbaceous plant, with creeping stems and grows close to ground level (Fig. 3).

Figure 3: The bambara groundnut plant (Source: ARC-VOPI Research farm Roodeplaat Pretoria, 2015).

The growth habit of this legume as recorded by Goli (1997) is either spreading, bunched or semi bunched. The spreading types are cross-pollinating while bunched types are self-

pollinating, and the latter usually matures earlier. The plant produces pods and seeds at the base of the plant at soil level similar to that of the groundnut (*Arachis hypogea*). The leaves are trifoliate (± 5 cm long), the petiole (up to 15 cm) is long, stiff and grooved, and the base is green or purple in colour (Fig. 4) (Swanevelder 1998). The pod is small (1.5 cm long), round or slightly oval shaped and wrinkled with mostly one or sometimes two seeds (Swanevelder 1998). According to Masindeni (2006), stem branching of the crop starts as early as about one week after germination and may produce up to 20 or more short branches on which the leaves are borne. Each lateral stem has nodes and internodes. The nodes give rise to the leaves and flower buds. The leaflets may be elliptic, lanceolate, round or oval and are attached to the rachis. The terminal leaflet is slightly larger than the lateral leaflets, with an average length of 6 cm and an average width of 3 cm (Goli 1997). Various leaf colours exist, from light green to dark green. Bambara groundnut has flowers that are papilionaceous and are attached to the peduncle by pedicels (Basu *et al.* 2007).

Figure 4: Bambara groundnut petiole pigmentation: purple (left) and green (right) (Source: ARC-VOPI Research farm Roodeplaat Pretoria, 2015).

Doku & Karikari (1971) have identified a hollow at the tip of the keel through which ants enter both opened and unopened flowers of the crop. Open flowers are mostly yellow in colour and occasionally white or red (Fig. 5). New flowers open in the early hours of the morning and they are yellowish-white, but towards the evening, the colour changes from yellow to brown. Older flowers can be light brown (Goli 1997). After a flower has been pollinated, and fertilization has occurred, the peduncle elongates to convey one or more ovaries just below the soil surface.

Flowering in bambara groundnut is thought to be day-neutral. However, continuous light has been shown to delay flowering by 6-11 days depending on the genotype (Nishitani *et al.* 1988).

During pollination and fertilization, the peduncle elongates to bring the ovaries at the soil level and after fertilization the pedicels penetrate the soil surface to form pods with either one or two seeds (Fig. 5). Bambara groundnut is an annual legume consisting of a well-developed compact tap root with many short (up to 20 cm long) lateral stems that grow geotropically (Massawe *et al.* 2002). The developed pod of bambara groundnut is a fruit; it attains its mature size within 30 days of fertilization, followed by seed development during the next 10 days (Mohammed 2014). Physiological maturity of bambara groundnut pods may be affected by temperature as reported by Goli (1997).

Figure 5: Bambara groundnut landraces showing yellow (A) and red (B) flowers respectively (Source: Mohammed 2014); and dried bambara groundnut pods showing one and two seeds (C).

Photo-insensitive cultivars among bambara groundnut landraces have been reported. The influence of photoperiod on fruit development was evaluated by Linnemann & Azam-Ali (1993). It was found that a long photoperiod delays or even prevents fruiting in certain cultivars. Single-seeded pods are common in bambara groundnut (Linnemann 1994), but pods with three seeds have been reported in the Congo (Goli & Ng 1988). Mature pods are indehiscent, often wrinkled, ranging from yellowish to reddish dark brown colour. The seeds are formed about 40 days after fertilization. At maturity, the seeds vary considerably in colour and size and are smooth and extremely hard when dry. Seed colour varies from cream white, brown, yellowish brown, red, spotted, purple and black (Fig. 6) (Stephens 2003). Goli (1997)

reported various testa patterns, including mottled, blotched or striped, in addition to the predominantly uniformly coloured seeds.

Figure 6: Types of seed colour present in bambara groundnut accessions. A: black seed coat landrace B: cream with spotted purple C: speckle brown seed coat D: cream brown seed coat E: red seed coat F: brown seed coat (Source: ARC-VOPI Research farm Roodeplaat Pretoria, 2015).

2.4 Genetic resources and diversity

The center of origin of bambara groundnut is likely to be in Africa (Hepper 1963). This crop is widely distributed throughout Africa with abundant landraces. According to Rassel (1960), and Goli (1997), the center of genetic diversity of the crop is believed to be in countries such as New Caledonia, Philippines, India, Indonesia, Malaysia, Sri-Lanka and South America and particularly Brazil. Bambara groundnut germplasm was first collected and evaluated in the early 19th century (Anonymous 1947). Massawe *et al*. (2005) reported that the major germplasm collection of bambara groundnut is held by the International Institute of Tropical Agriculture (IITA) in Nigeria. Although a number of scientists have collected bambara groundnut landraces from different parts of Africa and beyond, their valuable genetic resources have not been fully exploited (Massawe *et al.* 2005).

A wider range of phenotypic and genotypic diversity exists among bambara groundnut landraces than in the pure lines. Pure lines as reported by Zeven (1998) are excellent sources of genetic variation for breeding purposes. All cultivated bambara groundnut accessions are landraces that have evolved under domestication directly from their wild relatives (Hillocks *et al*. 2012). There are no improved varieties of bambara groundnut and as a result, landraces are still been cultivated in all the major growing regions particularly in sub-Saharan Africa (Massawe *et al.* 2005). Most national programmes in Africa reportedly have multiple accessions of bambara groundnut landraces in their germplasm collections (Goli 1997). Some of these collections have been evaluated for diversity, multiplication or for agronomic research such as seed yield and plant population (Mohammed 2014).

The Institute for Agricultural Research in Nigeria has a mandate for the genetic improvement of the bambara groundnut alongside other legumes including cowpea (Masindeni 2006). Its scientists organized the second germplasm collection mission where about 80 accessions were collected, multiplied and maintained, and the most promising lines were subjected to yield evaluation trials (Masindeni 2006). The IITA has over 2,000 accessions in stock. During the 1990s, the next largest collection was held at the Office of Scientific and Technical Research Overseas (ORSTOM) in France with over 1,000 accessions, while in Africa, the largest collection was at the University of Zambia (Hillocks *et al.* 2012). Other countries in Africa and Europe also have numerous bambara groundnut accessions (Table 2).

Table 2: Countries/Institutions holding bambara groundnut germplasm collections (Adopted from Goli 1997).

Country/Institution	**Number of accessions held**
Benin	3
Botswana	26
Burkina Faso	143
France, ORSTOM	1000
Ghana, University of Ghana	80
Ghana, Savanna Agricultural Research Institute (SARI)	90
Ghana, Plant Genetic Resources Centre (PGRC)	166
Guinea	43
Kenya, National Genebank	6
Kenya, Kenya Agricultural Research Institute (KARI)	2
Kenya, National Museums	2
Mali	70
Mozambique	12
Namibia	23
Nigeria, IITA	2035
Niger	79
South Africa, Grain Crops Institute	198
South Africa, Institute for Veld and Forage Utilization	117
South Africa, Department of Agriculture	20
Tanzania, The National Plant Genetic Resources Centre of Tanzania (NPGRC)	22
Zambia, University of Zambia	463
Zambia, The National Plant Genetic Resources Centre (NPGRC)	124
Zimbabwe	129

In South Africa, there are approximately 335 accessions of bambara groundnut, at the Agricultural Research Council in Potchefstroom (200), Mpumalanga Department of Agriculture (20) in White River and ARC in Roodeplaat (117). Off the 200 accessions in the

ARC-GCI only 20 have been evaluated during the 1996-97 growing season (Masindeni 2006). These collections possess a wide range of variation in shelling percentage, leaf colour and shape, seed size, seed colour, eye colour and testa pattern (Cilliers & Swanevelder 2002).

Several researchers have used morphological tools to evaluate bambara groundnut accessions for diversity studies. Goli *et al*. (1997) characterized 1384 out of more than 2000 accessions housed at IITA, and found significant genetic variation in growth habit and leaf shapes. Shegro *et al*. (2013) found significant variation among the bambara groundnut accessions evaluated using morphological quantitative traits. These reports, therefore, showed the importance of phenotypic markers for bambara groundnut in genetic studies and improvement. Similar observations were made by Ntundu *et al.* (2006) on the morphological diversity among bambara groundnut landraces in Tanzania. Ofori *et al*. (2009) characterized bambara groundnut landraces and observed that variation in the primary leaf colour of emerging seedlings to be 29% green and 71% purple. This report indicated that variability among yield parameters may be related to variation among leaf shape, stem length, pod and seed production. Sufficient genetic variation in bambara groundnut was also reported by Onwubiko *et al.* (2011).

The knowledge of genetic diversity of bambara groundnut accessions is an important tool for their efficient use in breeding programmes, for studies on crop evolution, and for conservation purposes (Masindeni 2006). Bambara groundnut shows a considerable amount of variability for various morphological, physiological and agronomic traits. Thus far, the full genetic diversity of bambara groundnut accessions remains largely unexploited in Africa. Hence, only farm level selection has been practiced wherein existing landraces are evaluated and their seeds multiplied for production (Massawe *et al.* 2005). Estimating the variation within and between populations of species according to Hayward & Breese (1994), is a useful tool for analyzing the genetic structure of crop germplasm.

2.5 Evaluation of genetic diversity using molecular marker techniques

Molecular markers have been used by a number of researchers, as a tool for assessing bambara groundnut genetic diversity (Pasquet *et al.* 1999; Amadou *et al.* 2001; Massawe *et al.* 2003; Singrün & Schenkel 2004). Assessment of genetic diversity using molecular markers has been identified as a powerful tool in crop improvement programmes since they are independent of environmental conditions as opposed to morphological marker assessments ((Mondini *et al.* 2009).

2.5.1 DNA techniques

Various types of DNA markers have been used. Some of the more commonly used systems are; RFLPs (Restriction fragment length polymorphism), AFLPs (Amplified fragment length polymorphism) RAPDs (Random amplification of polymorphic DNA), VNTRs (Variable number tandem repeat), Microsatellites (Simple sequence repeat; SSR), SNPs (Single nucleotide polymorphism), STRs (Short tandem repeat), SFP (Single feature polymorphism), and DArT (Diversity arrays technology) (Ahmad 2012). The groundwork for genetic improvement of bambara groundnut was conducted at the University of Nottingham, UK and Technical University of Munich, Germany between 1997 and 2003 to improve the understanding of the diversity between and within accessions (Massawe *et al.* 2003).

Studies conducted by Massawe *et al.* (2002) and Singrün & Schenkel (2004) using randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers, revealed high levels of polymorphism, indicating genetic variations among bambara groundnut landraces studied. Lagercrantz *et al.* (1993) reported that simple sequence repeat (SSRs) DNA markers are found to be markers of choice in diversity studies of bambara groundnut landraces. Genetic characterization of a crop offers the capacity to detect genetic diversity that exceeds that of phenotypic methods (de Vicente *et al.* 2005). DNA markers linked to agronomic traits can increase the efficiency of classical breeding by significantly reducing the number of backcross generations. Assessing the morphology of a plant is the oldest and considered the first step in description and classification of germplasm (Hedrick 2005). According to Mohammed (2014), molecular markers should not be seen as an alternative to the phenotypic characterization of cultivars, rather they are supporting tools to conventional breeding.

2.5.2 Proteomics study

Liebler (2002) defined proteomics as the study of the proteome, the protein complement of the genome. The terms “proteomics” and “proteome” were coined by Wilkins *et al.* (1995) and mirror the terms “genomics” and “genome,” which describe the entire collection of genes in an organism. The production of plant proteins is of growing interest to developing and developed countries because of its increasing interest in both food and non-food applications (Marcello & Gius 1997). Proteomics technology encompasses four principal applications. These are: i) mining, ii) protein-expression profiling, iii) protein-network mapping, and iv) mapping of protein modifications (Liebler 2002). Plant protein characterization is important to improve

nutritional quality and functionality of food. There is the need to intensify research efforts aimed at identifying new vegetable protein sources (Yemisi *et al.* 2011).

Polyacrylamide gel electrophoresis is one of the most commonly used protein separation and purification techniques. SDS-PAGE is the most commonly practiced gel electrophoresis technique used for proteins (Davey & Lord 2003). This method is regarded as the easiest way to estimate the number of polypeptides in a sample and evaluates the complexity of the sample or the purity of a preparation. SDS-PAGE is particularly useful for monitoring the fractions obtained during purification procedures. It also allows samples from different sources to be compared for protein content. The most important features of SDS-PAGE are that it is a simple, reliable method for estimation of molecular weights of proteins (Hames 1998).

SDS-PAGE is widely used to analyze the proteins in complex extracts. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (1970). The system actually consists of two gels - a resolving or running gel in which proteins are resolved on the basis of their molecular weights (MWs) and a stacking gel in which proteins are concentrated prior to entering the resolving gel. Differences in the compositions of the stacking gel, resolving gel and electrophoresis buffer produces a system that is capable of finely resolving proteins according to their MWs.

Proteins are usually separated using 1D- and 2D-SDS-PAGE and preparative isoelectric focusing (IEF) before digestion. In 1D-SDS-PAGE and preparative IEF, proteins are separated into a relatively small number of fractions and into many fractions (spots) as in 2D-SDS-PAGE (Liebler 2002). Another powerful and efficient tool that has been successfully used for proteomic studies of plants, animals, microbes, and humans is Mass spectrometry (MS) (Natarajan *et al*. 2009; Finehout & Lee 2004). Mass spectrometry permits one to not only measure the mass of pure peptides and proteins, but to measure the masses of mixtures of peptides and proteins (Jurinke *et al.* 2004). There are little information available on protein composition of bambara seeds; many of the proteins located in the beans remain uncharacterized, if not unknown. Therefore, one of the objectives of this study was to extract proteins from 30 bambara groundnut seeds in various solvents, separate and characterize them using 1D SDS–PAGE.

2.6 Cultivation and growth requirements

2.6.1 Cultivation

Bambara groundnut is cultivated in many semi-arid African countries such as Ghana, Nigeria and South Africa with a secondary center of cultivation in South East Asia, namely, Thailand, Indonesia and parts of Malaysia (Mabhaudi *et al.* 2013). Bambara groundnut is mainly grown by rural women in their home gardens for consumption or as a cash crop for their own economic benefit (Masindeni 2006). Traditionally, it was cultivated in extreme, tropical environments by small-scale farmers with little guidance on improved practices and without access to irrigation and fertilizers (Mabhaudi *et al.* 2013). A global mapping report for bambara groundnut was published in 2001 by Food and Agriculture Organization (FAO), in which crop modelling was used for the first time to predict potential areas of production and as well as potential yields. Several researchers have used crop modelling to predict the growth, development and yield of bambara groundnut (Collinson *et al.* 1996; Azam-Ali *et al.* 2001; Bannayan 2001; Karunaratne 2009). The report by Azam-Ali *et al*. (2001), revealed that beyond its two current cultivation centers, there is a potential for cultivating bambara groundnut in many countries with a Mediterranean climate such as Lebanon and Israel as well as European countries such as Italy, Portugal, Spain and Greece.

2.6.2 Growth requirements

2.6.2.1 Climate

Bambara groundnut is a fast growing plant, which requires a moderate rainfall and warm temperatures and does not tolerate freezing temperatures at any stage of growth (Bamishaiye *et al*. 2011). Bambara can be cultivated in an area up to 1 600 m above sea level and an average day temperature of 20 to 28°C is ideal for the crop (Swanevelder 1998). According to Bamishaiye *et al*. (2011), the optimum temperature for germination of bambara groundnut seed is 30-35°C and extreme temperatures causes death of the leaves, resulting in the reduction of the yield biomass. Wych *et al.* (1982) indicated that cool temperatures are conducive to longer seed filling periods and as a result increases grain yield. Under less favourable growing conditions such as limited water supply and infertile soil, bambara beans grow well and out-yields other legumes such as groundnut (National Research Council 1979). The production of bambara groundnut usually occurs under rainfall of 600–700 mm per annum but optimum growth occurs with 900–1200 mm per annum (Gibbon & Pain 1985). The crop as reported by Doku & Karikari (1969), is the most drought resistant pulse, producing seeds under conditions of high temperature and low rainfall.

2.6.2.2 Soil requirements

The seeds of bambara groundnuts are borne below the soil surface and therefore the choice of soil type is very important (Masindeni 2006). The crop adapts to a wide range of soils and performs better on low fertile soils than do groundnuts (Tweneboah 2000). According to Swanevelder (1998), bambara beans will grow on any well-drained soil, but light, sandy loams with a pH of 5.0 to 6.5 are most suitable. Borget (1992) reported that the crop is the least demanding for mineral elements and thrives in soils which are considered too marginal for groundnut. Generally, the crop performs better on poor soils which are low in nutrients. An abundance of nitrogen favours vegetative growth and bambara beans grow poorly in calcareous soils (Swanevelder 1998). The cultivation of bambara groundnut is of particular importance in semi- arid areas. In such regions, the crop has been found to thrive and produce a yield under adverse conditions, such as limited water supply and low soil fertility (Wassermann *et al.* 1983).

2.7 Agronomical practices and crop managements

During land preparation, no tillage is required when growing bambara groundnut in a well-drained, loose, aerated soil as reported by Masindeni (2006). But for compacted soil and weed infested areas, ploughing, followed by about two times of harrowing is recommended to ensure good germination and stand. Bambara gives the best yields on a deeply ploughed field with a fine seedbed, eventually allowing the plant to bury its developing fruits (Bamishaiye *et al*. 2011). A level seedbed is best, but ridging is advisable if the soil is shallow or prone to water logging (Brink *et al.* 2006). Proper loosening of the soil according to Baudoin & Mergeai (2001), helps pod penetration during fructification and improves the yield. Tweneboah (2000) also mentioned that a well prepared friable seed bed is required to enable the plants bury their pods after fertilization. Bambara groundnut is mostly grown from seed by women, and is intercropped with major commodities such as maize, millet, sorghum, cassava, yam, peanut and cowpea or in pure stand (Goli 1997; Ocran *et al*. 1998; Bamishaiye *et al.* 2011).

Different plant spacings are used in bambara groundnut cultivation. Swanevelder (1998) indicated that the recommended spacing between the plants is 10-15 cm and between rows is 45-90 cm to obtain optimum yield. The author further reported that the highest yield was recorded in Swaziland using a 50 cm spacing between rows. One seed is usually sown per hole. In conditions of high moisture levels and in heavy soils (which is not recommended) seed can be planted 2.5 to 3.0 cm deep and 5.0 to 7.5 cm in sandy soil (Swanevelder 1998). Seed rate

varies in several locations, such as, 35 kg/ha in Tanzania; 25-45 kg/ha in Kenya; a higher rate of 60-75 kg/ha was used in South Africa (FAO 1961). Gibbon & Pain (1985) indicated that the normal seed rate is 30-60 kg/ha of shelled nut giving 150,000 plants/ha.

Variation in planting dates has been reported for different locations. Bambara groundnut produces good yields when planted in October and November, especially after good rains in South Africa (Swanevelder 1998). In Zambia and Botswana, sowing takes place from November to February (Bamishaiye *et al.* 2011). In the derived savanna zone of Ghana, two cultivations of the crop is possible in one cropping season, the first crop is sown in May - June and the second crop in October. In the Guinea savanna zone, the crop is usually grown during the minor season (September-November) when the rainfall is reliable (Doku 1995). In 2011, Sinefu evaluated planting dates as a tool for managing water stress in bambara groundnut in the KwaZulu-Natal area of South Africa. The conclusions from that study showed that bambara planted at the optimum planting dates (November) had the best yields compared with late planting dates (January).

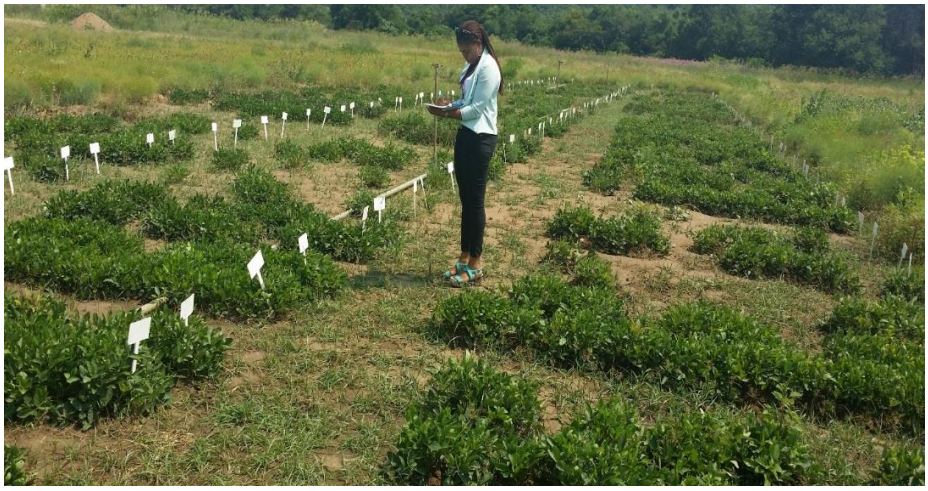

Figure 7: Data collection of bambara groundnut at Roodeplaat research farm Pretoria.

Bambara groundnut has been reported to take between 7 to 15 days (Swanevelder 1998) or 5-21 days (Bamishaiye *et al*. 2011) to emerge. However, recent studies using local South African landraces have reported slow emergence of up to 35 days after planting (Mabhaudhi *et al*. 2013). Seeds stored for about 12 months germinate well, but longer storage results in a loss of viability (Ayamdoo *et al.* 2013). Bambara groundnut is a short day plant and planting during long days results in delayed or no flowering (Swanevelder 1997). He also reported that flowering in bambara groundnut is cultivar dependent. Flowering starts 30 to 35 days after sowing and may continue until the end of the plant's life (Brink *et al.* 2006). Pod and seed

development takes place approximately 30 to 40 days after fertilization. The fruit of bambara groundnut develops above or below the soil surface, although in practice few varieties are surface bearers (Fig. 8). Seeds reach maturity when the parenchymatous layer surrounding the embryo has disappeared (Gqaleni 2014). The maturity of the bambara groundnut crop is dependent on the type of cultivar and climatic conditions and therefore on an overall basis it takes between 100-180 days to mature (Baudoin & Mergeai 2001). The days to maturity are influenced by photoperiods. Linnemann *et al.* (1995) reported that under long photoperiods, maturity is delayed in the bambara groundnut crop.

Figure 8: Development of pod above ground level (yellow arrow) (Source: Mohammed 2014).

Harvesting usually starts about four months after sowing when the pods are mature and the leaves are beginning to yellow (Bamishaiye *et al*. 2011). The tap root can be cut with a groundnut harvester or ploughed out, or the beans can be lifted or hoed out. The pods break off very easily and up to half of the pods can remain in the soil. The detached pods left in the ground are collected manually (Swanevelder 1997). Karikari (1998) mentioned that in Botswana, immature pods are usually harvested about two months before the pods dry completely. A farmer may harvest the crop while it is immature for immediate use, but for commercial purpose, only mature dry seeds are harvested. Harvested pods are air-dried for several days before threshing. The raw product is sold in the markets, as pods or seeds. In dry areas, materials for planting the following season are usually kept by farmers as pods and this reduces or eliminates attacks by insects (Goli 1997). Bambara groundnut seeds can also be stored directly at 4°C (Fig. 9).

Figure 9: Bambara groundnut seeds ready for storage at 4°C (Source: ARC-VOPI Research farm Roodeplaat Pretoria, 2015).

.Earthing up or ridging is a common practice performed by farmers in the whole of Africa and the main reason given for this was that it has a positive influence on yield; but scientists at the ARC in South Africa did not find supporting evidence relating to those results (Swanevelder 1998). Weeding of bambara groundnut takes place 1-3 times, often with a hoe. Tweneboah (2000) stated that the plants are hand weeded when they are 10 cm high and mounded or earthed up at flowering time to encourage development of the pods underground.

2.8 Drought tolerance

With the potential risk of drought associated with climate change, drought tolerance is likely to become even more important in African agriculture. According to Mabhaudhi (2009), drought occurs in plants when there is insufficient soil moisture to meet the needs of a particular crop at a particular time. This may be as a result of meteorological drought, uneven rainfall distribution, mid-season drought, inefficient irrigation and/or poor crop husbandry. Drought stress has a tremendous negative effect on agriculture (Sazares *et al*. 2011). Bambara groundnut is a drought tolerant crop, and has the potential to provide improved food security in the dry areas of Africa (Doku & Karikari 1969; Harris & Azam-Ali 1993; Tweneboah 2000; Berchie *et al.* 2012). With the potential risk of increased drought associated with climate change (Hassan 2006), drought tolerant crops are likely to become even more important in African agriculture (Berchie *et al.* 2012). Underutilized crops such as bambara groundnut have been reported by Harris & Azam-Ali (1993), to have possibly evolved to become drought tolerant due to years of cultivation under severe conditions.

The plant is mainly suited to hot dry areas, due to the ability to adapt and tolerates harsh conditions (Karunaratne *et al.* 2011). The crop will yield in unfavourable environments but there are few reports of its productivity in relation to water stress. It is generally accepted that bambara groundnut is tolerant to drought but little research has been conducted to establish what degree of stress the crop is able to tolerate (Linnemann 1991). Although the mechanisms that allow bambara groundnut to still produce some yield during severe droughts are poorly understood. Begemann (1986) suggested that the strong root system with a compact tap root enhances the resistance of this plant to drought. The nitrogen requirement is met by natural N_2 fixation, as indicated by several nodulation studies (Fig. 10) (Doku 1969; Somasegaran *et al*. 1990; Goli 1997). The roots form nodules for nitrogen fixation, in association with suitable rhizobia especially strains of *Bradyrhizobium* which may be useful in intercropping and rotation systems (Linnemann & Azam-Ali 1993).

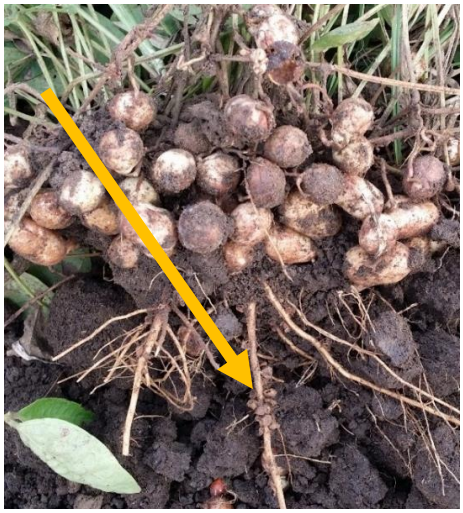

Figure 10: A freshly harvested bambara groundnut showing root nodules (Source: ARC-VOPI Research farm Roodeplaat Pretoria, 2015).

Collinson *et al.* (1997), also reported that bambara groundnut is apparently able to maintain turgor through a combination of osmotic adjustment, reduction in leaf area index and effective stomatal regulation of water loss enabling good yield under harsh conditions. However, due to the noted variability that exists between and within bambara landraces (Massawe *et al*. 2005), it is important to assess local germplasm for drought tolerance. One key step to achieving this is by understanding the mechanisms and the crop adaptations to drought.

2.9 Nutritional value

Bambara groundnut is one of the leguminous crops whose seeds are referred to and used as a complete food because they contain protein, carbohydrate and fat in sufficient proportions to provide a nutritious meal (Poulter & Caygill 1980). The crop provides an important source of proteins (16-25%), carbohydrates (42-60%), and fat (5-6%) for human and animal nutrition (Linnemann 1987; Arora 1995). Several types of food (Fig. 11) are produced from bambara groundnut seeds.

Figure 11: Different types of food obtained from bambara groundnut seeds: A: general seed features of bambara groundnut landraces, B: Bambara flour, C: okpa, D: boiled bambara seed (Source: ARC-VOPI Research farm Roodeplaat Pretoria, 2015).

The gross energy value of bambara groundnut seed as reported by FAO (1982) is greater than that of other common pulses such as cowpea, lentil and pigeon pea. According to Obizoba (1991), bambara groundnut mixtures (BG-Corn) showed a nutritional superiority to pigeon pea when cooked. In the study of nutritive value of the crop, the author observed that bambara groundnut and pigeon pea had a protein content of 14.85% and 18.39%, respectively, when compared to cowpea variety which had the highest protein content of 22.87%. The author further indicated that the cowpea and bambara groundnut mixture have acceptable characteristics as sole sources of nutrients for infants or supplements for adults. Bambara

groundnut according to Ihekoronye & Ngoddy (1985), is richer than groundnuts in essential amino acids such as isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine (Table 3). The red seeds of the crop could be useful in areas where iron deficiency is a problem, as they contain almost twice as much iron as the cream seeds (Hillocks *et al*. 2012).

Table 3: Amino acid composition of bambara groundnut seeds. Source: (Belewu *et al*. 2008).

Amino acids	**Average (% protein)**
Alanine	4.4
Arginine	6.8
Aspartic acid	11
Cystine	1.5
Glutamic acid	16.9
Glycine	3.7
Histidine	3.1
Isoleucine	4.1
Leucine	7.6
Lysine	6.7
Methionine	1.3
Phenylalanine	5.5
Serine	4.7
Threonine	3.5
Tryptophan	1.2
Tyrosine	3.4
Valine	4.9

Purseglove (1992) also reported that the ripe seeds contain protein 16-21%; fat 4.5-6.5% and carbohydrate 50-60%; thus providing a completely balanced food. Brink *et al.* (2006) mentioned that dried leaves used for fodder contains: crude protein 15.9%, crude fibre 31.7%, ash 7.5% and fat 1.8%. The high lysine content of bambara groundnut seed makes it a high quality protein source and a good supplement to maize-based diets (Masindeni 2006).

2.10 Production and yield potentials of Bambara groundnut

There is little information available on the amount of bambara groundnut produced around the world, but PROTA (Plant Resources of Tropical Africa) reported that the annual world

production in 2006 was 330,000 tons of which 45-50% was produced in West Africa (Nigeria, Niger, Burkina Faso, Chad, Cote d'Ivorie). Most of the world's bambara groundnut is grown in West Africa and the crop is most prominent in the rural communities (Hillocks *et al.* 2012). About one third of the world's annual production (10,000,000 kg) comes from Nigeria (Swanevelder 1998), followed by Burkina-Faso with 44,000,000 kg per annum.

The FAO (2009) has production data from only four countries: Burkina Faso, the Democratic Republic of Congo, Cameroon and Mali. Production statistics for individual countries are scarce. In Zimbabwe, during the 1990s, approximately 50 tons were produced a year by 3,500 smallholders on an area of 2,300 ha, with an average yield of 650 kg/ha. Karikari *et al.* (1995) reported that bambara is grown over an estimated area of 1500 ha, producing about 400 tons of seed annually in Ghana. In Kenya, bambara is a minor crop and is used as a traditional food only by the Luhya, Giriama and Kambe at the coast, and to a lesser extent by the Luo (Ngugi 1995). The main producing areas of bambara in South Africa are Limpopo, Mpumalanga and KwaZulu-Natal by few smallholder farmers (Swanevelder 1998). In the literature, yields vary from 50 up to 4,000 kg/ha. Yields of over 3,000 kg/ha were obtained in a cultivar trial conducted by the ARC at Potchefstroom. The worldwide demand for bambara is greater than the amount produced (Swanevelder 1998).

The average yield of bambara groundnut is rather low compared to other cultivated *Vigna* crops. This is due mainly to the fact that all of bambara groundnut cultivars grown are landraces. No improved varieties were developed by a selective breeding programme because of the lack of an efficient hybridization technique (Suwanprasert *et al.* 2006). Variation in yields of bambara groundnut has been recorded. For instance, a yield of 400-1,400 kg/ha unshelled pods was reported in Zimbabwe by Heller *et al.* (1997). In Swaziland yields of 2,600 kg/ha was recorded in the field (Sesay *et al.* (2004), and over 3,000 kg/ha have been obtained in South Africa by Swanevelder in 1998. In Côte d'Ivoire, Kouassi & Zoro (2010) recorded a seed yield as high as 4,000 kg/ha.

The crop has the potential of yielding greater than 3,000 kg/ha in both greenhouse and field trials (Collinson *et al*. 1996; Hillocks *et al*. 2012). However, performance varies under farmer's management (Goli 1997), probably due to the prevailing agronomic conditions such as planting density, soil and genotype differences. Late planting was found to reduce seed yield drastically in Tanzania (Collinson *et al*. 2000). The low yields in bambara groundnut might be associated with poor seed germination and variable germination rates, which often lead to poor crop

establishment in dry regions (Linnemann & Azam-Ali 1993). The high yield potential of the crop can be exploited through breeding (Mohammed 2014).

2.11 Production constraints

Production of bambara groundnut is widely affected by both biotic and abiotic factors. Environmental factors play a major role in plant adaptation, because of their ability to influence the reproductive development of a genotype. There are various degrees in which these factors affect the crop and this depends on the genetic components of the crop (Masindeni 2006). Ngugi (1995) stated that individual cultivars are not very adaptable. He also mentioned that although the crop is found in vastly different environments, careful cultivation is needed, as the flower stalks are much weaker in comparison to those of groundnuts, and cannot penetrate hard soil crusts. Tanimu & Aliyu (1995) mentioned that both haulm and seed yields are invariably low since all cultivated bambara groundnut are local varieties and therefore, their genetic potential is limited. According to Swanevelder (1998), high rainfall prior to harvest can be detrimental and leads to significant yield losses. Sowing date has been reported to influence the yield and yield variability, through the effects of temperature and day length on plant development (Collinson *et al.* 1996; Sesay *et al.* 2004). The development and yield of bambara groundnut is affected by photoperiod and temperature. The onset of flowering and podding are both photoperiod sensitive (Linnemann 1991; Linnemann & Craufurd 1994). Nishitani *et al*. (1988) reported that bambara groundnut is a short day plant and adverse variations could be observed as a result of long days. Linnemann *et al.* (1995) found that some varieties had more pods under photoperiods of 10 to 12 hours than the same varieties under 14 hours. Therefore, it was concluded that in some varieties the shorter the photoperiod the higher the number of pods.

2.11.1 Pests and diseases

Although several researchers (Gibbon & Pain 1985; Purseglove 1992; Doku 1995; Tanimu & Aliyu 1995) reported that the bambara groundnut crop appears to be remarkably free from serious pests and diseases, Thottappilly & Rossel (1997) stated that pests, diseases and nematodes are the major yield limiting factors of the crop. Since bambara groundnut is grown during the rainy season, a period of high temperatures and humidity, it is highly susceptible to fungal diseases, which are common during this period (Tanimu & Aliyu 1995). The most important fungal diseases are cercospora leaf spot (*Cercospora* spp.), powdery mildew (*Erysiple polygoni*) and Fusarium wilt (*Fusarium oxysporum*) (Brink *et al.* 2006). According

to Goli (1997), in dry weather, pod attacks by termites have been consistently observed and the root knot nematode (*Meloidogyne javanica*) also attacks the roots of the plant in sandy soils.

Attacks of rust and leaf blight, caused by *Puccinia* sp. and *Colletotrichum* sp., respectively, have been reported in the crop and isolated cases of rosette disease has also been observed on bambara groundnut in Nigeria (Tanimu & Aliyu 1995). The sap-sucking leafhopper, *Hilda patruelis* and termite damage was recorded on bambara groundnut in Zimbabwe (Hillocks *et al.* 2012). Fusarium wilt diseases has been reported in Kenya as one of the major diseases limiting yields of the crop (Cook 1978). In South Africa, most farmers experience wilting problems in their fields (Masindeni 2006). Hillocks *et al.* (2012), also reported that in Botswana, the main disease of the crop is *Fusarium* wilt, which attacks young seedlings in wet weather, particularly under waterlogged conditions. In dry weather and during storage, bambara groundnut pods may be attacked by termites and as a result, local farmers store their seeds with various substances, including tobacco, peppers and sand (Linnemann 1988), to protect them from storage pests which represent a major problem. These limiting factors on bambara groundnut production varies from location to location and from year to year, and their effect is reflected in the crop yield. Bambara groundnuts tolerate a wide range of agro-ecological conditions Therefore, identifying the most stable and adapted cultivars is an important consideration for bambara groundnut production (Collinson *et al.* 1996).

2.12 Uses, consumption and economic importance of bambara groundnut

Bambara groundnut is an African crop essentially grown by subsistence farmers for human consumption (Swanevelder 1998). Additionally, the crop can also contribute towards food security. The seeds have been regarded as a complete balanced diet for human nutrition by Purseglove (1992), since it contains sufficient amount of protein, fat and carbohydrate. The seed is consumed in different ways and at different stages of maturity as a vegetable or snack (Mohammed 2014). They can be eaten fresh or grilled while still immature. At maturity, they become very hard, and therefore require boiling before any specific preparation (Goli 1997). Consumption of bambara groundnut seeds varies from one location to another. The immature seeds are consumed fresh or grilled and they can also be boiled, either shelled or unshelled, and eaten as a meal or mixed with immature groundnut and maize. The boiled seeds can also be pounded and mixed with local South African dish ‘samp’ (Swanevelder 1998). In some countries like South Africa and Swaziland, bambara groundnut is used to add variety to daily diets and as a mainstay in time of starvation and it can also be used to make soup (Masindeni

2006). In the eastern part of Nigeria, Okpuzor *et al.* (2009) reported that the seed is made into a pudding (or steamed-paste) called Okpa. Another common use of bambara groundnut according to Obizoba (1983), is to make a paste out of the dried seeds, which is then used in the preparation of various fried or steamed products, such as 'akara' and 'moi-moi' in Nigeria.

Doku & Karikari (1969) reported that in Ghana, the nuts are boiled with pepper and salt in the preparation of "Aboboi" which, when served with "gari" (grated and roasted cassava) or "tatare" (mashed fried ripe plantain), makes a very delicious meal. Canning of bambara seeds into sauce has also been reported in Ghana. The product was thus available throughout the year, and over 40,000 cans of various sizes were produced annually (Doku & Karikari 1971a; Begemann 1986a). In Côte d'Ivoire, the seed is used to make flour, which makes it more digestible and in East Africa, the beans are roasted, then pulverized, and used to make a soup, with or without condiments (Goli *et al.* 1997). Bread made from bambara groundnut flour has been reported in Zambia (Linnemann 1990). Flour may be prepared from roasted or unroasted seeds, which can be used for livestock feeding (Oluyemi *et al.* 1976). The haulms were found to be palatable (Doku & Karikari 1971) and an important source of livestock feed during the dry season. The leaves are reported to be suitable for animal grazing because they are rich in nitrogen and phosphorus (Rassel 1960). The leaves which are also rich in protein are used as fodder for livestock (Drabo *et al.* 1995). Some medicinal benefits of bambara groundnut has also been reported. The leaves are used in Senegal to treat abscessed and infected wounds (Directorate Plant Production 2011), while the leaf sap is applied to the eyes to treat epilepsy, and the roots are sometimes taken as an aphrodisiac.

The Zybo tribe in Nigeria uses the plant to treat venereal diseases (Brink *et al.* 2006). Seeds can be pounded and mixed with water and taken for eye cataracts. In South Africa, raw seeds are chewed to cure nausea experienced by pregnant women (Directorate Plant Production 2011). The Luo tribe in Kenya uses bambara groundnut to cure diarrhoea. Water from the boiled maize and pulse mixture is drunk to treat diarrhea (Ngugi 1995). The leaves are pounded with those of *Lantana trifolia* L. ('nyabend winyo', 'nyamrithi'), then water is added to make a solution used to wash livestock as a preventative against ticks. This solution is used as a pesticide on vegetables too (Ngugi 1995). It is also a cheap source of vitamin B to prevent beriberi and is a superior source of vitamin B to many other legumes, including mungbean (*Vigna radiata* [L.] Wilczek) (Basu *et al.* 2007). In agriculture, bambara groundnut has

beneficial use because they fix atmospheric nitrogen thereby directly increasing the level of the soil nitrogen and in turn increasing crop yields.

2.13 Antinutritional Factors

Bambara groundnut is an important source of protein in many developing countries. However, this protein may not be readily bio-available because of the presence of antinutrients. Antinutrients are substances that reduces the nutritional value of the food by interfering with mineral bioavailability and digestibility of vital nutrients (Ames *et al.* 1990). The antinutrients are mainly located in the testa of legumes. Brown coloured seeds of cowpeas and bambara groundnuts contain more tannins than those that are cream coloured (Nwokolo 1996).

Low toxic substances in legumes as a result of antinutrients has been reported to produce serious pathological conditions. A serious outbreak of lathyrism disease which is associated with consumption of kesari dhal has been reported in India (Bora 2014). Lathyrism is a paralytic disease affecting the lower limbs and the incidence of this disease is higher in males than females and recovery from the condition does not usually occur. In lathyrism, the toxic substance interfaces with the formation of normal collagen fibre in the connective tissue (Bora 2014). Another disease called *'Favism* disease' was also reported by Dmello *et al* (1991). This disease is characterized by haemolytic anaemia which affects certain individuals following the ingestion of fresh or uncooked broad beans. The victims suffer from an inherited biochemical abnormality which affects the metabolism of glutathione in red blood cells and is the result of decreased activity of the enzyme glucose-6-phospate dehydrogenase (Dmello *et al.* 1991).

There have been several reviews in recent years about the antinutritional factors found in foods. Francis *et al*. (2001) & Agbo (2008) divided antinutrients into four groups. These incudes:

I. Factors affecting protein utilization and digestion (e.g., protease (trypsin) inhibitors, tannins and lectins).

II. Factors affecting mineral utilization (e.g., phytates, gossypol pigments, oxalates and glucosinolates).

III. Antivitamins.

IV. Miscellaneous (e.g., mycotoxins, mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytoestrogens and saponins).

These factors in legumes interfere with food utilization and affect the health of human and animals either through their metabolic products in living systems or by themselves (Makkar 1993).

2.13.1.1 Condensed tannins

According to Apata & Ologhobo (1997), the digestion and bioavailability of the nutrients in bambara seeds for animal and human nutrition is limited by antinutrients such as condensed tannins. Condensed tannins (CTs) are polyphenolic substances widely distributed in plants, especially in legumes. Due to their large structure, they are known to inhibit protein digestibility by forming irreversible complexes with proteins, thereby reducing the bioavailability of amino acids. Tannins have molecular weights ranging from 500 to over 3,000 Da (Muzquiz *et al.* 2000). Tannins are heat stable and they decrease protein digestibility in animals and humans, probably by either making protein partially unavailable or by inhibiting digestive enzymes and increasing fecal nitrogen (Gemede & Ratta 2014). Felix & Mello (2000) reported that tannins are known to be present in food products and inhibit the activities of trypsin, chemotrypsin, amylase and lipase enzymes. They also mentioned that tannins decreases the quality of protein in foods and interfere with dietary iron absorption. Tannins are also known to be responsible for decreased feed intake, growth rate, feed efficiency and protein digestibility in experimental animals. If tannin concentration in the diet becomes too high, microbial enzyme activities including cellulose and intestinal digestion may be depressed (Aletor 2005). However, recent research has also indicated that condensed tannins in low concentrations have beneficial effects in animal and human health and nutrition (Champ 2002; Akindahunsi & Salawu 2005).

2.13.1.2 Trypsin inhibitors

Protease inhibitors are widely distributed within the plant kingdom, including the seeds of most cultivated legumes and cereals (Akande *et al.* 2010). Protease inhibitors have the ability to inhibit the activity of proteolytic enzymes within the gastrointestinal tract of animals (Liener & Kakade 1980). According to Gemede & Ratta (2014), protease inhibitors may be easily denatured by heat processing due to their particular nature of protein. The two groups of heat and acid sensitive protease inhibitor includes trypsin and chymotrypsin inhibitor which occurs in raw legume seeds (Akande *et al.* 2010). Protease inhibitors reduce trypsin activities and to a lesser extent chymotrypsin; therefore, impairing protein digestion in monogastric animals and some young ruminant animals (Friedman *et al.* 2003).

Liener (1976) mentioned that protease inhibitors have been implicated in reducing protein digestibility, growth inhibition and in pancreatic hypertrophy. The potential beneficial effects of protease inhibitors remains unclear, although lower incidences of pancreatic cancer have been observed in populations where the intake of soybean and its products is high (Giri & Kachole 2004). This protease inhibitor may also act as anticarcinogenic agents in animals. The Bowman-Birk inhibitors derived from soybean have been shown to inhibit or prevent the development of chemically-induced cancer of the liver, lung, colon, oral and esophagus (Finotti *et al.* 2006). Protease inhibitors in foods are mostly inactivated by heating especially moist heat (Bressani & Sosa 1990; Liener 1995).

2.13.1.3 Phytate

Phytate (also known as Inositol hexakphosphate (IP_6)), is one of the most powerful anti-nutritional factors in plant feedstuffs and is present in considerable quantities within major legumes and oilseeds (Akande *et al*. 2010; Gemede & Ratta 2014). Matyka *et al.* (1993) reported that about 62-73% and 46-73% of the total phosphorus within cereal grains and legume seeds are in form of organically bound phytin and phosphorus, respectively. Phytate is the salt form of phytic acid. According to Erdman (1979), as phytic acid accumulates in seed storage sites, other minerals apparently chelates to it forming the complex salt phytate. Akande *et al.* (2010) also reported that phytic acid breaks down phytate and releases nutrients; therefore, they pass through the gut undigested and the major part of the phosphorus contained within phytic acid are largely unavailable to animals due to the absence of the enzyme 'phytase' within the digestive tract of monogastric animals. Phytase enzyme releases phosphorus, minerals and amino acids from phytate, paving the way for maximum utilization of nutrients.

Phytic acid also inhibits the action of gastrointestinal tyrosinase, trypsin, pepsin, lipase and amylase (Liener 1980; Hendricks & Bailey 1989; Khare 2000). Erdman (1979), stated that the greatest effect of phytic acid on human nutrition is its reduction of zinc bioavailability. Phytic acid contents in food can be lowered by addition of enzymes which hydrolyze them **(**Bora 2014).

2.13.2 Other antinutritional factors

Hemagglutinin or lectins in legumes can bind to intestinal epithelial cells, where they may impair nutrient absorption and cause damage that may allow infiltration of bacteria into the blood stream (Jansman *et al.* 1998). Oxalate is an insoluble salt formed from oxalic acid and has the tendency to precipitate (or solidify) in the kidneys or in the urinary tract, thus forming

kidney stones in the urinary tract (Nachbar *et al.* 2000). Saponins are secondary compounds that was recognized as antinutrient constituents, because it reduces the bioavailability of nutrients and decreases enzymatic activities. It also affects protein digestibility by inhibiting various digestive enzymes such as trypsin and chymotrypsin (Liener 2003). Saponins in high concentrations impart a bitter taste and astringency in dietary plants (Bora 2014). In addition, saponins were treated as toxic because they seemed to be extremely toxic to fish and cold-blooded animals. Despite these antinutritional activities of saponins, Bora (2014) reported that it has found wide applications in beverage and confectionery industries, as well as in cosmetics and pharmaceutical products. Another antinutrient, cyanogenic glycosides, which are found in cassava, produces hydrogen cyanide on hydrolysis and when consumed, is converted to thiocyanate which can interfere with iodine metabolism giving rise to goiter and cretinism (Ames *et al*. 1990). Other anti-nutrients such as phenols may also play beneficial roles in human diets by acting as anti-carcinogens or by decreasing the risk of heart disease or diabetes (Holloway & Bradbury 1999).

It is important to gain knowledge of the antinutritional contents in bambara groundnut seeds and find effective methods to inactivate them especially since the seeds are used in weaning formulae (Ohiokpehai 2003). Most of the antinutrients found in food crops can be reduced by post-harvest processing. There are many proteinase inhibitors that are denatured easily by heating (Osagie 1998). Offor *et al*. (2011) reported that most of the antinutrients found in food crops can be reduced by post-harvest processing. Oxalic acid in food, the levels of poisonous alkaloids and steroids can be reduced through post-harvest processing (Pearson 1994). Ijarotimi & Esho (2009) showed that fermentation improved mineral composition with minor effect on the amino acid profile. This procedure significantly reduced the antinutritional factors present in the bambara groundnut seed studied, including phytic and tannic acids, as well as oxalate and trypsin. Condensed tannins, phytic acid, polyphenols, trypsin inhibitors and free phosphate contents was assessed in this study to determine the levels of these anti-nutrients in 30 bambara groundnut accessions.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Assessing the extent of morphological variation among 30 selected landraces of bambara groundnut landraces.

3.1.1 Experimental material and study site

30 accessions of bambara groundnut landraces were obtained from the germplasm bank of Agricultural Research Council-Vegetable and Ornamental Plant Institute (ARC-VOPI), Roodeplaat, South Africa. The list of bambara groundnut genotypes, their seed morphology, leaf shape and growth habit used in this study is given in Table 4. The accessions were planted under open field conditions at the ARC-VOPI Roodeplaat research farm during the 2014/2015 summer cropping season. Roodeplaat lies at 25º59’ S latitude and 28º 21’ E longitudes at an altitude of 1164 meters above sea level. The soil type is a clay loam with the pH of 7.08 (ARC-VOPI 2015).

Table 4: The list of bambara groundnut accessions with their seed colours, leaf shape and growth habit used in this study.

No	Accessions name	Seed colour	Leaf shape	Growth habit
1	SB1-1	Brown/spotted purple	Lanceolate	Erect
2	SB7-1C	Dark red	Oval	Semi-erect
3	SB2-1B	Cream brown	Round	Semi-erect
4	SB4-1	Dark red	Oval	Semi-erect
5	SB4-2	Cream brown	Elliptic	Erect
6	SB4-4	Black	Oval	Spreading
7	SB4-4C	Cream	Oval	Erect
8	SB7-1	Dark red	Round	Spreading
9	SB7-1A	Light red	Oval	Semi-erect
10	SB7-2	Light Red	Lanceolate	Semi-erect
11	SB8-1	Brown	Round	Spreading
12	SB8-3A	Dark red	Round	Semi-erect
13	SB9-1A	Cream brown	Oval	Spreading
14	SB10-1	Dark red	Elliptic	Spreading
15	SB10-1A	Brown	Lanceolate	Spreading
16	SB10-1C	Black	Oval	Spreading
17	SB10-2	Dark red	Elliptic	Semi-erect
18	SB11-1A	Cream	Elliptic	Erect
19	SB11-5	Speckle brown	Elliptic	Erect
20	SB12-3B	Cream brown	Lanceolate	Erect
21	SB16-5A	Speckle brown	Elliptic	Erect
22	SB17-1	Dark red	Lanceolate	Spreading
23	SB17-1A	Light red	Oval	Semi-erect
24	SB19-1A	Brown	Lanceolate	Spreading
25	SB19-3	Black	Lanceolate	Spreading
26	SB19-3B	Black	Lanceolate	Semi-erect
27	BAMBARA-6	Cream	Elliptic	Semi-erect
28	BAMBARA-7	Speckle brown	Lanceolate	Erect
29	BAMBARA-9	Cream	Lanceolate	Semi-erect

30	BAMBARA12	Cream black	Lanceolate	spreading

3.1.2 Methods

The seeds of the 30 bambara groundnut accessions were evaluated in an open field experiment. The accessions were sown in a plot size of 3 x 1.5 m with three replications in a randomised complete block design. Each entry was planted in two rows, keeping plant to plant distances of 0.3 m and the distances between blocks 1.5 m. Uniform crop management practices were applied to all entries in the trial as recommended for the area. Two seeds were hand sown per hole and the seedlings thinned to one at two weeks after planting when they were fully established. Five randomly selected plants were selected from each plot to estimate the genetic variability using the morphological traits among the accessions evaluated.

3.1.3 Parameters measured and data analysis

Eighteen quantitative characters (Table 5) were measured among the thirty bambara groundnut accessions. The descriptors for bambara groundnut was used in measuring the morphological traits (IPGRI 2000).

Table 5: List of quantitative morphological characters recorded from 30 bambara groundnut accessions at a weekly interval.

Quantitative characters	Code	Description
Days to 50% flowering (count)	D50%F	Number of days from emergence to when 50% of the plants have started flowering in the plot
Leaf length (cm)	LL	Length of the middle leaf
Leaf width (cm)	LW	Width of the middle leaf
Leaf area (mm^2)	LA	Area of the middle leaf
Initial plant stand (count)	IS	Number of plant after 50% emergence in the plot
Panicle length (cm)	PL	Length of panicle from its base to the tip
Plant Height (cm)	PH	Height of main stalk from the ground to the tip of the main panicle
Number of leaves per plant (count)	NLPP	Count of total number of leaves from the plant
Number of branches per plant (count)	NBPP	Count of total number of branches from the main stem
Days to 50% maturity (count)	D50%M	Number of days from emergence to when 50% of the plants have matured in the plot
Days to harvest (count)	DH	Total number of days from planting to harvest
Final plant stand (count)	FS	Number of plant after 50% maturity in the plot
Fresh weight (g)	Fwt	Average weight of five harvested fresh seed
Dry weight (g)	Dwt	Average weight of five harvested dried seed
Number of seed per plant (count)	NSPp	Total count of number of seed per plant (average of five plants)
Yield per plant (g)	YPP	Weight of seed per plant (average of five plants)
Hundred seed weight (g)	Hswt	Weight of hundred seed counts at 12% moisture content
Yield per plot (g)	YPPlot	Total weight of seed per plot

The leaf area was measured using a leaf area meter (AM300 ADC BioScientific Limited, Hoddesdon UK). The quantitative traits for all the accessions of the three replications were computed and subjected to analysis of variance (ANOVA), using Agrobase statistical software (Agrobase 2008). Means were compared by the least significance difference (LSD) at 0.01

probability level. Cluster and Principal Component Analyses were conducted to determine similarities and dissimilarities among the genotypes using SPSS (IBM SPSS Statistics 2015). A similarity matrix was used and a dendrogram constructed to describe similarities and differences among the bambara groundnut accessions based on the traits recorded.

3.2 Characterization of major seed proteins in bambara groundnut accessions using one dimensional gel electrophoresis.

3.2.1 Proteins extraction

4 different methods were employed to extract seed proteins from 30 bambara groundnut accessions (Table 4) in order to ascertain the best method for protein extraction. These methods included: 10%-80% isopropanol, 10% trichloroacetic acid (TCA) in acetone solution, sonication and 2x Lammeli buffer extraction methods.

3.2.1.1 Isopropanol extraction (method no. 1)

Day 1

Proteins were extracted using the method described by Natarajan *et al*. (2009). 500 mg of bambara seed was ground into powder with liquid nitrogen and placed into 15 ml eppendorf tubes containing 5 ml (10-80%) isopropanol. The mixture was shaken for 1 hour in an orbital shaker (FINE PCR, United Kingdom). The samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was removed and 10 ml of ice cold acetone added to the tube and vortexed (VELP Scientifica, United Kingdom) thoroughly. The extract was incubated at -20°C overnight.

Day 2

The samples were centrifuged at 8000 rpm for 20 minutes at 4 °C and the supernatant discarded. The pellet was dried at room temperature for 30 minute. The pellets were re-suspended in 0.5 ml solubilizing buffer (7 M urea, 4% CHAPS, 2 M thiourea) and vortexed until completely dissolved and used for protein assay.

3.2.1.2 Ten percent TCA- acetone extraction (method no. 2)

Proteins were extracted using the method described by Cilia *et al*. (2009). 1 g of sample was ground in liquid nitrogen and was placed in 10 ml of TCA solution (10 % TCA in Acetone, 2% β-mercaptoethanol) and mixed by inverting the tubes. The mixture was centrifuged and the supernatant was placed into a new tube that was stored at -20°C overnight. The precipitated

protein was centrifuged at 5000 rpm for 30 minutes. The pellets were washed 3 times with ice-cold acetone with vigorous disruption of the pellet and air dried. The pellets were re-suspended in 1 ml of solubilising buffer (7 M urea, 4% CHAPS, 2 M thiourea) and vortexed until completely dissolved and used for protein assay.

3.2.1.3 Sonication method (method no. 3)

Proteins were extracted according to the method adopted from Bio-Rad bulletin (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf). About 0.5 ml of hot SDS (95°C) sample solubilizing buffer (1% SDS, 100 mM Tris-HCl (pH 9.5)) was added to 50 ml of seed flour and vortexed thoroughly. The mixture was sonicated for 20 minutes at high speed and incubated at 95°C for 5 minutes. The extract was stored at -20°C overnight. The sample was diluted with 0.5 ml 2x SDS-PAGE sample buffer (62.5mM Tris-HCl (pH 6.8), 2% SDS, 0.01% bromophenol blue and 25% glycerol,) and incubated at room temperature for 30 minutes. The mixture was centrifuged at 20°C for 30 minutes at 14,000 rpm. The supernatant was harvest and used for protein assay.

3.2.1.4 2x Laemmeli buffer method (method no. 4)

Protein extractions were done according to the method described by Nakamura *et al.* (2002). Equal volume of 10% SDS and 2x Laemmeli buffer (62.5mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue) was added to 50 mg of sample flour. The mixture was vortexed thoroughly and placed in an orbital shaker (FINE PCR) for 30 minutes. The extract was boiled for 10 minutes at 100°C and centrifuged at 20°C for 20 minutes at 13,300 rpm. The supernatant was harvest and store at -80°C until used.

3.2.2 Protein quantification

The quick start Qubit® fluorometer protein kit from Thermo Fisher Scientific (file:///E:/Chapter%203/lgen_Fluorometer_Qubit-3.0-manual.pdf) was used to determine the protein concentration in each sample. Three standard assay tubes were set up with one tube for each sample. Qubit working solution was prepared by diluting the Qubit reagent (1:200) in Qubit buffer. Approximately 200 µl of working solution was prepared for each standard and sample (Table 6). All the tubes were vortexed thoroughly for 2-3 seconds and incubated for 15 minutes at room temperature. The absorbance of the standards and unknown samples were measured using Qubit® fluorometer 3.0 at 595 nm. A standard curve was generated and the sample concentrations calculated thereafter.

Table 6: Calculations for 200µl Qubit® fluorometer Standard Assay.

	Standard Assay Tubes	Sample Tubes
Volume of working solution added	190 µl	190 µl
Volume of standard solution added	10 µl	0
Sample volume	0	10 µl
Total volume of each assay	200 µl	200 µl

3.2.3 Protein separation

3.2.3.1 Preparation of samples for SDS-PAGE

The protein samples were adjusted to a concentration of 30 ug/ul. Equal volumes of 2x sample buffer (2x Laemmli buffer) and protein samples were mixed and vortexed briefly. The samples were heated at 95ºC for 10 minutes and allowed to cool immediately on ice before loading.

3.2.3.2 Gel preparation

Separation of proteins from bambara groundnut seeds were resolved in SDS-PAGE according to Hopkins & Barker (2008). Gels were prepared (Table 7) without adding ammonium persulfate and N, N, N, N-tetramethylethylenediamine (TEMED). The Bio-Rad mini-PROTEAN Tetra cell was assembled according to the manufacturer's instructions. Freshly prepared 10% ammonium persulfate and 2 µl TEMED was added to the resolving gel solution and quickly pipetted into the assembled gel. Ice cold 100% isopropanol was poured across the top of the resolving gel to produce a straight edge. The gel was left to stand for approximately 45 minutes to polymerize. The isopropanol was thoroughly rinsed from the gel with distilled water and let to dry prior to pouring the stacking gel.

Table 7: Gel Preparation (Hopkins & Barker 2008).

12 % Resolving Gel Total Volume = 5 ml		5 % Stacking Gel Total Volume = 1 ml	
Reagent	**Volume (ml)**	**Reagent**	**Volume (ml)**
Distilled water	1.65	Distilled water	0.68
30% Acrylamide mix	2	30 % Acrylamide mix	0.17
1.5 M Tris, pH 8.8	1.25	1.5 M Tris, pH 8.8	0.13
10 % SDS	0.05	10 % SDS	0.01
10 % Ammonium persulfate	0.05	10 % Ammonium persulfate	0.01
TEMED	0.002	TEMED	0.001

Exactly 10% ammonium persulfate and 0.001 ml TEMED was added to the stacking gel solution and quickly pipetted into the assembled gel. A comb was quickly placed into the stacking gel. The stacking gel was left for 45 minutes to polymerize after which the comb was removed. The wells were rinsed with the running buffer (1 x Tris-glycine gel buffer) to remove any unpolymerised gel.

3.2.3.3 Loading and running (1D) gel electrophoresis

The upper chamber was filled with 1 x Sigma-Aldrich Tris-glycine running buffer. About 10 µl of protein marker was loaded into the first lane and 10 µl of each sample was loaded into each well carefully. The lower chamber was filled with 1 x Tris-glycine gel buffer and the gel was run at 200 V for 1 hour.

3.2.4 Image analysis

The gel images were viewed or scanned under the Bio-Rad UV Spectrometer (scan), using Bio-Rad image lab 3.0 software and labelled accordingly. The gels were analyzed by comparing with mobilities of the marker.

3.3 Determination of the anti-nutritional factors in the seeds of selected bambara groundnut landraces.

3.3.1 Sample preparations

Seeds of 30 bambara groundnut landraces (Table 4) were ground to a fine powdery flour (granulometry from 20 to 200 mm) in a mixer mill type MM 200 (Retch, Germany) for 30 seconds with the frequency set at 1/ 30. 3 replicates of all the samples were ground for each assay evaluated. The flour was then immediately extracted and used for the different assays. All data obtained in the analyses carried out were expressed on the basis of the flour fresh weight.

3.3.2 Determination of condensed tannins

Condensed tannins (proanthocyanidins) were determined by the butanol/HCl method, as described by Porter *et al.* (1986). 500 mg of sample were extracted using 10 ml of 70% acetone. The mixture was incubated at 60℃ for 1 hour in the water bath, sonicated at 4℃ for 2 minutes, centrifuged at 4℃ for 10 minutes and filtered using 0.45 m Millipore filter. A 0.5 ml aliquot of each extract (three replicates) was mixed with 3 ml of butanol/HCl (95:5, v/v) solution in screw capped test tubes and incubated for 60 minutes at 95℃. A red coloration was developed, and the absorbance of the samples was then read at 550 nm versus a prepared blank and compared

with a known concentration range of delphinidin standards prepared similarly. All results were expressed as mg delphinidin equivalents/g dry material. The data are reported as mean ± standard deviation for three replications. A linear response was obtained between 0.9 mg and 3.5 mg delphinidin/ mL solution, i.e. a detection limit of 5 mg delphinidin equivalent/g of dry material.

3.3.3 Determination of free phosphate and phytic acid contents in seeds

3.3.3.1 Phytic acid phosphate content

Phytic acid phosphate (PAP) content was determined according to the method described by Pilu *et al*. (2003). 50 mg of sample flour was subsequently mixed with 2 ml of extraction buffer (0.4 M HCl and 0.7M Na_2HPO_4). The mixture was vortexed thoroughly and incubated at 4℃ overnight.

Day 2

The mixture was centrifuged for 10 minutes at 13000 rpm. 1 ml of supernatant was collected and 500 µl of reagent mixture (15 mM $Fecl_3$, 0.2 M HCl) and 1 ml dH_2O was added. The extract was incubated in the dry bath at 100℃ for 30 minutes and allowed to cool. The content was then transferred into 15 ml tubes and centrifuged at 13000 rpm for 10 minutes (observed white pellet). The supernatant was then discarded and pellet suspended twice using 400 µl of 0.2 M HCl and centrifuged each time at 13000 rpm for 10 minutes. The pellet was re-suspended the third time using 400 µl of concentrated H_2SO_4 and quickly transferred into small test tubes. The sample mixture was left in the dry bath at 98℃. About 50-100 µl of 3% H_2O_2 was continuously added at a 3 hours intervals until the colour is cleared for 24 hours without further addition of hydrogen peroxide. 400 µl of the sample was then used for the phosphate assay.

3.3.3.2 Sample preparation for phosphate assay

About 50 µl of the prepared sample from day 2 was diluted with 200 µl of distilled H_2O. About 50 µl of the diluted sample extract was then added to 950 µl of a freshly prepared Chen's reagent (6 N H2SO4: 2.5% ammonium molybdate: 10% ascorbic acid: H_2O (1:1:1:2, v/v/v/v) and incubated at 50℃ for 1 hour before reading the absorbance at 650 nm of the blue reaction mixture (Chen *et al*. 1956). A reference standard line was routinely prepared using a series of Na_2HPO_4 solutions (without sample) and Chen's reagent within the linearity range (from 10 to 60 nmol phosphate). The limit of detection was around an absorbance value of 0.2 and expressed as mg/g of sample flour.

Table 8: Preparation of 2 ml phosphate assay.

Reagents	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Chen's	2000 µl	1980 µl	1960 µl	1920 µl	1880 µl
Na_2HPO_4	0	20 µl	40 µl	40 µl	120 µl

3.3.3.3 Free phosphate determination

In order to measure the free phosphate content in each sample, 50 mg of each sample flour (three replicates) was extracted using 1 ml of 12.5% TCA and 25 mM $MgCl_2$ solution for 30 minutes at room temperature and then left stirring overnight at 4℃ (Pilu *et al*. 2003). After centrifugation (6500rpm) at 4℃ for 15 minutes, 100 µl of the supernatant was added to 900 µl of a freshly prepared Chen's reagent (6 N H_2SO_4: 2.5% ammonium molybdate: 10% ascorbic acid: H_2O (1:1:1:2, v/v/v/v) and free phosphate was determined in a similar way as phytic acid using the equation y = 404.02x – 0.0098 and expressed as mg/g of sample flour.

3.3.4 Quantification of total phenolics

The total phenolic content was determined using Folin–Ciocalteu reagent (Taga *et al*. 1984). A 500 mg of flour sample of each bambara groundnut landrace (three replicates) was extracted with 2.5 ml methanol/water (60:40, v/v; 0.3% HCl). The mixture was vortexed thoroughly at room temperature for 30 minutes and centrifuged at 6500 rpm for 10 minutes. The supernatant was collected and the pellet re-suspended in 2.5 ml 70% acetone. The mixture was centrifuged and filtered through a 0.45m Millipore filter. Sample extracts were stored at -20℃ until analysis. Approximately 100 µl of filtrate was mixed with 100 µl of 0.2 N Folin–Ciocalteu reagent and 2.4 ml of dH_2O and mixed thoroughly. After 3 minutes, 300 µl of 0.25 M sodium carbonate was added to the mixture and incubated for 2 hours at 50℃ (colour changes from yellow to blue). The absorbance of the solution was measured at 750 nm with a spectrometer. Quantification was based on the standard curve of garlic acid (0–0.5 mg/ml), which was dissolved in methanol/water (60:40, v/v; 0.3% HCl). The total phenolic content was calculated using the equation y = 7.1507x – 0.0533 and expressed as mg/g of sample.

3.3.5 Trypsin inhibitor analysis (TIA)

The trypsin inhibitory activity was determined according to the improved colorimetric method by Liu & Markakis (1989).

3.3.5.1 Reagents

The assay buffer was 50 mM Tris buffer at pH of 8.2, containing 10 mM $CaCl_2$. A stock trypsin solution was prepared by dissolving 10 mg of crystalline porcine trypsin (Type IX, Sigma Chemical Co., St. Louis, MO) in 50 ml of 1 mM HCl solution, pH about 2.5, containing 2.5 mM $CaCl_2$. The solution was kept at 5℃. To prepare a working solution, 2 ml of stock solution was diluted to a total volume of 25 ml, using the above HCl solution.

A stock of N-α-benzoyl-DL-arginine-pnitroanilidehydrochloride (BAPNA) which was used as the trypsin substrate was prepared by dissolving 400 mg of BAPNA (Sigma) into 10 ml of dimethyl sulfoxide. The solution was very stable at room temperature. A working BAPNA solution was prepared by diluting 0.25 ml of stock BAPNA solution to a total volume of 25 ml, using the assay buffer pre-warmed at 37℃. BAPNA solution was freshly prepared for each assay.

3.3.5.2 Inhibitor sample preparation

100 mg of sample flour (three replicates each) was weighed into 14 ml eppendorf tubes and extracted with 10 ml of dH_2O for 30 minutes with mechanical shaking at a speed of 200 rpm. Approximately 10 ml of sample suspension was then destabilized by adding an equal volume of the assay buffer and vigorously shaking for 2-3 minutes before filtering through a 0.45m Millipore filter. The filtrate was then further diluted with water to the point where 1 ml gave 30-70% trypsin inhibition. This was done to keep the relative standard deviation (RSD) of TIA measured within $\pm$ 3.5%. A suitable final concentration of samples was around 0.1 mg/mm of dry sample.

3.3.5.3 Assay procedure

The procedure for assaying Trypsin Inhibitor Activity (TIA) is shown in Table 9. The assay reaction mixture was incubated at 37℃. Exactly 10 minutes after adding the trypsin solution, the reaction was stopped by injecting 0.5 ml of 30% acetic acid solution. The absorbance at A^s_{410} (sample reading), was a measure of the trypsin activity in the presence of inhibitors. The assay reaction mixture was also run in the absence of an inhibitor by replacing the sample with 1 ml of water. The corresponding absorbance was symbolized as A^r_{410} (reference reading). Distilled water was used as a blank.

Table 9: Procedure for Assaying Trypsin Inhibitor Activity.

Mixing sequences	Reactants	Concentrations in working solutions	Volume Needed for assay
1st	BAPA	0.92 mM	2.0 ml
2nd	Sample	causing 30-70% inhibition	1.0 ml
3rd	Enzyme	16 µg /ml	0.5 ml
4th	Acetic acid	30%	0.5 ml
Total volume			4.0 ml

3.3.5.4 Calculation of TIA Values

Defining a trypsin unit as an A410 increase of 0.01 under the conditions of the assay, the trypsin inhibitory activity is expressed in trypsin units inhibited (TIA) per milligr am of sample and calculated as follows:

$$\text{TIA/mg sample} = \frac{[(A^{r}_{410} - A^{s}_{410})\ X100]/\ \text{ml diluted extract}}{\text{(mg sample/ml diluted extract)}}$$

3.3.6 Data analysis

The raw data collected from the anti-nutritional levels were subjected to analysis of variance (ANOVA) using SPSS (IBM SPSS Statistics 2015). The level of significance in the biochemical data was accepted at $p< 0.05$ and Duncan multiple range test was used to separate means.

CHAPTER 4

4 RESULTS

4.1 Assessing the extent of morphological variation among 30 selected landraces of bambara groundnut.

4.1.1 Analysis of variance

The analysis of variance (ANOVA) revealed significant differences for the phenotypic traits evaluated (Table 10). The mean number of days to 50% flowering (D50% F) ranged from 42 to 67, with a mean value of 59 days. The earliest flowering accession was SB11-1A (42 days) and was followed by accessions SB7-1 and SB7-1A (52 days), respectively. There were two late flowering accessions that included Bambara-12 (66 days) and SB2-1B (67 days).

Accession SB11-1A had the longest leaf with a mean value of (10.82 cm) and width (4.34 cm), while accession SB4-2 had the largest leaf area (1553.10 mm^2). Accessions SB7-1 (5.25 cm) had the shortest leaf, while accession SB10-1 (1.70 cm) had the narrowest leaf. The lowest leaf area was found in SB10-1 (883 mm^2). Accession SB19-1A was the tallest plant with a height of 27.48 cm and longest petiole length (18 cm). Furthermore, the accession SB12-3B had the highest number of branches (161) and the highest number of leaves per plant (482). Accession SB11-1A was the shortest plant (12.67 cm) with the shortest petiole (8.83 cm). The fewest number of leaves (180.73) and branches (60.24) per plant was recorded for accession SB7-1. There was a significant difference ($p \leq 0.05$) between plant height and petiole length in the accessions that were evaluated. The number of days to harvest ranged from 128 to 152 with an overall mean of 137. Early maturing varieties included SB7-1A (128 days) and SB7-2 (130 days), while Bamara-9 matured very late (152 days). There was a highly significant ($p \leq 0.01$) difference among all accessions for grain yield and other yield related traits.

The highest fresh (188.77 g) and dry weight (55.04 g) was observed in accession SB7-1, while SB8-3A had the lowest fresh (12.67 g) and dry (5.39 g) weight. Accession SB4-4C (67.80) produced the highest number of seeds per plant, while SB16-5A (7.90) produced the fewest seeds. The highest seed yield per plant was obtained from SB19-1A (37.81 g), while SB8-3A (2.97 g) had the lowest yield. The mean values for hundred seed weight ranged between 20.45 g and 68.19 g with a grand mean of 43.03. The highest mean hundred seed weight was observed in SB16-5A while the lowest was found in SB11-1A. Bambara-12 (333.00 g) produced the highest mean value for yield per plot while SB11-1A (17.48 g) had the lowest compared to the rest of the accessions.

4.1.2 Correlational matrix

The phenotypic correlation matrix among the 18 quantitative traits of 30 bambara groundnut is presented in Table 11. A significant positive correlation (r = 0.96) was observed between number of branches and leaves per plant. A moderate correlation (r = 0.37) was found between the number of leaves per plant, and the number of branches per plant with yield. The days to 50% flowering showed a positive correlation with plant height (r = 0.38), days to harvest (r = 0.41) and yield per plot (r = 0.42). Leaf length was negatively correlated with initial plant stand (r= -0.41)**,** days to 50% maturity (r= -0.38), finial plant stand (r= -0.37) and hundred seed weight (r= -0.42).

Conversely, a significant negative correlation was observed between initial plant stand and days to harvest (r= -0.44), fresh weight (r= -0.51), dry weight (r= -0.45) and number of seeds per plants (r= -0.43). There was a strong positive correlation of plant height and petiole length (r = 0.85) and between petiole length and hundred seed weight (r = 0.49). Yield per plot was moderately correlated with days to 50% flowering (r = 0.42), petiole length (r = 0.43), plant height (r = 0.43), days to 50% maturity (r = 0.36), days to harvest (r = 0.44), final plant stand (r = 0.42), number of seed per plant (r = 0.39) and yield per plot (r = 0.56). A very strong significant positive correlation coefficient was obtained between days to 50% maturity and days to harvest (r = 0.75). Days to 50% maturity correlated moderately with number of seeds per plant (r = 0.39), yield per plant (r = 0.50) and yield per plot (r = 0.36). A significant positive correlation was observed between days to harvest and fresh weight (r = 0.49), dry weight (r = 0.53), number of seed per plant (r = 0.65) and yield per plant (r = 0.68). There was a strong significant and positive correlation (r = 0.95) between fresh and dry weight. Similarly, yield per plant had a very strong positive correlation with number of seeds per plant (r = 0.88), fresh (r = 0.71) and dry weight (r = 0.77). Finally, fresh weight was strongly and positively correlated with the number of seed per plants (r = 0.68).

Table 10: Mean values of the quantitative traits evaluated on 30 bambara groundnut accessions during the 2014/2015 cropping season. CV= coefficient of variation, LSD= least significant difference. $p \leq 0.05$= significant (*), $p \leq 0.01$= significant (**).

No	Accessions	D50%F	LL	LW	LA	IS	PL	PH	NLPP	NBPP	D50%M	DH	FS	Fwt	Dwt	NSPp	YPP	Hswt	YPPlot
1	SB19-1A	62.33	6.27	3.00	1394.70	16.67	18.00	27.48	407.40	135.80	117.33	136.67	11.33	152.50	54.11	51.70	37.81	52.32	221.87
2	SB8-1	60.00	5.64	2.79	1229.70	14.67	13.58	19.12	248.60	82.87	119.33	139.33	10.33	20.50	6.91	8.60	6.36	41.08	150.50
3	BAMBARA-9	64.00	6.31	2.53	1222.90	5.33	16.42	23.48	307.27	102.42	125.00	151.67	5.00	135.17	43.93	56.60	31.19	42.62	128.30
4	SB7-1C	57.33	5.58	2.44	1164.00	17.00	16.08	22.71	291.80	97.27	109.00	137.33	10.00	58.00	24.15	42.60	15.24	41.20	108.40
5	SB19-3B	63.33	6.05	2.59	1192.00	13.00	12.50	23.40	360.10	126.70	119.00	139.33	4.00	94.00	30.60	51.10	16.61	40.20	104.70
6	SB10-2	59.33	5.70	2.81	1188.30	15.00	15.85	21.61	254.73	87.13	110.67	137.00	12.33	24.50	8.85	13.20	3.23	36.08	185.10
7	BAMBARA-7	65.33	6.35	2.83	1313.30	7.00	16.68	25.80	366.80	122.27	114.00	138.67	7.67	122.00	32.75	34.75	20.90	49.52	131.30
8	SB4-1	59.00	6.05	2.57	1219.30	13.00	15.01	21.15	388.13	114.00	115.00	136.00	12.33	78.50	30.67	43.10	20.27	45.97	138.00
9	SB17-1A	52.33	5.86	2.97	1290.70	17.67	16.93	23.63	264.00	88.00	112.67	131.67	13.67	39.00	26.64	18.00	4.03	45.28	153.40
10	SB16-5A	57.33	5.93	2.23	1177.60	17.00	16.17	22.90	348.00	116.00	112.67	130.67	10.33	43.00	13.70	7.90	4.56	68.19	56.80
11	SB10-1	57.33	5.49	1.70	883.80	17.67	12.38	19.91	340.00	113.33	109.33	130.33	13.00	37.50	17.89	23.20	8.33	37.11	48.75
12	SB4-4C	57.00	5.94	2.23	1106.70	6.67	14.49	20.38	364.17	121.40	119.00	144.33	5.67	101.00	40.43	67.80	27.13	40.98	154.13
13	SB10-1A	59.33	5.91	1.93	1043.40	11.67	12.91	20.83	248.40	82.80	108.33	131.00	7.00	16.50	5.98	33.40	15.55	48.75	139.77
14	SB4-2	55.00	6.72	2.52	1553.10	14.67	14.77	22.13	340.80	113.60	113.67	137.67	13.67	35.50	13.65	26.50	13.46	39.85	65.82
15	SB8-3A	64.00	5.98	2.46	1136.70	15.67	13.67	19.05	251.60	73.87	117.00	134.00	7.67	12.67	5.39	13.20	2.97	43.57	66.98
16	SB11-5	62.33	5.98	2.52	1112.00	12.00	17.11	24.73	338.60	112.87	116.20	140.67	15.67	45.00	14.44	17.70	6.26	49.54	42.34
17	SB7-2	59.00	6.01	2.47	1196.20	17.67	16.59	22.88	325.93	108.60	114.00	129.67	15.00	24.50	5.76	14.60	3.39	37.58	60.06
18	SB2-1B	67.33	6.08	2.52	1240.70	10.00	13.75	21.25	332.90	110.97	114.90	134.00	4.67	53.75	18.40	18.00	6.77	37.45	38.67
19	BAMBARA-6	64.67	5.85	3.09	1345.00	10.67	16.33	23.53	264.80	88.27	120.33	138.67	14.00	48.00	26.86	39.00	17.58	37.64	242.47
20	SB9-1A	58.67	5.53	2.49	1069.30	18.67	15.64	21.83	354.33	118.11	119.00	133.67	15.00	41.17	11.89	18.67	7.88	38.53	83.17
21	SB11-1A	41.67	10.82	4.34	1016.70	1.67	8.83	12.67	360.27	103.15	103.33	132.00	1.33	100.33	31.51	47.00	10.55	20.45	17.48
22	SB19-3	64.33	6.33	2.68	1287.80	3.00	13.80	21.16	352.53	117.50	120.33	145.33	9.33	91.00	35.53	57.25	23.59	45.21	143.77
23	SB12-3B	61.67	5.86	2.75	1254.00	13.67	16.41	24.53	482.40	160.80	114.33	140.00	11.67	51.45	22.11	29.60	15.06	54.48	253.10
24	SB10-1C	64.00	5.76	2.73	1173.00	12.33	12.21	19.28	351.60	117.20	111.67	134.33	13.67	55.50	15.89	18.40	12.17	33.72	121.00
25	SB1-1	62.33	6.24	2.47	1199.70	6.67	14.15	21.64	305.20	101.72	111.00	136.00	6.00	38.00	18.87	16.00	7.61	36.65	38.01
26	BAMBARA-12	66.00	5.75	2.42	1140.30	17.00	16.01	22.32	341.00	113.67	119.33	147.33	17.67	121.00	43.70	50.00	29.30	42.61	333.00
27	SB7-1	51.67	5.25	2.57	1029.00	1.67	14.17	21.33	180.73	60.24	114.00	136.67	1.33	188.77	55.04	30.00	11.78	51.14	40.07
28	SB17-1	64.67	6.07	2.52	1241.40	10.33	14.55	23.14	331.77	110.60	120.33	143.67	8.00	96.50	32.68	61.40	26.64	46.10	124.53
29	SB4-4	53.00	5.83	2.34	1137.70	5.00	10.82	19.82	304.30	101.43	120.00	143.33	4.67	46.88	16.96	27.63	13.66	47.21	54.15
30	SB7-1A	51.67	5.96	2.18	1130.30	17.67	15.71	22.77	304.60	113.53	108.00	128.00	11.67	27.50	9.49	9.90	5.97	41.47	62.03
	Grand Mean	59.53	6.10	2.59	1189.65	12.02	14.72	21.88	324.96	107.20	114.97	137.30	9.79	66.66	23.82	31.56	14.20	43.03	116.92
	Mean squares	92.59	2.65	0.59	47294.58	81.70**	12.48*	20.55*	10122.94	1138.29	69.41	94.51	56.95**	5870.16**	597.03**	926.07**	262.41**	201.74**	16678.30**
	CV (%)	15.47	25.26	23.00	26.30	11.62	17.51	15.26	29.17	30.96	8.24	6.04	20.15	8.38	18.01	16.52	21.73	15.55	5.00
	LSD	15.05	2.52	0.97	511.29	2.28	4.21	5.46	154.92	54.24	15.49	13.55	3.22	9.13	7.01	8.52	5.04	10.94	9.55

Table 11: Pearson correlation coefficients (r) among 18 quantitative traits of 30 bambara groundnut accessions.

	D50F	LL	LW	LA	IS	PL	PH	NLPP	NBPP	D50M	DH	FS	Fwt	Dwt	NSPp	YPP	Hswt	YPPlot
D50F	1.00																	
LL	-0.49**	1.00																
LW	-0.30	0.75**	1.00															
LA	0.33	0.03	0.24	1.00														
IS	0.13	-0.41*	-0.34	0.07	1.00													
PL	0.38*	0.47**	0.16	0.45*	0.45*	1.00												
PH	0.51**	0.54**	0.28	0.50**	0.33	0.85**	1.00											
NLPP	0.18	0.23	0.12	0.21	0.10	0.11	0.22	1.00										
NBPP	0.24	0.08	-0.01	0.25	0.17	0.20	0.37*	0.96**	1.00									
D50M	0.57**	-0.38*	-0.14	0.33	-0.13	0.26	0.36	0.06	0.10	1.00								
DH	0.41*	-0.10	0.02	0.25	-0.44*	0.11	0.19	0.14	0.15	0.75**	1.00							
FS	0.24	-0.37*	-0.19	0.22	0.74**	0.55**	0.37*	0.20	0.24	0.04	-0.13	1.00						
Fwt	0.03	0.15	0.26	0.04	-0.51**	0.08	0.19	0.14	0.11	0.30	0.49**	-0.39*	1.00					
Dwt	0.04	0.12	0.27	0.12	-0.45*	0.14	0.23	0.17	0.14	0.33	0.53**	-0.30	0.95**	1.00				
NSPp	0.13	0.24	0.17	0.11	-0.43*	-0.05	0.07	0.31	0.26	0.39*	0.65**	-0.29	0.68**	0.76**	1.00			
YPP	0.33	0.04	0.06	0.29	-0.28	0.18	0.33	0.37*	0.37*	0.50**	0.68**	-0.10	0.71**	0.77**	0.88**	1.00		
Hswt	0.21	-0.48**	-0.41*	0.17	0.14	0.49**	0.60**	0.11	0.17	0.26	0.10	0.08	0.15	0.13	-0.05	0.16	1.00	
YPPlot	0.42*	-0.25	0.10	0.33	0.22	0.43*	0.38*	0.22	0.25	0.36*	0.44*	0.42*	0.21	0.35	0.39*	0.56**	0.17	1.00

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.1.3 Principal component analysis

The principal component analysis of agronomic characters (Table 12) of 30 bambara groundnut accessions revealed that principal components 1 to 6 accounted for 86.59% of the variability among the accessions. Table 13 represents the principle component analysis for 18 quantitative traits of 30 bambara groundnut genotypes showing Eigen vectors, Eigen values, individual and cumulative percentage of variation accounted by the first six principle component (PC) axes. The first six principle components cumulatively explained 86.59% of the genetic variation. The first principal component (PC1) accounted for 30.18% of the total variation with an eigen value of 5.43. Yield per plant was the primary source of variation in PCA 1 with the largest negative coefficient of -0.37. This was followed by days to harvest (-0.32), dry weight (-0.30) and number of seeds per plant (-0.30). PC2 contained 23.94% of variation which was contributed by initial plant stand (0.39), final plant stand (0.36), leaf length (-0.35), and petiole length (0.31). The characters that contributed more strongly to PC3, which accounted for 12.14% of total variation included number of leaves per plant (0.52), number of branches per plant (0.48), leaf length (0.36), and leaf width (0.31).

Table 12: The principal component analysis for 18 quantitative characters associated with thirty bambara groundnut genotypes.

Variables	PC1	PC2	PC3	PC4	PC5	PC6
D50%F	-0.24	0.20	-0.11	-0.09	-0.37	-0.19
LL	0.10	-0.35	0.36	-0.12	0.07	-0.11
LW	0.01	-0.27	0.31	-0.45	0.23	-0.12
LA	-0.20	0.10	0.17	-0.40	0.14	-0.51
IS	0.06	0.39	0.18	-0.04	0.02	0.33
PL	-0.22	0.31	0.02	-0.13	0.38	0.01
PH	-0.27	0.28	-0.01	0.07	0.30	-0.15
NLPP	-0.18	0.01	0.52	0.33	-0.18	-0.11
NBPP	-0.20	0.07	0.48	0.36	-0.17	-0.12
D50%M	-0.29	0.03	-0.25	-0.15	-0.30	-0.21
DH	-0.32	-0.13	-0.18	-0.13	-0.30	-0.08
FS	-0.04	0.36	0.23	-0.23	-0.07	0.33
Fwt	-0.27	-0.27	-0.09	0.12	0.31	0.13
Dwt	-0.30	-0.25	-0.06	0.06	0.29	0.21
NSPp	-0.30	-0.27	0.02	0.05	-0.11	0.22
YPP	-0.37	-0.15	0.02	0.05	-0.04	0.19
Hswt	-0.17	0.21	-0.16	0.36	0.33	-0.22
YPPlot	-0.28	0.10	0.09	-0.31	-0.07	0.41
Eigenvalue	5.43	4.31	2.18	1.36	1.29	1.01
Individual%	30.18	23.94	12.14	7.56	7.19	5.59
Cumulative %	30.18	54.12	66.26	73.81	81.00	86.59

PC3, PC4, PC5 and PC6 accounted for 32.49% of the variability and was contributed by the quantitative characters.

Table 13: Eigen values, individual and cumulative percentage of the 18 principle components for 30 accessions Bambara groundnut.

No.	Eigenvalue	Individual (%)	Cumulative (%)
1	5.43	30.18	30.18
2	4.31	23.94	54.12
3	2.18	12.14	66.26
4	1.36	7.56	73.81
5	1.29	7.19	81.00
6	1.01	5.59	86.59
7	0.50	2.76	89.35
8	0.44	2.43	91.78
9	0.42	2.35	94.13
10	0.28	1.55	95.68
11	0.24	1.33	97.01
12	0.19	1.08	98.09
13	0.12	0.64	98.73
14	0.08	0.44	99.17
15	0.07	0.41	99.58
16	0.04	0.22	99.80
17	0.03	0.14	99.95
18	0.01	0.05	100.00

4.1.4 Principal component biplot

The principal component analysis loading plot of 18 quantitative traits of 30 bambara groundnut accessions is shown in Figure 12. The PCA grouped the characters into four quadrants. Quadrant 1 included ten characters: days to 50% flowering, leaf area, plant height, petiole length, number of leaves and branches per plant, days to 50% maturity, final plant stand, yield per plant and hundred seed weight. Initial plant stand was the only character found in quadrant 2 while quadrant 3 included five characters (days to harvest, fresh and dry weight, number of seeds per plant and yield per plot). Finally, two characters including leaf length and leaf width were in quadrant 4.

Figure 13 shows the plot obtained from the first two eigenvectors of the PC analysis. The PC biplot clustered the accessions into four quadrants. The accession SB11-1A was placed at the extreme end of the fourth quadrant. The first quadrant grouped accessions SB12-3B, SB19-1A, SB4-1, Bambara-12 and Bambara-6 together, while SB11-5 was between the borders of the first and second quadrant. The second quadrant contained SB16-5A, SB7-2, SB9-1A, SB7-1A,

SB17-1A, SB10-1, SB8-3A, SB10-1A, SB10-1C, SB10-2, SB8-1, SB7-1C and SB4-2. Accessions Bambara-7, Bambara-9, SB17-1, SB19-3 and SB19-3B were grouped in the third quadrant along with SB4-4C. The fourth quadrant accommodated accessions SB7-1, SB4-4, SB2-1B, SB1-1 and SB11-1A.

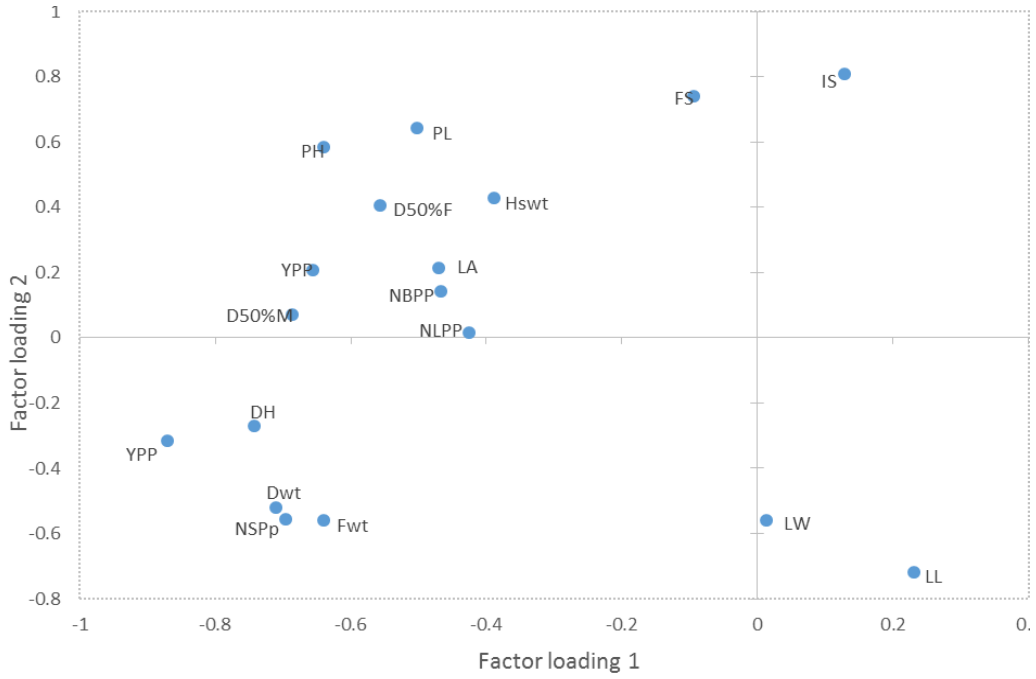

Figure 12: The principal component analysis loading plot of 18 quantitative traits of 30 bambara groundnut accessions.

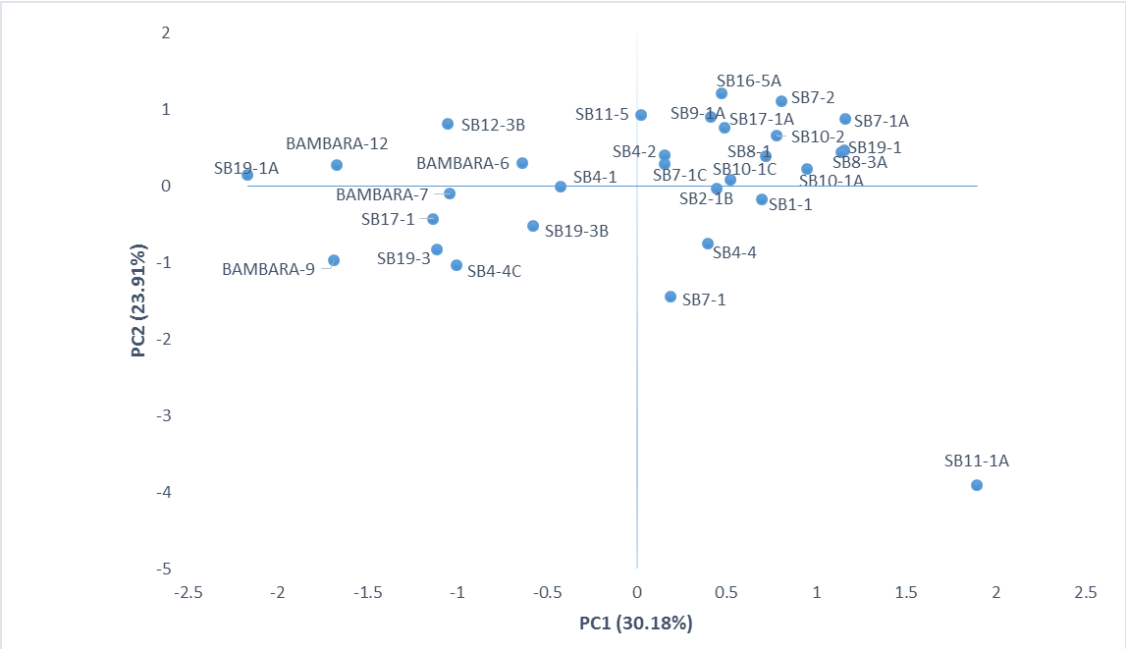

Figure 13: The principal component analysis plot of first and second PC scores of 30 bambara groundnut accessions.

4.1.5 UPGMA cluster analysis

The cluster analysis of the 30 bambara groundnut accessions are presented Figure 14. With the exception of SB11-1A, the dendrogram clustered the accessions into four clusters. Cluster I consisted of six accessions including SB7-1, SB4-4, SB16-5A, SB10-1A, SB10-1 and SB4-2. The second cluster comprised of twelve accessions, namely SB7-1A, SB9-1A, SB7-2, SB11-5, SB17-1A, SB7-1C, SB10-1C, SB1-1, SB2-1B, SB10-1C, SB8-3A, andSB8-1. The third cluster included nine accessions including Bambara-12, Bambara-6, Bambara-7, Bambara-9, SB19-3, SB4-4C, SB4-1, SB19-3B and SB17-1. The last cluster contained two accessions, namely, SB12-3B and SB19-1A.

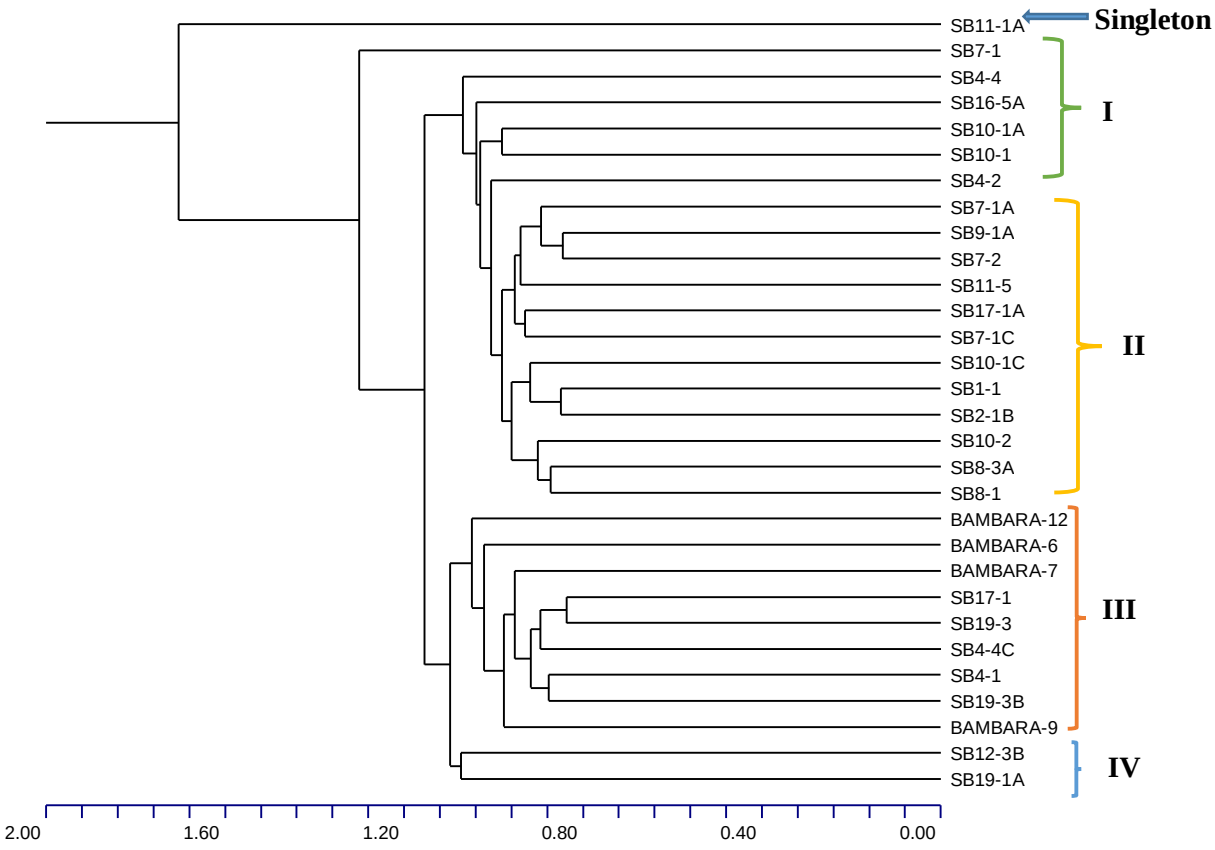

Figure 14: A pair-wise genetic distance matrix of 30 bambara groundnut accessions generated by UPGMA using the data set.

4.2 Characterization of major seed proteins in bambara groundnut accessions using one dimensional gel electrophoresis.

4.2.1 Isopropanol extraction

The preliminary studies of total seeds proteins extracted with various concentrations (10% to 80%) of isopropanol is shown in Figure 15. The result showed that 10% isopropanol (lane 1) provided the best resolution of the extracted proteins from the bambara seeds. Although more proteins were extracted by increasing the concentration of isopropanol the banding patterns became less clear with increasing concentrations. Very little proteins were extracted with 50% to 80% isopropanol. The isopropanol method produced a wide range of protein bands both high and low molecular proteins (Fig. 15). The high molecular weight proteins ranged from about 25 to 250 kD while the low molecular weight proteins ranged from 10 to 24 kD.

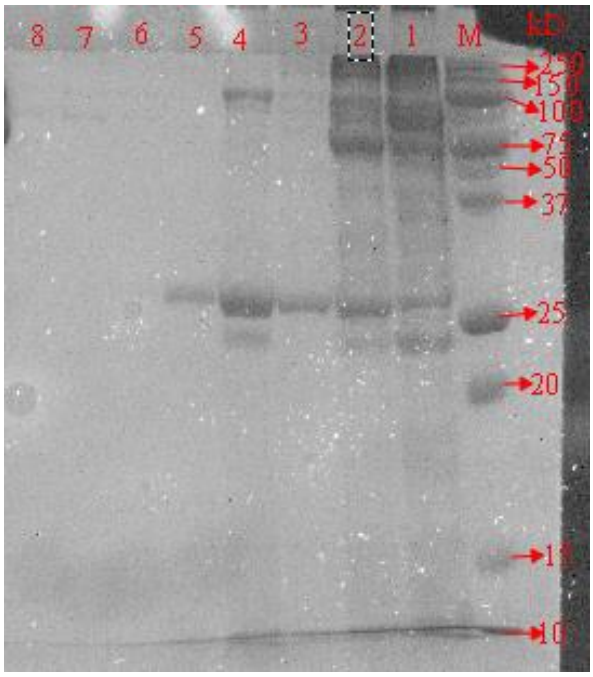

Figure 15: One-dimensional 12% SDS-PAGE of bambara groundnut proteins extracted using various concentrations of isopropanol. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 1, 10%; lane 2, 20%; lane 3, 30%; lane 4, 40%; lane 5, 50%; lane 6, 60%; lane 7, 70%; and lane 8, 80% isopropanol extracted proteins. Lane 1-8 represents sample numbering in Table 4.

However, when this method was employed for extraction of total proteins in 30 bambara groundnut accessions, the resolution of the proteins was very faint in some accessions (data not shown). Most of the abundant proteins were above 30 kDa in size and did not produce very clear banding profiles.

4.2.2 Ten percent TCA- acetone extraction

The 10% TCA-Acetone method (Fig. 16) revealed high molecular weight proteins ranging from 10 to 37 kD in size. Some high molecular weight protein bands were observed in sample 2 (SB7-1C) at 37 to 250 kD. Only one sample (SB7-1C) was resolved with this extraction method and hence could not be exploited for total protein extraction for the rest accessions under study.

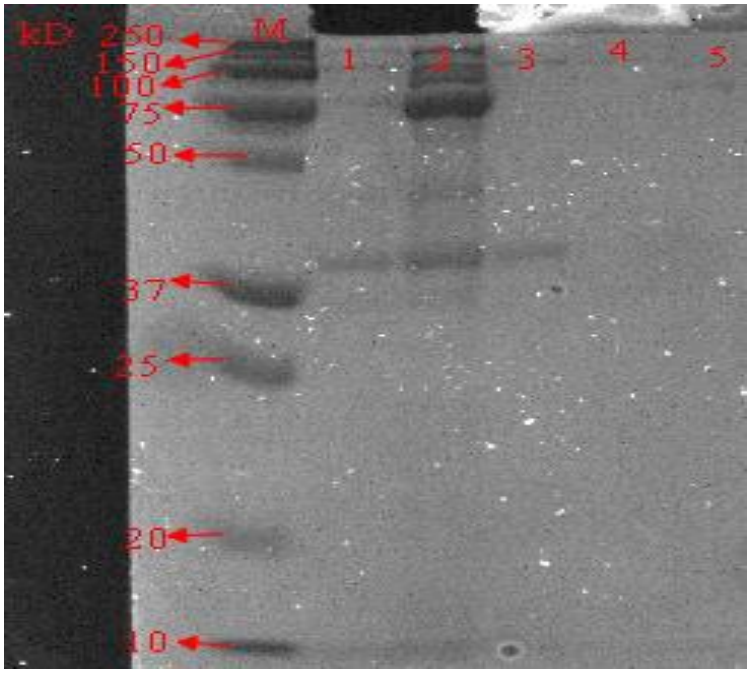

Figure 16: One-dimensional SDS-PAGE (12%) of bambara groundnut proteins extracted using ten percent TCA- Acetone extraction method. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 1-5 represents sample numbering in Table 4.

4.2.3 Sonication method

The total proteins extracted from eight bambara seeds using the sonication extraction method revealed identical protein banding patterns in all the samples (Fig. 17). The proteins ranged in size from 10 kD to 250 kD. The resolution of the protein bands were not very clear.

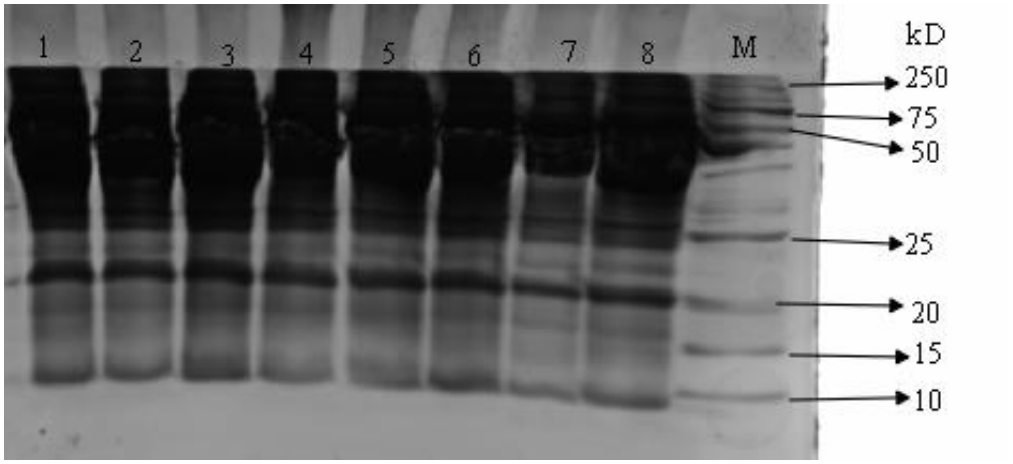

Figure 17: One-dimensional 12% SDS-PAGE of bambara groundnut proteins extracted using the sonication extraction method. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 1-8 represents sample numbering in Table 4.

4.2.4 2x Laemmli buffer method

The protein patterns (Fig. 18) were identical in all the samples. The proteins ranged in size from 10 kD to 250 kD. Abundant proteins were observed at 20, 75, 100, and 150 kD. A slight variation was observed in the banding intensity within all the samples. Due to the band resolution from this method, it was exploited for total protein extraction for the rest accessions under study.

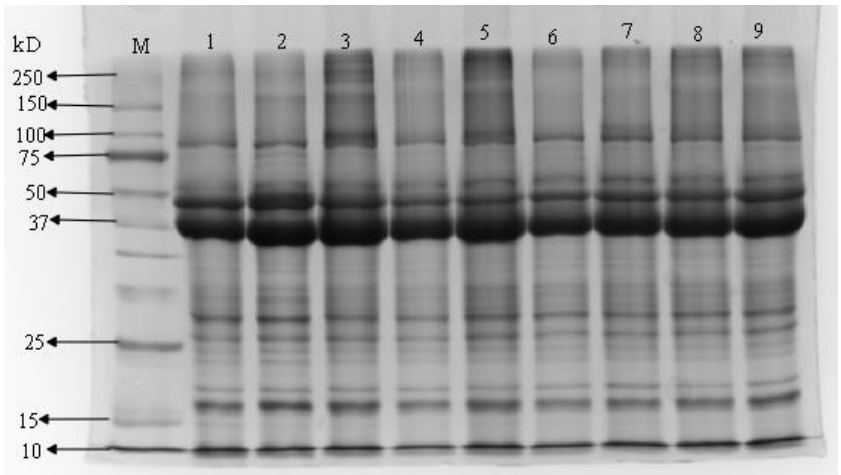

Figure 18: One-dimensional 12% SDS-PAGE of bambara groundnut proteins extracted using 2x Laemmli buffer method. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 1-8 represents sample numbering in Table 4.

4.2.5 Protein extractions of 30 bambara groundnut accessions using 2x Laemmli buffer method

Identical protein banding patterns were observed in all the accessions (Figs. 19, 20, 21 and 22). The banding patterns only varied in their intensity among the accessions. Protein extraction using 2x Laemmli buffer method yielded a large number of identical proteins bands in all the samples evaluated. This method gave a better resolution for both higher and lower molecular weight of protein and was used for extraction of total proteins in all the accessions studied.

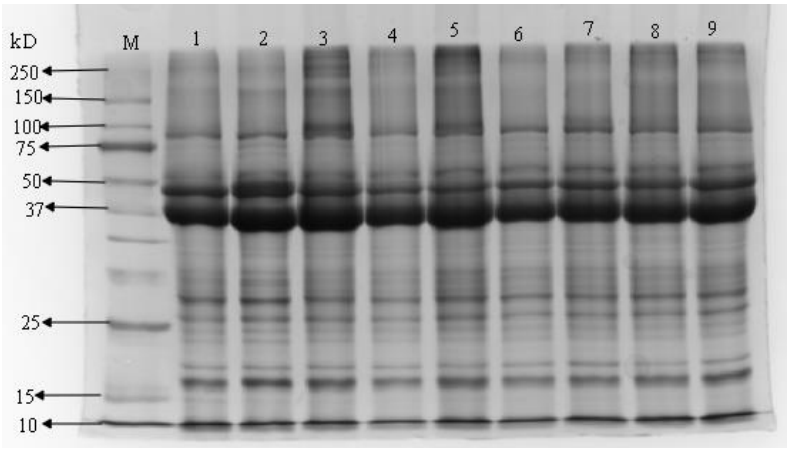

Figure 19: One-dimensional 12% SDS-PAGE of bambara groundnut proteins extracted using 2x Laemmli buffer method. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 1-9 represents sample numbering in Table 4.

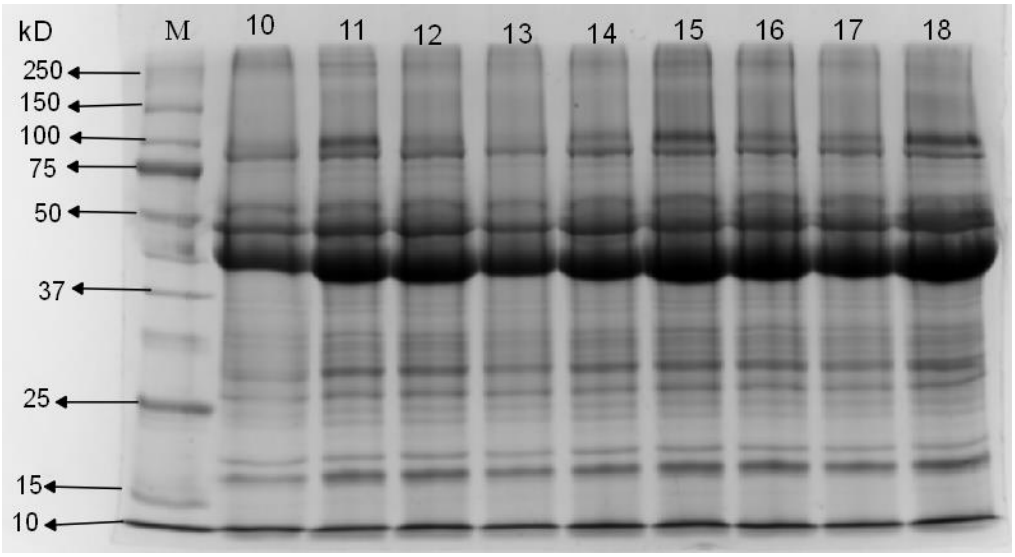

Figure 20: One-dimensional 12% SDS-PAGE of bambara groundnut proteins extracted using 2x Laemmli buffer method. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 10-18 represents sample numbering in Table 4.

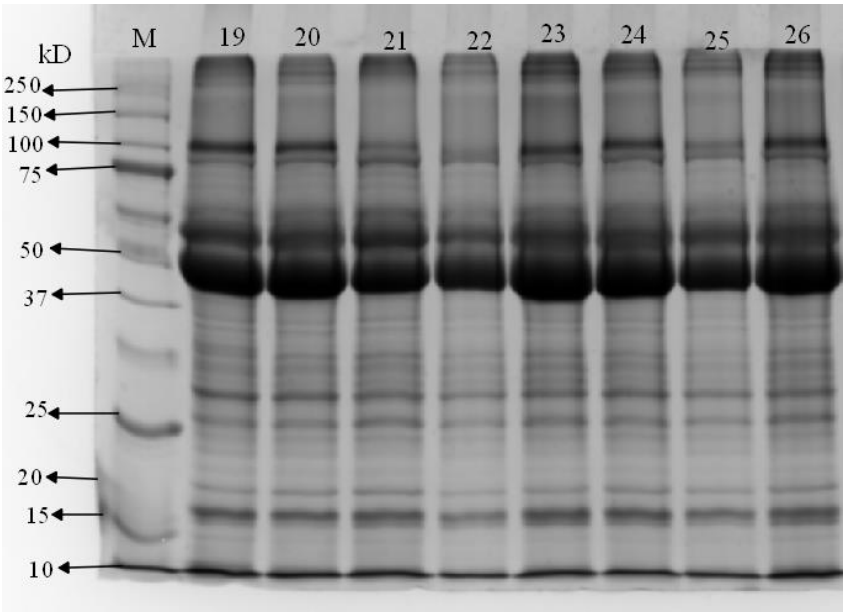

Figure 21: One-dimensional 12% SDS-PAGE of bambara groundnut proteins extracted using 2x Laemmli buffer method. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 19-26 represents sample numbering in Table 4.

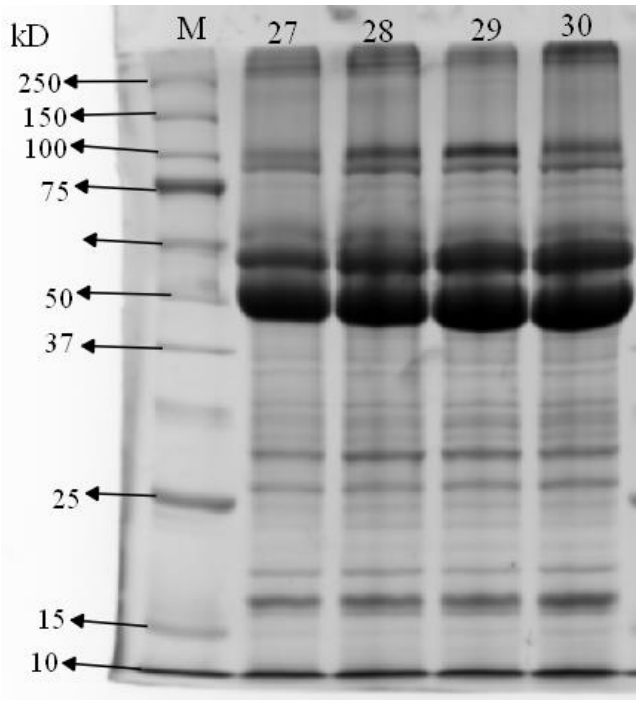

Figure 22: One-dimensional 12% SDS-PAGE of bambara groundnut proteins extracted using 2x Laemmli buffer method. Gels were stained with Coomassie blue stain G-250. M is molecular marker. Lane 27-30 represents sample numbering in Table 4.

4.3 Determination of the anti-nutritional factors in the seeds of selected bambara groundnut landraces.

4.3.1 Analysis of variance (ANOVA)

Analysis of variance (Table 14) and graphical representations (Figs. 23, 24, 25, 26 and 27) of condensed tannins, free phosphate, phytic acid phosphate, polyphenol and trypsin contents among 30 bambara groundnut accessions depicted a highly significant ($p<0.01$) variation among all the quantitative characters.

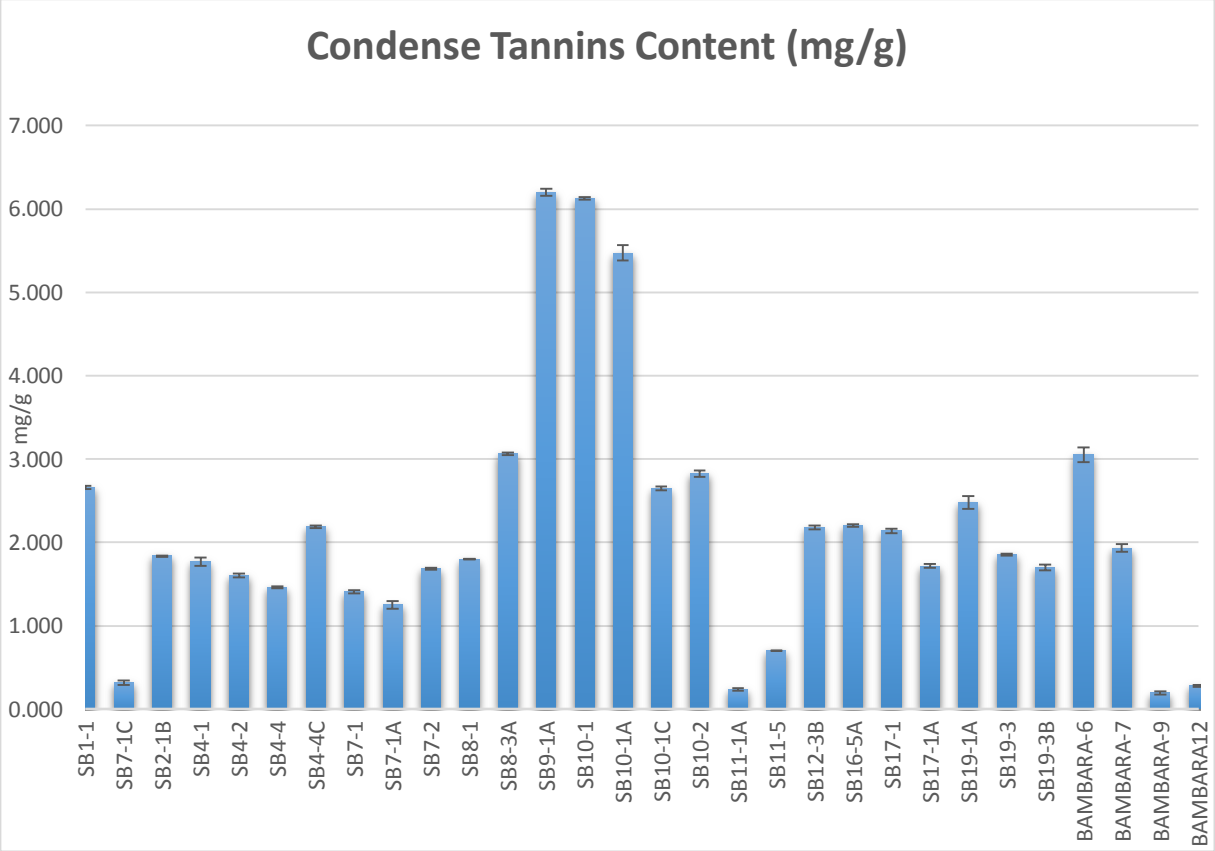

Figure 23: The condensed tannin content in mg/g of thirty bambara groundnut accessions.

The mean value for condensed tannins (Table 14) ranged between 0.20 mg/g to 6.20 mg/g with an average mean of 2.17 mg/g. Among the accessions, SB10-1A (5.47 mg/g), SB10-1(6.12 mg/g) and SB9-1A (6.20 mg/g) recorded the highest mean values (Fig. 23) for the condensed tannins content (CTc) while lowest CTc was observed in Bambara-9 (0.20 mg/g), SB11-1A (0.24 mg/g) and Bambara-12 (0.28 mg/g).

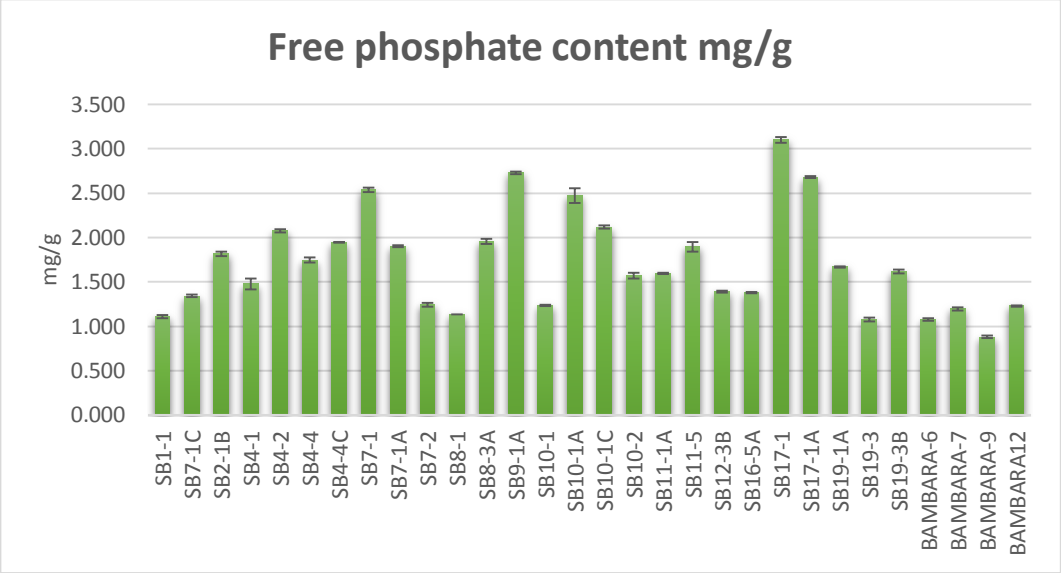

Figure 24: The free phosphate content in mg/g of p (phosphate) in 30 bambara groundnut accessions.

A highly significant ($p<0.01$) difference was observed for the presence of phosphates (Table 14) in all the accessions. Bambara-9 (0.88 mg/g) showed the lowest free phosphate content (Fig. 24) compared to the rest of the accessions and the highest was recorded by SB17-1 (3.10 mg/g).

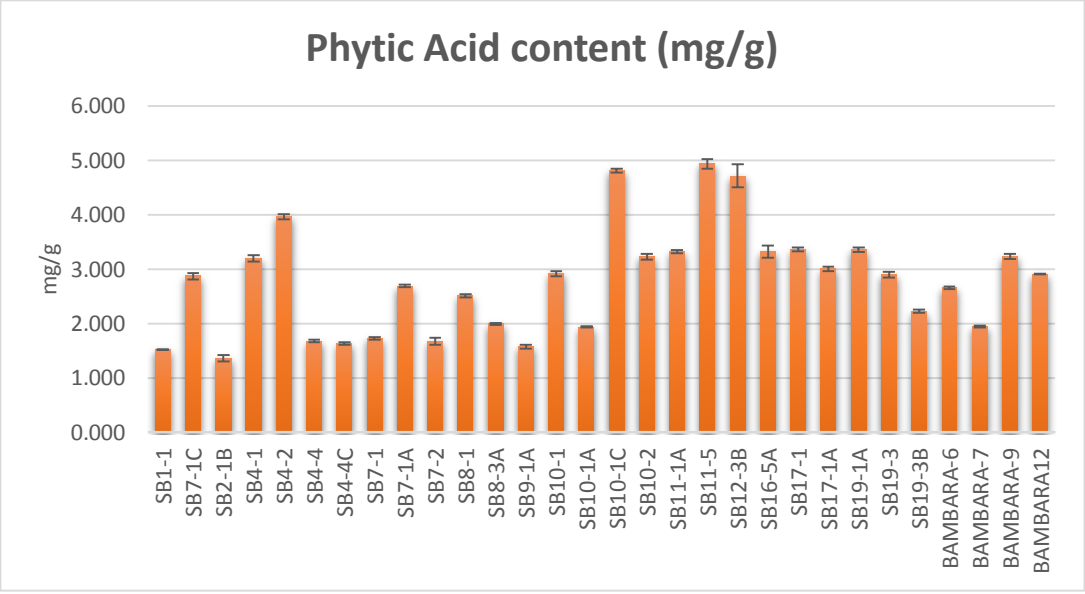

Figure 25: The phytic acid phosphate content in mg/g of thirty bambara groundnut accessions.

Furthermore, SB2-1B (1.35 mg/g) contained lesser amount of phytic acid phosphate (Fig. 25) compared to other accessions. While the highest value was recorded by SB12-3B (4.71 mg/g), SB10-1C (4.81 mg/g) and SB11-5 (4.93 mg/g).

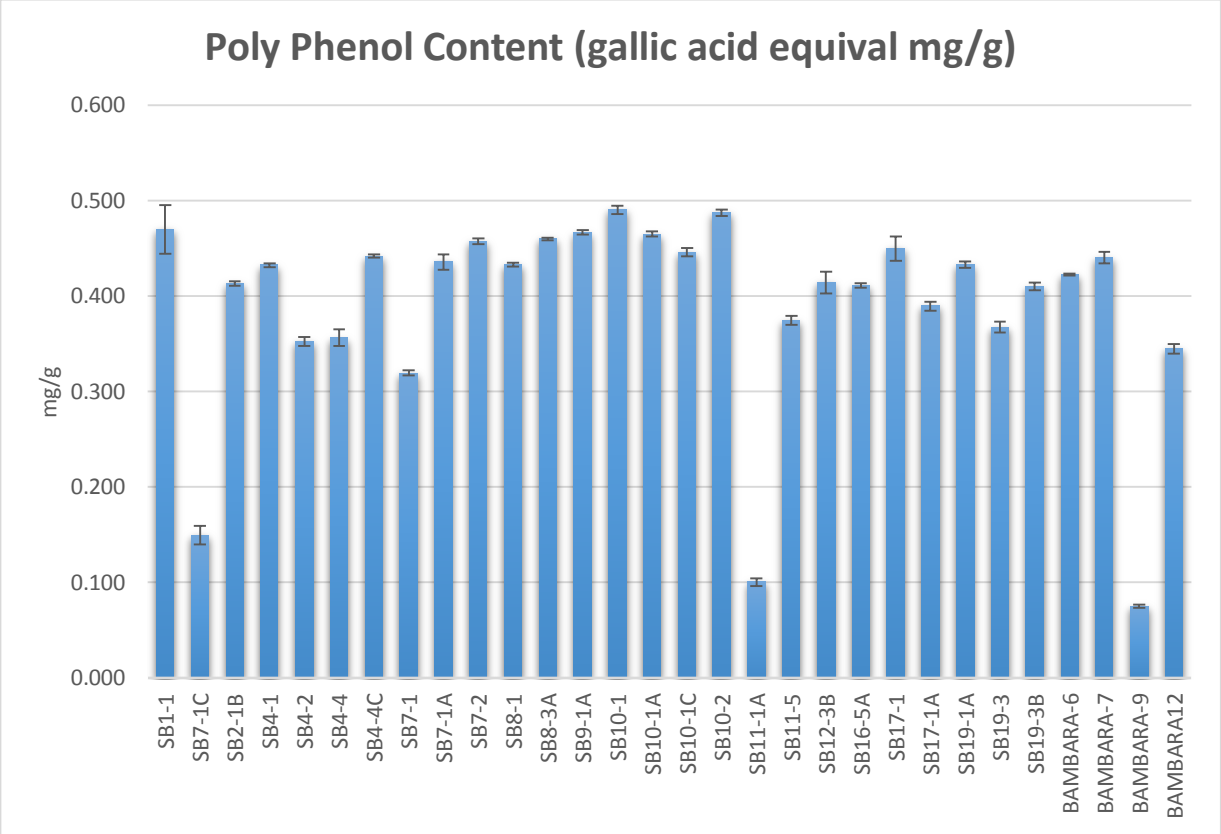

Figure 26: The polyphenol content in mg/g of thirty bambara groundnut accessions.

A highly significant ($p<0.01$) difference was also observed for the polyphenol content (Table 14) among 30 bambara groundnut accessions. The mean value for the polyphenol content (Fig. 26) ranged between 0.08 mg/g to 0.49 mg/g with a mean of 0.39 mg/g. The highest polyphenol content was obtained by SB10-1 and SB10-2 (0.49 mg/g) respectively, while the smallest was recorded in Bambara-9 (0.08 mg/g).

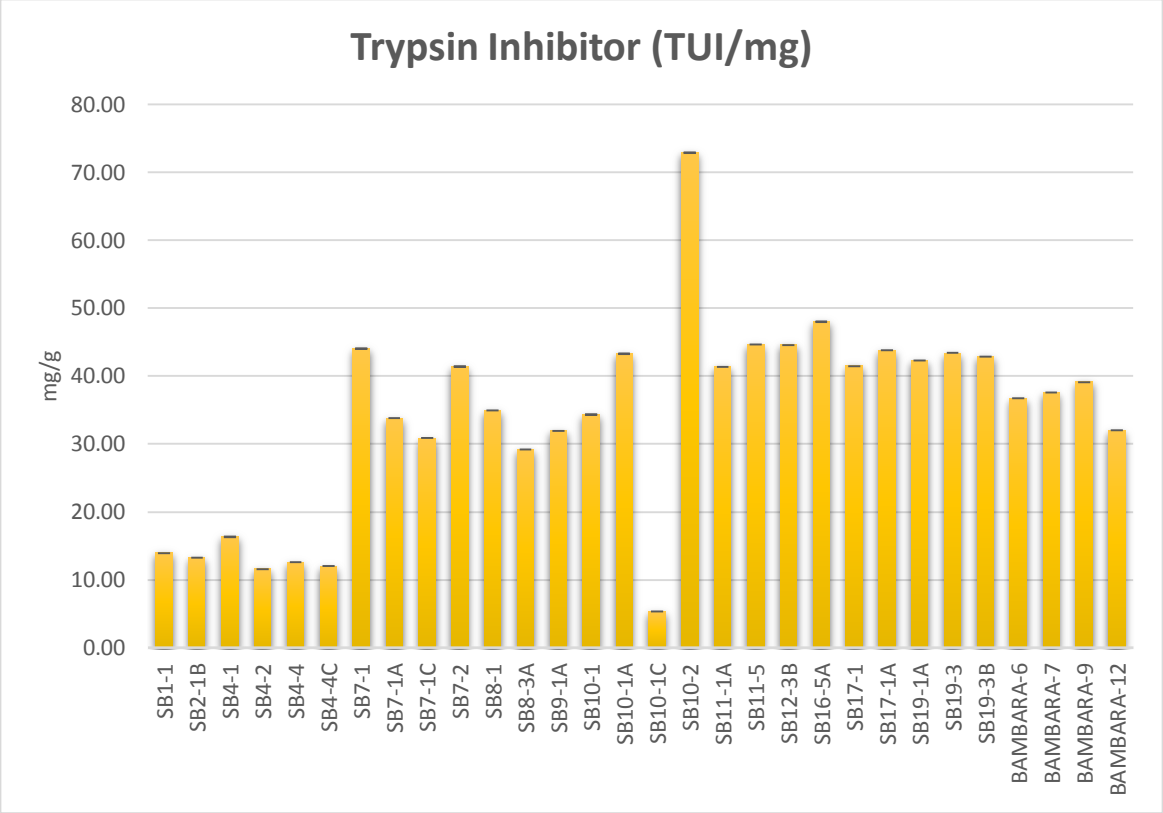

Figure 27: The trypsin inhibitory activities in TUI/mg of thirty bambara groundnut accessions.

Finally, a highly significant ($p<0.01$) difference was obtained for the presence of trypsin inhibitors (Table 14) in all the accessions. Accession SB10-1C (5.30 mg/g) had the lowest mean for trypsin inhibitory activities (Fig. 27) while the highest trypsin activity was present in SB10-2 (73 mg/g).

Table 14: Mean values of the anti-nutritional analysis on 30 bambara groundnut accessions. p≤ 0.01= significant (**).

Accession names	Condensed Tannins (mg/g)	Free Phosphate (mg/g)	Pytic Acid Content(mg/g)	Poly Phenol Content (mg/g)	Trypsin Inhibitor (TUI/mg)
SB1-1	2.66±0.11	1.11±0.08	1.52±0.07	0.470±0.04	13.90±1.10
SB7-1C	0.317±0.03	1.34±0.06	2.87±0.64	0.15±0.02	13.50±0.90
SB2-1B	1.83±0.05	1.82±0.11	1.35±0.62	0.41±0.00	17.90±2.70
SB4-1	1.77±0.30	1.48±0.26	3.20±0.62	0.43±0.00	12.00±1.00
SB4-2	1.61±0.31	2.08±0.08	3.96±0.51	0.35±0.01	13.20±0.40
SB4-4	1.46±0.07	1.75±0.13	1.68±0.22	0.36±0.02	12.30±0.30
SB4-4C	2.19±0.09	1.95±0.01	1.63±0.26	0.44±0.00	40.10±5.10
SB7-1	1.41±0.12	2.54±0.11	1.72±0.28	0.32±0.01	34.30±0.50
SB7-1A	1.25±0.26	1.90±0.05	2.69±0.29	0.44±0.01	32.40±2.60
SB7-2	1.69±0.07	1.25±0.10	1.67±0.67	0.46±0.00	44.50±14.10
SB8-1	1.80±0.02	1.13±0.01	2.51±0.29	0.44±0.00	31.00±1.40
SB8-3A	3.07±0.09	1.96±0.12	1.99±0.19	0.46±0.00	28.70±1.50
SB9-1A	6.20±0.45	2.73±0.07	1.57±0.40	0.46±0.00	33.70±1.30
SB10-1	6.12±0.16	1.24±0.04	2.91±0.46	0.49±0.01	36.00±5.00
SB10-1A	5.47±0.98	2.47±0.35	1.94±0.14	0.47±0.00	43.30±2.70
SB10-1C	2.65±0.12	2.12±0.06	4.81±0.37	0.45±0.01	5.30±0.30
SB10-2	2.83±0.23	1.57±0.14	3.23±0.51	0.49±0.01	73.40±0.00
SB11-1A	0.24±0.02	1.60±0.03	3.32±0.27	0.10±0.01	41.40±1.00
SB11-5	0.70±0.02	1.89±0.23	4.93±0.92	0.37±0.01	44.70±0.30
SB12-3B	2.18±0.14	1.39±0.05	4.71±2.17	0.41±0.02	44.60±0.40
SB16-5A	2.20±0.09	1.38±0.03	3.32±1.16	0.41±0.00	48.00±2.20
SB17-1	2.14±0.16	3.10±0.14	3.36±0.38	0.45±0.02	41.50±0.10
SB17-1A	1.72±0.15	2.68±0.04	3.00±0.41	0.39±0.01	43.20±0.00
SB19-1A	2.48±0.45	1.67±0.02	3.35±0.46	0.43±0.01	42.30±1.50
SB19-3	1.85±0.07	1.08±0.10	2.90±0.55	0.37±0.01	43.40±1.00
SB19-3B	1.70±0.20	1.62±0.09	2.23±0.25	0.41±0.01	42.90±1.10
BAMBARA-6	3.05±0.52	1.08±0.06	2.66±0.25	0.42±0.00	36.70±0.50
BAMBARA-7	1.93±0.27	1.19±0.07	1.95±0.19	0.44±0.01	37.60±0.80
BAMBARA-9	0.20±0.02	0.88±0.07	3.23±0.44	0.08±0.00	39.10±0.50
BAMBARA12	0.28±0.01	1.23±0.03	2.91±0.07	0.34±0.01	32.00±1.00
Grand Mean	2.17	1.71	2.77	0.39	34.10
Mean squares	6.79**	0.96**	2.92**	0.03**	415.85**
Standard Deviation	1.54	0.58	1.31	0.11	14.64

4.3.2 Pearson's correlation

Table 15: Matrix showing the Pearson correlation coefficients among five antinutritional contents in bambara groundnut accessions.

	Condensed Tannins	Free Phosphate	Phytic Phosphate	Polyphenol	Trypsin Inhibitor
Condensed Tannins	1				
Free Phosphate	0.27	1			
Phytic Phosphate	-0.24	-0.03	1		
Polyphenol	-0.24	0.09	0.02	1	
Trypsin Inhibitor	0.63**	0.22	-0.18	-0.04	1

**. Correlation is significant at the 0.01 level (2-tailed).

The Pearson correlation coefficient among the content of 5 anti-nutritional factors in bambara groundnut accessions is presented in Table 15. A positive correlation (r = 0.27) was observed between the free phosphate and condensed tannin contents. CTc observed an equal negative correction (r = -0.24) with phytic phosphate and polyphenol among all the accessions evaluated. CTc was highly significant ($p<0.01$) and positively correlated with (r = 0.63) trypsin inhibitory activities (r = 0.63). There was a positive correlation (r = 0.22) between free phosphate content and the trypsin inhibitor. There was a negative correlation (r = -0.18) between trypsin inhibitory and phytic phosphate contents.

CHAPTER 5

5. DISCUSSION

5.1 Assessing the extent of morphological variation among 30 selected landraces of bambara groundnut.

5.1.1 Anova analysis

Characterization of bambara groundnut germplasm can provide useful information for a breeding programme aiming to genetically improve the crop. The results of this study showed that there was wide genetic variability among the bambara groundnut evaluated for 18 morphological quantitative characters recorded (Table 10). Days to 50% flowering was quite variable among the bambara groundnut accessions ranging from 42 to 67 days. Similarly, a study by Massawe *et al.* (2005) showed that days to flowering in bambara groundnut ranged from 64 to 76 days in Loughborough, United Kingdom, while Masindeni (2006) recorded 43-80 days in the Free State Province in South Africa.

The mean days to flowering (60 days) obtained in this study were higher than the values reported in previous studies. Ouedraogo *et al.* (2008) observed that flowering ranged from 32 to 53 days among bambara groundnut germplasm accessions from Burkina Faso. Similarly, Shegro *et al.* (2013), reported that flowering occurred between 36-53 days among 20 bambara groundnut accessions assessed under similar conditions to this study in Pretoria, South Africa. A number of environmental factors such as temperature, altitude and soil conditions as well as genotypic factors can affect flowering in bambara groundnut (Shegro *et al.* 2013). Similar factors may be responsible for the variation in days to flowering in this study. Swanevelder (1997) also reported variation in days to flowering among bambara groundnut accessions in South Africa. Bambara groundnut is a short day plant and when planted during long days there is either delayed or no flowering, a trait which is also cultivar dependent. Generally, first flowering in bambara groundnut occurs about 30 to 45 days after planting (DAP) and might continue until it reaches maturity. It has been reported that 50% flowering may take from 60 up to 80 DAP depending on the cultivars. Early flowering implies early maturity (Shegro *et al.* 2010) and it is an important trait in bambara groundnut cultivation in South Africa. In this study, the early flowering accessions (SB11-1A, SB7-1C and SB7-1A) could be selected for early maturity.

Days to physiological maturity was highly significant ($P \leq 0.05$) between genotypes and days to harvest ranged from 128 to 152. A similar maturity range was observed by Masindeni (2006) among eight bambara groundnut accessions. Days to maturity are quite variable in bambara groundnut and could range from 90 to 165 (Goli *et al.* 1997). According to Swanevelder (1998), maturity of the bambara groundnut is dependent on cultivar and climatic conditions and ranges from three to six months. The days to maturity in bambara groundnut is also influenced by photoperiod. Linnemann *et al.* (1995) reported that under long photoperiods maturity is delayed. It was observed that the earlier a genotype flowers, the earlier is the physiological maturity (Shegro *et al.* 2010). A similar finding was observed in this study. Assessing days to maturity of crops facilitates escape from drought-stressed environmental conditions and may enable selection for adaptation to drought-prone areas of South Africa (Shegro *et al.* 2010).

No significant difference was observed in the morphological traits such as terminal leaf length and width, leaf area, number of leaves and branches per plant. The mean values obtained for these traits in this study are congruent with those reported by Mohammed (2014) for the same characters. There was a significant difference ($P \leq 0.05$) between petiole length and plant height among the accessions. This is similar to the results reported in a diversity study of bambara groundnut landraces in Tanzania (Ntundu *et al.*2006). Shegro *et al.* (2013) showed that there was a highly significant ($P \leq 0.01$) difference with regards to petiole length and plant height in bambara groundnut genotypes in South Africa

A highly significance difference ($P \leq 0.01$) was obtained between grain-yield and yield related traits such as fresh and dry weight, number of seeds per plant, yield per plant and hundred seed weight indicating high genetic variation among these traits. This result is in contrast with those of Ntundu *et al.* (2006) who reported that there was no significant difference among bambara groundnut accessions for the number of seeds per plant, seed number per pod, hundred seed weight and yield per plot over two seasons in Tanzania. However, variation in yield related traits was also reported by Shegro *et al*. (2013) who suggested that there may be environmental influence on yield in bambara groundnut.

The yield per plant ranged from 2.97 g to 37.81 g. The accession SB19-1A produced the highest yield and was followed by Bambara-9 and Bambara-12. The accession SB8-3A produced the lowest yield which was due to the relatively small size and number of seeds per plant. The mean

yield value obtained from this study was significantly higher than those reported by Massawe *et al*. (2005) and Ouedraogo *et al*. (2008). Slightly higher yields ranging from 4.0 g to 57.52 g was reported by Shegro *et al.* (2013) and Mohammed (2014).

Hundred seed weight has been reported as a tool for the assessment of morphological traits (Massawe *et al.* 2005; Masindeni 2006; Ntundu *et al*. 2006; Ouedraogo *et al.* 2008; Mohammed 2014; Shegro *et al.* 2015). The hundred seed weight in this study ranged from 20.45 g to 68.19 g. The phenotypic coefficients of variation for the traits such as number of seeds per plant, plant height, petiole length and hundred seed weight in this study were almost similar, suggesting that the variations for these traits might be due to genetic rather than environmental factors (Kulkarni *et al*. 2002). In other to assess the real extent of genetic diversity of bambara groundnut accessions, agronomic, physiological, biochemical and molecular evaluation and characterization should be done since morphological genetic markers may be influenced by environmental conditions (Kumar 1999). Nevertheless, agronomic evaluation and characterization is the first step in assessing the genetic diversity of a crop for the breeding programmes. The wide genetic variation observed in this study may be useful for breeders planning to enhance the germplasm of bambara groundnut.

5.1.2 Correlation among the traits

Pearson correlation analysis of vegetative growth, grain yield and yield-related traits revealed that there was a significant ($P \leq 0.01$) difference among some of the quantitative traits (Table 11) in 30 bambara groundnut accessions. Days to 50% flowering showed a positive correlation with all the traits evaluated except for leaf length and leaf width where a negative correlation was observed. It appears that plant height, petiole length and the number of days to 50% maturity were most significant in affecting the days to 50% flowering. The positive correlation between plant height and days to 50% flowering observed in this study was also observed in previous studies (Zongo *et al.* 1993; Kebede *et al.* 2001) suggesting that these traits are heritable and can be transferred into desired genotypes. According to Shegro e*t al.* (2015), the simple correlations between each pair of phenotypic traits clearly depicts the close association between some of the traits and selection of associated traits can be used to improve important traits of interest.

Plant height was positively associated with fresh weight, dry weight, number of seeds per plants, yield per plant, hundred seed weight, yield per plot and the final plant stand was also positively and significantly associated with plant height. This suggests that selection based on these

characters may be effective for improving seed yield. As expected, leaf area was positively correlated with leaf length and width. Ayana and Bekele (2000) also reported a functional relationship for these traits in sorghum. Correlation coefficient is an important parameter in plant breeding since it measures the degree of association, genetic or non-genetic between two or more characters (Jonah *et al.* 2014). Correlation studies between traits have been of great value for selection of superior genotypes (Adebisi *et al.* 2004). The highly significant ($P \leq 0.01$) correlation between the number of leaves and branches per plant coupled with the high correlation ($r = 0.95$) between fresh and dry weight may suggest that selection based on these traits may be useful for breeding bambara groundnut for fodder production. The highly significant ($P \leq 0.01$) correlation observed among fresh weight, dry weight and the number of seeds per plant and the moderate positive association among the number of leaves and branches per plant, days to 50% maturity and days to harvest are congruent with the results obtained by Karikari (2000) and the findings of Jonah *et al.* (2014). These characters may be useful to plant breeders for selecting parents that could improve yield in bambara groundnut. However, the hundred seed weight was negatively correlated with the number of seeds per plant. This was due to the variation in seed size among the different accessions.

Panicle length had a very strong positive significant correlation with plant height and a moderate positive correlation with days to 50% flowering, leaf length and width, leaf area and initial plant stand. The positive correlations among and between the various traits recorded in this study clearly indicate that selecting for any of these traits will have a positive effect on selecting for associated traits in a bambara groundnut improvement programme.

5.1.3 Principal component analysis

Wiley (1981) defined Principle Component Analysis as "a data reduction method to clarify the relationships between two or more characters and to divide the total variance of the original characters into a limited number of uncorrelated new variables". According to Johnson (2012), principal component analysis is perhaps the most useful statistical tool for screening multivariate data with highly significant correlations. It is the most common techniques used in variability studies and numerical classification; useful in grouping varieties of crops based on their similarities (Bello 2004; Dum 2007). Das (2000) reported that the information obtained from PCA assists breeders in identifying quantitative characters that contribute great genetic variation among genotypes for selection of potential parents for crossing the traits of interest. In this study,

the multivariate analysis (Table 12) showed that approximately 87% of the variance in the bambara groundnut accessions was accounted for by the first six PCs. Therefore, the first six principal component axes were retained. Yield-related traits including yield per plot, days to harvest, dry weight and number of seeds per plant were the main factors accounting for the variation in PC1. Similar results were obtained by Jonah *et al*. (2014). This suggests that these characters could be selected for improvement of yield in bambara groundnut.

The initial and final plant stand, leaf and petiole length explained most of the variation in PC2. PC1 and PC2 explained most of the variation among the genotypes suggesting a high degree of association and interrelationships among the traits studied. This result was evident with the report by Jolliffe (1986) who suggested that the first PC usually summarizes most of the variability present in the original data relative to all remaining PCs and the second PC explains most of the variability not summarized by the first PC and uncorrelated with the first PC. The utilization of agronomic and seed yield traits has been used in bambara groundnut improvement programme (Shegro et al. 2013). Furthermore, the characters that contributed more strongly to PC3 and PC4 were leaf length and width and number of leaves and branches per plant. These characters could contribute towards the breeding of bambara groundnut genotypes for use as fodder. Loading characters on PC5 included: petiole length, days to 50% flowering, hundred seed weight, fresh weight, plant height, days to 50% maturity and days to harvest. The sixth principle component comprises of leaf area, yield per plot, fresh weight and initial plant stand. Jonah *et al*. (2014) stated that all principal components are not required to summarize the data adequately because the sample characters are generally inter-correlated to some degree of variations. Both positive and negative loading were observed in the present principle component analysis. Earlier report by Lezzoni & Pritts (1991) reveals that variables which have high positive loading on a principal component are positively inter-correlated as a group. Variable having a high negative loading are also correlated as a group; however, they are negatively correlated with those variables having positive loading.

5.1.4 Principal component biplot

The scattering of the accessions within the four quadrants in PC1 and PC2 (Fig. 12 and Fig. 13) in the biplot analysis indicated that there is wide genetic variation among the traits. The close affinity of the accessions in the first quadrant (Fig. 13) were due to the following ten phenotypic traits: days to 50% flowering, leaf area, plant height, petiole length, number of leaves and branches per plant, days to 50% maturity, final plant stand, yield per plant and hundred seed

weight (Fig. 12). Initial plant stand was the only character that grouped genotypes in the second quadrant while the accessions in the third quadrants were clustered due to days to harvest, fresh and dry weight, number of seeds per plant and yield per plot. Two characters including leaf length and leaf width grouped the genotypes in quadrant four.

The results obtained from the biplot analysis showed that eight genotypes including: SB4-1, SB4-2, SB7-1C, SB10-1C, SB10-1A, SB2-1B, SB1-1 and SB8-1 were clustered around the origin indicating strong associations among these genotypes. Conversely, SB-11-1A was placed as a distinct entity when compared to the other accessions. Similarly, the accessions SB7-1, Bambara-9 and SB19-1A were scattered independently from the other accessions (Fig. 13). According to Shegro *et al.* (2015), genotypes that are closer to each other and/or located near to the origin in the score plot are genetically similar, whereas those genotypes that appear away from the origin are distinct genotypes. The results imply that there is sufficient genetic diversity in bambara groundnut for breeding purposes. This study showed that there was a strong agreement between the grouping of the accessions based on the PC analysis and PCA biplot. Similar observations were made by Shegro *et al*. (2013) and Mohammed (2014). Further research using other methods may be necessary to confirm the relationships among the accessions.

5.1.5 UPGMA cluster analysis

The Cluster analysis for phenotypic traits showed that there was a high level of genetic diversity among the bambara groundnut accessions (Fig. 14). Cluster analysis according to Hair *et al*. (1995), refers to a group of multivariate whose primary purpose is to group individuals or objects based on the characteristics they possess, so that individuals with similar descriptions are mathematically gathered into the same cluster. Based on these traits, the dendrogram divided the accessions into four main clusters and a singleton.

The separation of accession SB11-1A as the most divergent is perhaps due to its late flowering, leaf length and width, shortest petiole length and plant height and lowest hundred seed weight and yield per plot. Clustering of the accessions (Fig. 14) were similar to the grouping of the accessions based on the PC analysis (Fig. 12) and the PCA biplot (Fig. 13). For instance, the two accessions SB7-1 and SB4-4 that feature in cluster I were also closely related in the same quadrant in the PC biplot (Fig. 13).

Cluster I was characterized by early days to flowering, narrowest panicle, average leaf length and narrowest leaf width, shortest plant, smallest number of leaves and number of branches, early

days to 50% maturity, average days to harvest, average number of seed per plant and yield per plant, lowest hundred seed weight and yield per plot (Table 13). Some of the accessions formed relationships on the basis of shared morphological traits. The separation of accession SB7-1 in cluster 1 is perhaps due to the fact that it had the lowest leaf length, lowest number of branches and leaves per plant. A sister relationship was observed between accessions SB10-1A and SB10-1 and both genotypes were closely associated in the second quadrant in the principle component biplot. This sister relationship indicates that these accessions may have a common ancestry.

The grouping of the accessions in cluster II may be due to the presence of similar morphological traits in these genotypes. This cluster consisted of accessions with an intermediate days to 50% flowering and number of leaves and branches per plant, smallest leaf area, lowest fresh and dry weight, lowest number of seeds and yield per plant. The close grouping of accessions SB8-1 and SB8-3A, SB1-1 and SB2-1B, SB17-1A and SB7-1, and SB9-1A and SB7-2 in this cluster may be due to the similarities in their morphological traits including same value for intermediate plant height and panicle length, lowest fresh and dry weight and lowest yield per plant. The rest of the accessions in this cluster were grouped independently.

The clustering of nine accessions in the third cluster appears to be due to shared morphological traits such as days to 50% flowering, smallest leaf length and average leaf width, average panicle length and plant height, late maturing, highest number of seed per plant and medium yield per plant. The uniqueness of Bambara-12 within the cluster may be due to its greatest final plant stand and grain yield per plot. The accessions in this cluster were mainly found around the origin in the first and fourth quadrant in the PC biplot (Fig. 13).

The accessions SB12-3B and SB19-1A were the only accessions contained in cluster IV and were also close together in the first quadrant of the PC biplot (Fig. 13). This cluster consisted of accession with the longest and widest leaves, largest leaf area, tallest plant, highest number of leaves and branches per plant, medium number of seeds, highest yield and hundred seed weight. Characterization and clustering of accessions on the basis of their morphological traits and genetic similarity can help in the identification and selection of the best parents for hybridisation (Souza & Sorrells 1991). Therefore, the grouping of accessions by univariate and multivariate methods of analysis based on their similarity in the present study is an important information that will be valuable for bambara groundnut breeders.

5.2 Characterization of major seed proteins in bambara groundnut accessions using one dimensional gel electrophoresis.

Cilia *et al.* (2009) stated that methodological comparison of protein isolation methods is the first critical step for proteomic studies since protein extraction methods can vary widely in reproducibility and representation of the total proteome; therefore, a combination of extraction approaches increases proteome coverage when using gel-based separation techniques. Out of the four different seed protein extraction methods exploited for this study, 2x Laemmli buffer extraction method produced the best results with clear protein bands. Preliminary studies of total seeds proteins extracted with 10% and 20% isopropanol produced a large number of low and high molecular weight proteins (Fig. 21). However, when this method was employed for extraction of total proteins in 30 bambara groundnut accessions, the resolution of the proteins was very faint in some accessions. This result was in contrast with the study conducted by Natarajan *et al*. (2009) who reported that isopropanol extraction is efficient and suitable for the isolation and separation of abundant seed storage proteins in soybean. The authors hypothesized that this solvent could be used to enrich seed proteins that are different from the main storage proteins.

Using 10 % TCA-Acetone extraction methods (Fig. 22), only one sample (SB7-1C) produced high molecular weight protein fragments. Larger proteins greater than 25 kDa only appeared in the banding profiles while proteins lower than 25 kDa never appeared. This finding is not in agreement with the observations from Osborne (1924) who reported that 10 % TCA-Acetone produced identical protein banding profiles and only extracted abundant proteins of low molecular weight from vegetables. Recently, Wang *et al* (2006) reported that a TCA method combined with phenol was efficient for extracting leaf proteins from bamboo, lemon, olive, and redwood and from apple, pear, banana, grape, tomato, and orange fruits. Direct precipitation of protein from powdered tissues using TCA/acetone has been used to successfully extract proteins from soybean seeds and leaves (Friedman & Brandon 2001).

Furthermore, extraction with the Sonication method (Fig. 23) produced identical protein banding profiles for both abundant proteins of low and high molecular weight. This method yielded more abundant higher molecular weight proteins compared to abundant lower molecular weight proteins in all the bambara seed samples tested. Although the sonication method yielded sufficient protein bands, this method is more suitable for comparisons between higher and lower molecular weight

proteins in a given sample. Hence this protocol was not exploited further for analysis of protein banding patterns for the thirty bambara groundnut accessions evaluated in this study.

On the other hand, the 2x Laemmli buffer extraction method was found to be superior to the other three tested methods for bambara groundnut seed proteome analysis in terms of 1D-PAGE separation. According to Laemmli (1970), the beta 2-mercaptoethanol in Laemmli buffer is known to reduce the intra and inter-molecular disulfide bonds of the proteins to allow proper separation not by shape but by size. The SDS detergent in this buffer binds to all the proteins positive charges which occur at a regular interval, thus giving each protein the same overall negative charge so that proteins will separate based on size and not by charge (Laemmli 1970). For successful proteomics implementation, it is vital to employ an efficient protein extraction method (Chen *et al*. 2011). All extraction methods except 10 % TCA-Acetone resolved common banding patterns in all the bambara groundnut samples. This data suggests that there is a very little or no intraspecific genetic diversity in the seed proteins of bambara groundnut accessions evaluated. Although genetic variations were observed in the morphological characterization (Table 10) of bambara groundnut seeds used in this study, no relationship was obtained between the seed size and protein banding profiles. Lack of genetic variation observed in the protein profiles in this study has also been recorded by several studies. Very little variation was observed in the protein profiles of wild groundnut (Singh *et al.* 1993), suggesting low genetic variation among their accessions. Javaid *et al.* (2004) also reported that seed storage protein profiles in most groundnut accessions were similar suggesting low genetic diversity. In other studies, chloroform/methanol, phenol, TCA, SDS solution and isopropanol have been used to analyze and enrich both less abundant and abundant proteins in various crops (Krishnan 2004; Marmagne *et al*. 2004; Barbara *et al.* 2007; Sheoran *et al.* 2009; Chen *et al.* 2011). There appears to be no universal protocol for protein extraction from all plant tissues (Natarajan *et al*. 2009), but 2x Laemmli buffer method may be exploited further with two dimensional gel electrophoresis for proper identification of bambara groundnut seed proteins.

5.3 Determination of the antinutritional factors in the seeds of selected bambara groundnut landraces.

5.3.1. One-way analysis of variance

Antinutritional factors in foods are mainly responsible for the deleterious effects that are related to the absorption of nutrients and micronutrients which may interfere with the function of certain organs (Gemede & Ratta 2014). There was wide variation in the content of the five antinutritional compounds among the 30 bambara groundnut accessions (Table 14).

The tannin content (Fig. 23) reported for bambara groundnut is highly variable in different studies. The mean values for condensed tannin content in this study ranged between 0.20 - 6.20 mg/g. These values are slightly higher than the value (1 mg/g) obtained by Ohiokpehai *et al.* (1994) in Ghana. Nevertheless, some accessions including: SB7-1C, SB11-1A, SB11-5, BAMBARA-9 and BAMBARA-12 had similar values to those obtained by Ohiokpehai (2003). Samuel *et al*. (2014) observed higher values of tannin contents (18.61 mg/g) in bambara groundnut seeds obtained from local farmers in Nigeria. A lower mean value of 0.046 mg/g was reported by Mazahib *et al.* (2013) among bambara seeds in Sudan. Omoikhoje *et al*. (2006) and Ijarotimi *et al*. (2009) reported a mean value of 0.0011 mg/g and 0.039 mg/g, respectively, among the bambara seeds in Nigeria.

Tannins are heat stable and have been reported to reduce the protein digestibility in animals and humans by either making protein partially unavailable or inhibiting digestive enzymes and increasing fecal nitrogen (Yao *et al.* 2015). Tannins are known to be present in food products and to inhibit the activities of trypsin, chemotrypsin, amylase and lipase, decrease the protein quality of foods and interfere with dietary iron absorption (Felix & Mello 2000). If tannin concentration in the diet becomes too high, microbial enzyme activities including cellulose and intestinal digestion may be depressed (Aletor 2005).

A highly significant ($p<0.01$) difference was observed for the phosphate (free phosphate and phytic acid phosphate) contents among all the accessions evaluated in this study. The overall mean (1.71 mg/g) of free phosphate (Fig. 24) recorded for this study was higher than the mean (0.12 mg/g) obtained by Doria *et al*. (2012). The range (1.35 - 4.93 mg/g) of phytic acid phosphate (PAP) (Fig. 25) in this study was congruent with the values recorded for bambara groundnut in previous studies by Samuel *et al*. (2014) who obtained 4.29 mg/g among their accessions and a slightly lower range of 1.1 mg/g was observed by Yao *et al*. (2015) among ten bambara groundnut landraces from Côte

d'Ivoire. Lower values ranging between 0.130 mg/g to 0.132 mg/g were reported by Ijarotimi *et al*. (2009) and Omoikhoje *et al*. (2006) among the bambara seeds from Nigeria. The mean value recorded for phytic acid phosphate from this investigation was not in agreement with those reported by Mazahib *et al* (2013) who recorded a mean value of 14.78 mg/g among their landraces evaluated in Egypt. The major part of the phosphorus contained within phytic acid are largely unavailable to animals due to the absence of the enzyme phytase within the digestive tract of monogastric animals (Akande *et al*. 2010). Erdman (1979) stated that the greatest effect of phytic acid on human nutrition is its reduction of zinc bioavailability. Phytate content in food can be lowered by addition of enzymes (phytase) which hydrolyze them (Bora 2014).

The polyphenol (Fig. 26) content (0.39 mg/g) observed in this investigation is quite low when compared to the study of Mazahib *et al.* (2013) who obtained a value of 8.72 mg/g. Yao *et al.* (2015) also reported a mean value of 7.07 mg/g. Variation in polyphenol content has also been recorded by several researchers in other legumes (Bravo 1998; Doria *et al*. 2012). Although phenols are known for their antioxidant activities (Rice- Evans *et al*. 1997; Crozier *et al*. 2009) and positive health implications, a high phenol level in legumes is often correlated with poor iron and zinc bioavailability and consequent negative effects on diets of consumers in undernourished populations (Frossard *et al.* 2000; Guzman-Maldonado *et al*. 2000; Diaz-Batalla *et al*. 2006). The levels of polyphenol vary greatly even between cultivars of the same species (Ahn *et al.* 1989). Environmental factors such as light, germination, degree of ripeness, processing and storage, variety and genetic factors has been reported as a major influence on the levels of polyphenol in legumes (Ahn *et al.* 1989).

Trypsin inhibitors are protease inhibitors that occur in raw legume seeds (Gemede & Ratta 2014). The trypsin inhibitor (TIA) was quite variable among the bambara groundnut accessions ranging from 5.30 to 73.40 TIA/mg. The high levels of trypsin inhibitor (Fig. 27) observed in this study are similar to those recorded by Tibe *et al.* (2007) which ranged between 6.40 - 49.1 TIA/mg among bambara groundnut landraces grown in Southern Africa. The mean value observed in this study was higher than the values reported by Samuel *et al*. (2014) and Ijarotimi *et al*. (2009) who obtained values ranging between 0.07 TIA/mg and 0.06 TIA/mg of protein, respectively. Linnemann and Azam-Ali (1993) reported a much narrower range (6.75 to 15.44 TUI/mg) of trypsin inhibitor activity in eight bambara accessions. Trypsin inhibitors are sensitive to heat and

are destroyed completely by wet heat (Apata & Ologhob 1997; Gurumoorthi *et al*. 2003). The variation in trypsin activity among the different studies could be due to different genotypes and environmental conditions such as soil fertility, climate, seasonality, rainfall and light intensity (Champ 2002). According to Gemede & Ratta (2014), trypsin inhibitors inhibit the activity of the enzymes trypsin and chymotrypsin in the gut, thus preventing protein digestion.

Generally, a higher level of antinutrients was observed in this study compared to other studies. According to the observations made by Owolabi *et al*. (2012), the higher the level of antinutrient in legume seeds, the lower the bioavailability of the nutrient and minerals contained therein. Several studies have reported that antinutritional contents in bambara groundnut seeds was significantly reduced through soaking, dehulling, boiling/cooking, roasting, microwaving and autoclaving (Omoikhoje *et al*. 2006; Oluwole & Taiwo 2009; Mazahib *et al*. 2013; Samuel *et al*. 2014). The levels of trypsin inhibitor were significantly reduced by 53 and 64% in soaked and dehulled samples whereas tannin contents was reduced by 64% and 73% in soaked and dehulled bambara groundnut samples, respectively, compared to raw bambara seeds (Omoikhoje *et al*. 2006). A significant reduction in phytic acid contents by up to 74% in boiled bambara groundnut seeds was observed by Ene-Obong & Obizoba (1996) and Samuel *et al*. (2014). According to the work done by Mazahib *et al*. (2013), the polyphenol level in their samples were reduced after soaking and cooking to 26% and 60%, respectively. Therefore, it is evident that soaking, cooking and dehulling can significantly reduce the amount of antinutrient contents in bambara groundnut seeds compared to raw seeds. There are no FAO/WHO provisional standards to compare these results with.

5.3.2 Correction analysis.

With the exception of condensed tannins and trypsin inhibitor, no significant correlation was observed among the five antinutritional factors assessed in this study. The highly significant ($p<0.01$) positive correction observed between condensed tannins and trypsin inhibitor indicates that the higher the amount of trypsin, the greater the amount of condensed tannin content in the sample. The negative correction recorded between phytic acid phosphate and condensed tannins, trypsin inhibitor and free phosphate, as well as the negative correction observed between condensed tannins and polyphenols, trypsin inhibitor and polyphenols suggests that as one variable increases the other variable decreases. The antinutrients in bambara groundnut indicates that they could negatively affect the bioavailability of some vital minerals in the digestive system. However,

this negative effect can be significantly reduced through soaking, cooking and roasting (Samuel *et al.* 2014). These findings are in agreement with earlier work by Samuel *et al.* (2014) who suggested that some of the antinutrients in bambara groundnut negatively affect the availability of the nutrients and minerals; however, the effects were reduced through adequate processing.

CHAPTER 6

6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Characterization and evaluation of bambara groundnut germplasm and identification of the best parents is important for development of new cultivars. A total of 30 bambara groundnut accessions were evaluated for 18 quantitative traits to determine the extent of phenotypic diversity. ANOVA identified the relative importance of each of the quantitative traits. These phenotypic traits substantially contributed in differentiating the accessions. A moderate to high genetic variability was found among the 30 bambara groundnut genotypes which can be exploited for use in an improvement programme for the phenotypic traits of interest. Moreover, the simple correlations between each pair of quantitative characters, clearly depicted the close association between some traits. Selection of highly associated or related characteristics can be used to improve important primary characteristics.

Subjecting the 18 quantitative traits to multivariate analysis supported the results of the ANOVA and correlational analysis. Cluster analysis grouped the accessions into four main groups and a singleton. All the accessions were distinctly separated from each other and the accessions with similar morphological characters grouped together. These reports suggested that agronomic and seed traits are useful for the characterization of bambara groundnut and selection of genotypes suitable for breeding. The low yield obtained in this study could be attributed to higher rainfall received at the experimental site prior to harvest. There was higher rainfall during the final stages of growth, which resulted in waterlogged plots. Higher yield is crucial in crop improvement programmes but it is highly influenced by many contributing traits and environmental conditions both in positive and negative directions. Therefore, indirect selection for improved yield is desirable than direct selection due to its low heritability. Based on the observed variation for the quantitative traits, it could be concluded that the bambara groundnut accessions used in this study showed significant variation in phenotypic characters, indicating that the accessions had high genetic diversity which should allow development of new genotypes for desired traits through selection and crossing programmes.

The protein profiles of the four different extraction methods varied greatly among all the accessions and 2x Laemmli buffer extraction method was found to be the best. All the protein extraction methods produced protein bands ranging in size from below 10 kDa to 250 kDa. A unique feature from all extraction methods was the presence of a common protein band at~75 kDa. All extraction methods except 10% TCA-Acetone resolved common banding patterns in all the bambara groundnut samples. This data suggests that there may be very little or no intraspecific genetic diversity among the seed proteins in bambara groundnut accessions evaluated. For successful proteomics implementation, it is vital to employ an efficient protein extraction method. Although there was a moderate morphological variation among the bambara groundnut, there was no variation in the seed protein banding profiles from the extraction method used, suggesting that these proteins may have been conserved.

Antinutrients are chemicals which are present in plants for their own defense, among other biological functions. However, they reduce the maximum utilization of nutrients especially proteins, vitamins, and minerals, thus preventing optimal exploitation of the nutrients present in a food and decreasing the nutritive value. There was a highly significant ($p<0.01$) variation in the content of the five antinutritional factors evaluated in this study. The presence of these antinutritional factors (condensed tannins, free and phytic acid phosphate, polyphenol and trypsin contents) may pose a health risk if the bambara beans are not processed and may induce undesirable effects in humans if their consumption exceeds an upper limit.

Accessions Bambara-12, SB12-3B and Bambra-6 gave the highest grain yield per plot and can be evaluated further. Moreover, a tailored fermentation process could be optimized in an attempt to reduce antinutrients and, in turn, to improve the bioavailability of minerals and the overall nutritional quality. In general, too much emphasis is usually placed on the analyses of crude protein and fibre as indicators of feed value. More importance should be given to the presence of secondary plant compounds such as tannins and hydrolysable phenolics, which may interfere with the level of protein and fibre content which are used as indicators of high nutritional value.

6.2 RECOMMENDATIONS

The quantitative data collection should be replicated in two to three cropping seasons in other to obtain the real extent of morphological variation among and between accessions. Further research using other methods may be necessary to confirm the relationships among the accessions in the

PCA bioplot. Due to instability in the climatic conditions, other biochemical markers should also be explored in analyzing for genetic variation since they are independent of environmental conditions. Further investigation using 2D-PAGE and mass spectroscopy should be done to identify the type of proteins presence in all the bambara groundnut samples evaluated. Since one dimensional PAGE of the seeds proteins revealed little genetic variation compared to morphological characteristics, 2D-PAGE, other protein extraction methods and DNA based analysis should also be exploited to gain a better understanding of the genetic variation in bambara groundnut.

The abundance of antinutritional factors and toxic influences in plants used as human foods and animal feeds is a cause for concern. Therefore, ways and means of eliminating or reducing their levels to an acceptable minimum should be discovered. Several studies have reported that antinutritional contents in bambara groundnut seeds was significantly reduced through soaking, dehulling, boiling/cooking, roasting, microwaving and autoclaving. Further research in this regard is needed. Plant breeders should also look into producing cultivars with reduced antinutritional factors as they impede the bioavailablity of minerals and protein digestibility. Further investigation is needed to ascertain the harmful effects of antinutrients (condensed tannins, free and phytic acid phosphate, polyphenol and trypsin contents) in bambara groundnut landraces.

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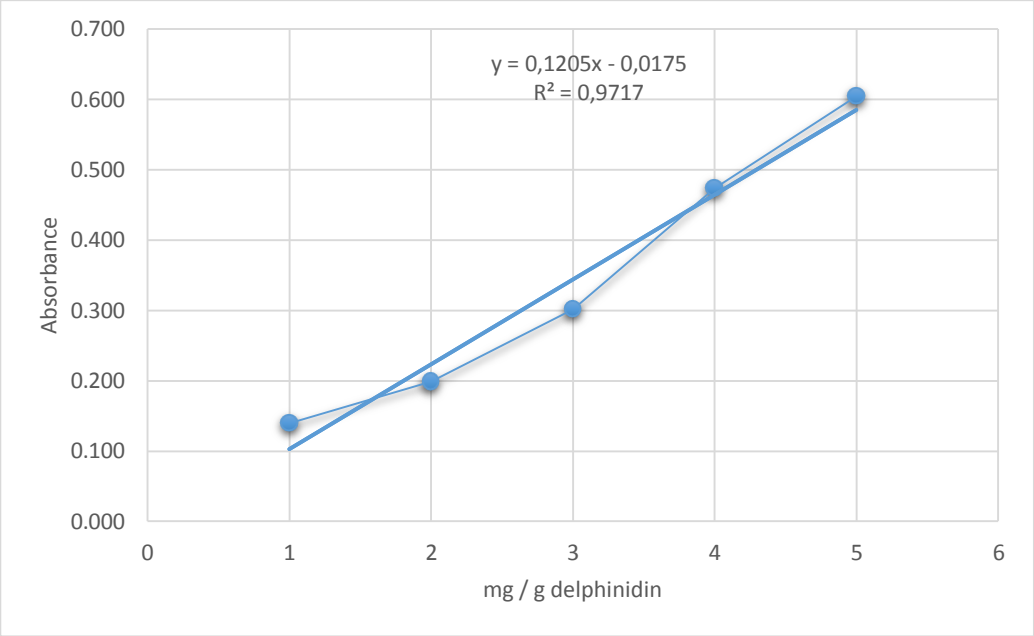
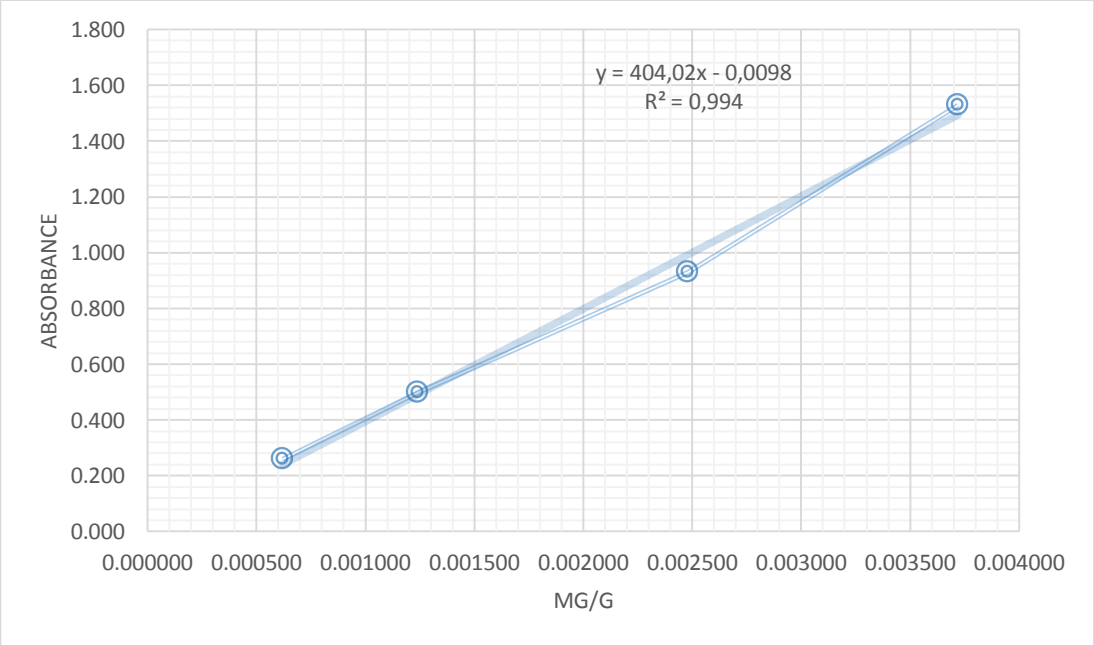
APPENDICES

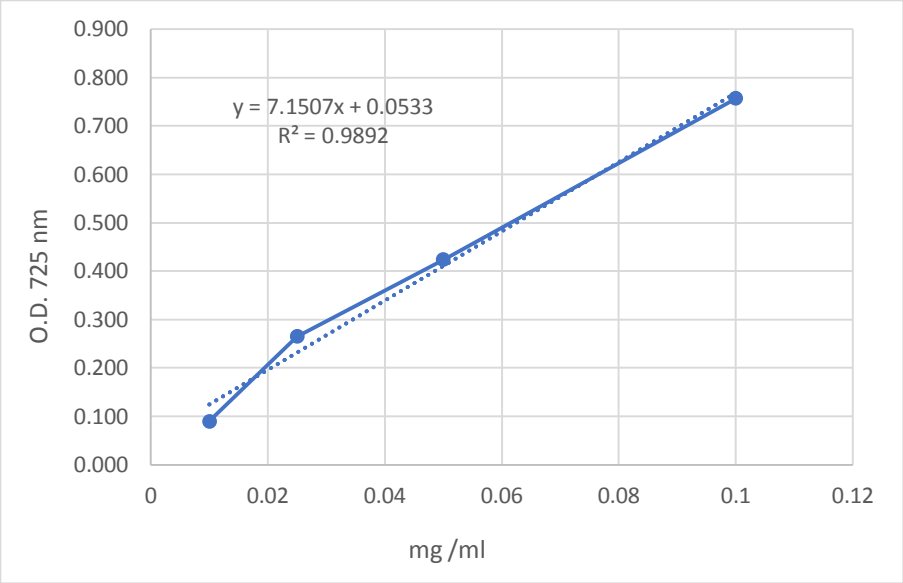
APPENDIX I: Standard curves for the Antinutrients analysis among 30 bambara groundnut accessions



Standard curve of condensed tannin contents.

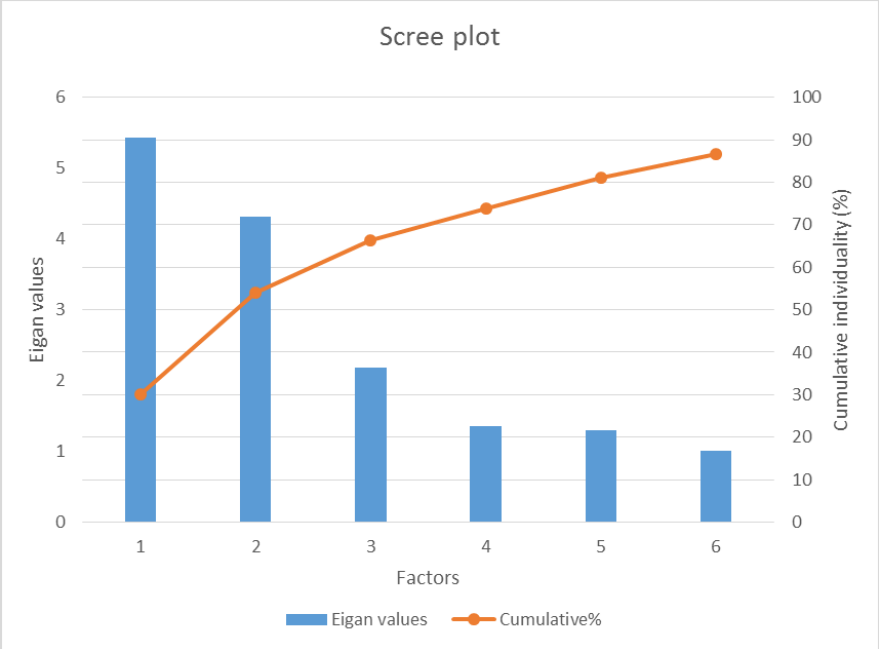


Standard curve for phytic phosphate content.



Standard curve of polyphenol content.

APPENDIX II: Scree plot for the Principal Component Analysis (PCA)



The scree plot of first six principle component with eigen values and cumulative percentage contributions to each PC loading.

APPENDIX III: Cluster means of 18 quantitative traits for the thirty bambara groundnut accessions evaluated.

The summary of cluster means of 18 quantitative traits for the bambara groundnut accessions based on data set.

Characters	Cluster means				
	I	II	III	IV	Mean
Days to 50% flowering	55.61	60.22	63.15	62.00	60.25
Leaf length	5.86	5.80	4.55	6.07	5.57
Leaf width	2.22	2.57	2.61	2.88	2.57
Leaf area	1137.43	1079.22	1263.19	1324.35	1201.05
Initial plant stand	11.28	13.25	9.56	15.17	12.32
Pennicle length	13.54	15.11	15.08	17.21	15.24
Plant Height	21.15	21.78	22.71	26.01	22.91
Number of leaves per plant	293.71	309.99	302.95	444.90	337.89
Number of branches per plan	81.23	101.01	112.98	148.30	110.88
Days to 50% maturity	96.33	114.01	118.99	115.83	111.29
Days to harvest	134.94	134.64	128.33	138.34	134.06
Final plant stand	8.33	11.95	9.30	11.50	10.27
Fresh weight	61.36	36.67	98.57	101.98	74.65
Dry weight	20.54	13.89	35.24	38.11	26.95
Number of seed per plant	24.77	17.41	51.22	40.65	33.51
Yield per plant	11.22	6.82	23.02	26.44	16.88
Hundred seed weight	32.51	41.06	43.43	53.40	42.60
Yield per plot	67.56	92.47	166.69	237.49	141.05

APPENDIX 1V: The amount of protein contained in thirty bambara groundnut using the quick start Qubit® fluorometer protein kit from Thermo Fisher Scientific.

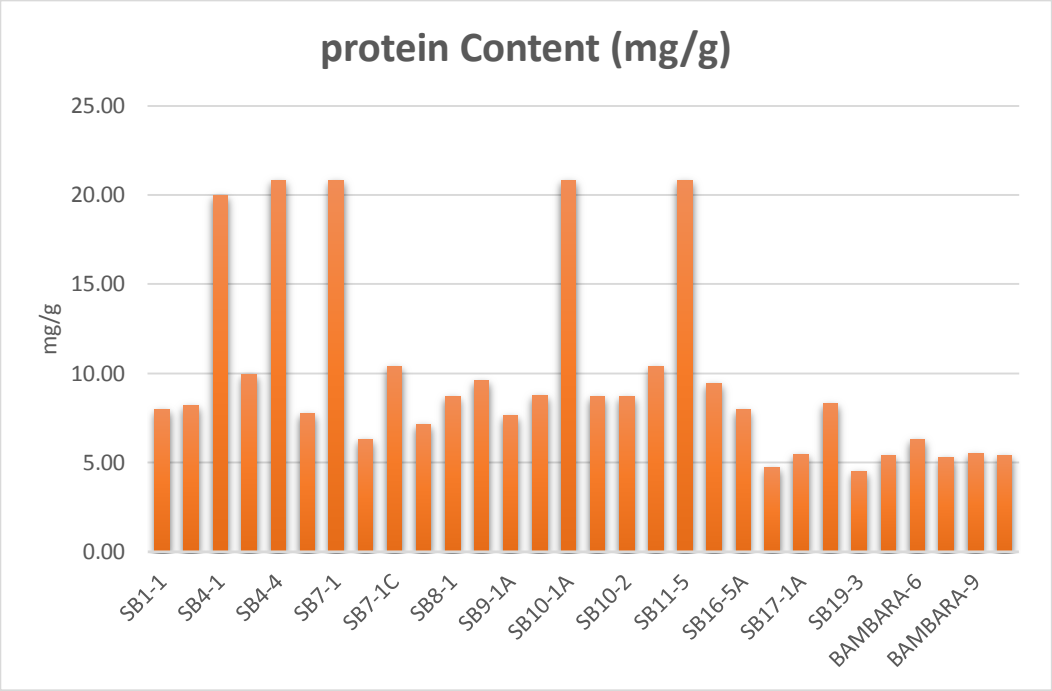


Figure 31: Concentrations of protein (mg/g) in thirty accessions of bambara groundnut.