EVALUATION OF THE POSSIBLE APPLICATION OF COWPEA GENOTYPES IN THE FARMING SYSTEMS OF THE EASTERN CAPE PROVINCE SOUTH AFRICA

ADEYEMI SAMSON ADEBOWALE

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Department of Biochemistry and Microbiology

Faculty of Science and Agriculture

University of Fort Hare, Alice 5700

SUPERVISOR: PROF. G. BRADLEY

CO-SUPERVISORS: PROF. AI OKOH & PROF. F LEWU

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DECLARATION

I, the undersigned, declare that this dissertation and the work contained herein being submitted to the University of Fort Hare for the degree of Master of Science in Biochemistry in the Faculty of Science and Agriculture, is my original work with the exception of the citations. I also declare that this work has not been submitted to any other university in partial or entirety for the award of any degree

ADEYEMI SAMSON ADEBOWALE

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SIGNATURE

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DATE

DEDICATION

With gratitude to God, this research work is dedicated to my grandmother: Mrs Morenike Christianah Adebisi who crafted in me the quest for academic excellence.

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LIST OF ABBREVIATIONS

97K = 97K-10169-8

ABS = Absorbance

AFLP = Amplified Fragment Length Polymorphism

ANOVA = Analysis of Variance

ARC = Agriculture Research Council

BICMV = Black eye Cowpea Mosaic Virus

CAPS = Cleavage Amplified Polymorphic Sequence

DAF = DNA Amplification Fingerprinting

DAF-PCR = DNA Amplification Fingerprinting Polymerase chain reaction

dATP = Deoxyadenosine triphosphate

dCTP = Deoxycytidine triphosphate

DG = Days of germination

dGTP = Deoxyguanosine triphosphate

DNA = Deoxyribonucleic acid

dTTP = Deoxythymidine triphosphate

EC = Eye colour

F = Fahari

FAO = Food and Agricultural Organization of the United Nations

FC = Flower colour

FD = Fahari dark

F-LSD = Fisher's Least Significant Difference Test

GH = Growth habit

GT = Growth pattern

IBPGR = International Board for Plant Genetic Resources

IITA = International Institute of Tropical Agriculture

IT = IT93K-73h

IV = Ivory grey

LC = Leaf colour

LG = Linkage Group

LM = Leaf marking

LT = Leaf texture

MAS = Marker Assisted Selection

MEGA = Molecular Evolutionary Genetics Analysis

mg/ml = Milligram per millimeter

OK = Okhalweni

PAGE = Polyacrylamide gel electrophoresis

PAST = Paleontological Statistics

PC = Pod colour

PCR = Polymerase Chain Reaction

PMSF = Phenylmethylsulphonylflouride

PP = Plant pigmentation

ppm = Part per million

QTL = Quantitative traits loci

RAPD = Random Amplified Polymorphic DNA

RFLP = Restriction Fragment Length Polymorphism

rmp = Revolutions per minute

SCC = Seed coat colour

SPSS = Statistical Package for Social Sciences

SS = Seed shape

SSCP = Single Strand Conformation Polymorphism

SSRP = Simple Sequence Repeat Polymorphism

TAE = Tris base, Acetic acid and Ethylenediaminetetraacetic acid

TLS = Terminal leaflet shape

Tris-HCL = Tris-aminomethane Hydrochloride

TT = Testa texture

TT = Twinning tendency

UPGMA = Unweighted pair group method of arithmetic average

UVP = Ultraviolet Product

VC = Vegetable cowpea

WHO = World Health Organization

µl = Microliter

TABLES OF CONTENTS

ABSTRACT

ABSTRACT

Characterization studies on the genetic diversity among cultivated cowpea (*Vigna unguiculata* (L.) varieties are valuable tools to optimize the use of available genetic resources by farmers, local communities, researchers and breeders. Eight cowpea (*Vigna unguiculata* (L.) genotypes (Vegetable cowpea, Ivory grey, Okhalweni, Fahari, Fahari dark, 97K-1069-8, IT93K-73h, and 129-3) were subjected to molecular, morphological and agronomical characterization. DNA amplification fingerprinting markers were used to evaluate the genetic diversity among the eight genotypes. Nine random arbitrary primers were used to screen the eight genotypes to assess their ability to reveal polymorphisms in cowpea, and seven of them were selected for use in characterizing the total sample.

A total of 43 bands were generated which are all polymorphic. On the average, the primers generated a total of 6.1 polymorphic bands. The resulting data-matrix included 43 analysed bands with a total of 344 characters. Neighbour joining analysis was used to generate the dendrogram, clustering the genotypes into two groups at an agglomerate coefficient of 0.30 irrespective of their geographical origins.

The results also showed the presence of significant differences in morphological and quality traits among the genotypes. Fahari yielded the highest concentration of crude protein (46.51 mg/mg dry leaf) while Vegetable cowpea yielded the lowest (24.41 mg/mg dry leaf). The influence of manure was also found to be effective by increasing the crude protein content of the genotypes as shown by Fahari dark with an average of 53.53 mg/mg dry leaf as opposed to 39.85 mg/mg dry leaf without manure application. Although some small clusters grouped accessions of the same growth habits, a general lack of agreement between clustering and morphological features was observed.

It can therefore be concluded that the significant differences between the molecular genetic analysis using DAF-PCR markers, morphologic characters and yield traits can be important tools to identify and discriminates the different cowpea genotypes.

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

TABLE OF CONTENTS

1.0 INTRODUCTION

Cowpea (*Vigna unguiculata*) is a leguminous species used as forage and vegetable crop mainly in the tropics (Steele, 1972). *V. unguiculata* is a plant source of protein; a vital nutrient for healthy growth in humans and livestock. Its leaves, green pods and grain are consumed as a dietary source of protein (Ghaly *et al.*, 2010). The cowpea seed contains about 23% protein and 57% carbohydrate, while the leaves contain between 27 – 34% proteins. The leaves and seed also serve as sources of high protein feed and fodder for livestock (Tarawali *et al.* 1997, 2002).

Cowpea production remains the most prominent food legume cultivated by farmers majorly in most of the Sub-Saharan African countries. This could be as a result of its ability to withstand drought and adapt to limited nutrient environments. The species is also able to fix atmospheric nitrogen in marginal soils where farmers are unable to adequately fertilize their crop due to income related limitations (Steele, 1972).

Over 16,000 genotypes of this species are held in trust for the World Bank by the International Institute of Tropical Agriculture, (IITA) Ibadan, Nigeria. This huge genotype bank has provided a wide range of information on the agronomy and potential nutritional benefits of growing this species especially in marginal soils.

A report by Asiwe, (2009) showed that investigation and efficient research in the cultivation of cowpea have reduced drastically in the last thirty years in South Africa. This could be as a result lack of funding by the government to boost its production as well as the lack of interest by researchers in promoting the improvement of the crop. It was further noted that lack of sound knowledge of effective agronomic practices, absence of good seeds for planting and discouraging poor marginal returns to farmers further worsen the limitations to

cowpea production in some of the provinces most especially Kwazulu-Natal, Limpopo and Mpumalanga. Acreage cultivated per farmer was also very small because grain yield was very low (Asiwe, 2009). These assessments confirmed that cowpea production in South Africa is still at subsistence level and needs a lot of improvements in terms of yield enhancement and reducing the constraints to its production.

Consequently, *Vigna Unguiculata* to a large extent is still one of the main leguminous crops which have not received an effective molecular transformation. Thus, the huge potential benefits in cowpea have been left untapped and not maximized. However, cowpea is yet one of the leguminous crops which has not been fully exploited for its large genetic gains in genetic improvement with minimum investment (Timko and Singh 2008). Due to the fact that cowpea is cultivated mostly by poor farmers in developing countries, it has received a very low attention from a research standpoint (Timko and Singh 2008). Hence, cowpea has been named as an ‘’orphan crop’’ that is recommended for increased public/donor support for biotechnology research (Naylor *et al.*, 2004).

Meanwhile, there are insufficient data on genetic diversity of landraces especially in South Africa. Quantitative analysis of genetic diversity in cultivated and wild crops has important consequences for further breeding and conservation of genetic resources (Khalighi *et al*., 2008). In hybridization process, plants breeders depend on the type and degree of genetic diversity to make proper selection of appropriate genotypic parent.

Investigations of genetic diversity in crop species are normally accessed as a result of variations in morphological characters and qualitative traits (Schut *et al*., 1997). However, evaluation of the genetic similarities using morphological markers among accessions is tasking and entails costly methods (Cooke, 1984).

Molecular markers are usually employed in characterizing the degree of genetic diversity among populations or groups of individuals owing to their ability to typically detect high levels of polymorphism (Kosman and Leonard, 2005). A number of researchers (Paterson *et al*., 1991; Weising *et al*., 1995; Karp *et al.*, 1996; Kumar, 1999) have also reported that molecular markers were still remain the most promising methods for differentiation among genotypes at both species and sub-species level.

Evaluation studies on the genetic diversity in cowpea genotypes would increase the development of culti-groups for targeted production constraints by generating diverse types of parental lines for breeding programs. A clear insight of the dimension, distribution and nature of differentiation would be helpful in the production of cowpea genotypes with both improved yield potential and better adaptation potential to environmental stresses.

The development of new genomics-based resources for cowpea will definitely assist in the future expansion of both marker-assisted breeding and marker-assisted-selection. This will also facilitate the development of transgenic plants that can be used in the developing world in a safe, rational, and controlled manner. Future developmental studies on cowpea will gain further insights from basic genomic research conducted on other legume crops as well as 'model species' (Timko and Singh 2008).

A few years ago, South Africa acquired some selected genotypes of cowpea for agronomic and nutritional characterization (Rhandzu, 2007). Evaluation studies on the performance of this species under different microclimates of the country are needed for the ultimate introduction of these genotypes into the farming systems of the country. Several studies have revealed nutritional differences among genotypes of different cultivars (Nielson *et al*., 1993; Hall *et al*. 2003). Evaluating the protein content is the first step at improving the protein quality of any crop. Although Sabetha *et al*., (2010) reported an average of 24.1-

30.7% leaf protein content for two cowpea varieties in Limpopo Province, there is currently no evaluation study on the protein content and the most appropriate time to introduce accessions of cowpea into the farming systems of the Eastern Cape Province of South Africa.

Protein deficiency has been reported as one of the main source of nutritional problems in the developing world (FAO, 1997). Ghaly *et al.*, (2010) reported that protein deficiency results in Maramus and Kwashiorkor which are majorly prevalent nutritional diseases in children. The production of important protein sources like single cell protein (Tennenhaum and Wang, 1975) and soybean protein (Mendez, 2002; Bhatia and Greer, 2008) has greatly influenced the alleviation of protein deficiency globally (Kuijer and Wielenga, 1999). Notwithstanding, about one billion people are still reported to be suffering from protein deficiency and malnutrition worldwide (WHO/ FAO, 2007). Breeding high yielding varieties of cowpea with quality traits such as high protein content will contribute to food security and improve income generation to alleviate poverty.

1.1 AIM AND OBJECTIVES

The broad aim of this research work is to investigate and discriminate eight accessions of *Vigna unguiculata* species under measured atmospheric parameters and to access the effects of these parameters on the leaf protein content of selected high performing genotypes with a view to introducing them into the farming systems of the Eastern Cape, South Africa.

1.1.1 SPECIFIC OBJECTIVES:

- To characterize eight accessions of *V. unguiculata* using molecular, morphologic and agronomic markers.
- To assess the effect of manure on leaf crude protein of the various genotypes.
- To identify promising genotypes for further agronomic studies.

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CHAPTER TWO

LITERATURE REVIEW

TABLE OF CONTENTS

LIST OF FIGURE

LITERATURE REVIEW

2.1. ORIGIN, DOMESTICATION AND GEOGRAPHICAL DISTRIBUTION.

Cowpea has been suggested to originate from Southern Africa, however it is not very clear where the crop was first planted in the region (Timko and Singh 2008). Baudoin and Marechal (1985) proposed Eastern and Southern Africa to be the original region of diversity and Western and Central Africa as secondary centers of diversity based on the distribution of various wild cowpeas across the entire length of Eastern to Southern Africa and Asia as the third centre of diversity.

Recent studies indicated that the highest genetic diversity of primitive wild forms of cowpea can be found in the southern region of the African continent currently encompassed by Namibia, Botswana, Zambia, Zimbabwe, Mozambique, Swaziland, and South Africa. Meanwhile the most primitive species were observed in Transvaal (comprising of Guateng, Limpopo and Mpumalanga Provinces), Western Cape, and Swaziland (Padulosi, 1993 and Padulosi *et al*., 1997). As a result of this observation, Padulosi and Ng (1997) proposed that southern Africa might be the centre of origin for cowpea with accompanied radiations of the wild types to other parts of Southern and Eastern Africa, followed by West Africa and Asia.

The small seed size of wild cowpeas likely enhanced their distribution by birds across the regions of Eastern and Western Africa contributing to the diversity and development of secondary wild forms. The choice for larger seeds and good growth habits from primary variants in wild cowpeas likely led to diverse cultigroups and their domestication in Asia and in Africa (Steele, 1972; Ng, 1995).

The report of Ng (1995) showed that evolution of *V. unguiculata* resulted in changes in its growth habit from perennial to annual and majorly from out breeding to inbreeding.

Cultivated cowpea (subsp. unguiculata) was documented to emanate primarily from the wild variety (var. dekindtiana) through selection (Nkouannessi, 2005). It was also reported that an increase in seed and pod size during the process of domestication and after the species was brought under cultivation through selection contributed to a loss in seed dormancy and pod dehiscence.

The same author however suggested Western Africa as the centre of maximum diversity of cultivated cowpea majorly in the savannah area of Nigeria, Southern Niger, part of Burkina Faso, Northern Benin, Togo, and the North-Western part of Cameroon.

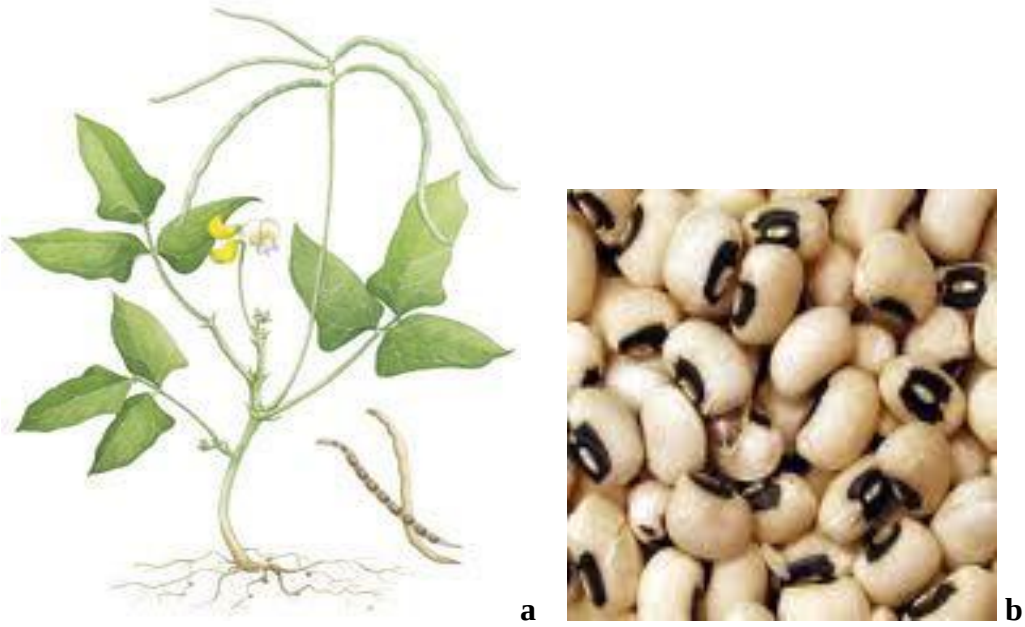
2.2 TAXONOMY OF COWPEA

Cowpea [*Vigna unguiculata (L) Walp*.] is a dicotyledonous crop in the order *Fabaceae*, subfamily *Faboideae* (Syn. *Papillionoideae*), tribe *Phaseoleae*, sub tribe *Phaseolinae*, genus *Vigna*, and section *Catiang* (Verdcourt 1970; Marechal *et al.*, 1978). It contain 22 chromosomes (2n=2×11=22). The genus *Vigna* is pan tropical and highly variable. *Vigna unguiculata* subspecies unguiculata includes four cultigroups; *unguiculata*, *biflora* (or cylindrical), *sesquipedalis*, and t*extilis* (Ng and Marechal, 1985).

2.3 BOTANY OF COWPEA

The cowpea plant is an annual warm-season herbaceous crop with a required growth temperature of at least 18^{o}C within its growth period and having a maximum growing temperature of about 28 ^{o}C (Craufurd *et al.*, 1997). The average weight of a cultivated cowpea seed ranges between 80 and 320 mg and varies in shape from round to kidney-

shaped. There are between 8 and 18 seeds within a pod which is cylindrical, curved or straight. The seed coat varies in texture (e.g., smooth, rough, or wrinkled), color (e.g., white, cream, green, buff, red, brown, black), and uniformity (e.g., solid, speckled, or patterned). Seeds of the most well-known cowpea varieties, such as “black eye pea” and “pinkeye,” are white with black or red coloured area surrounding the hilum that gives the seed the appearance of an eye as shown in figure 2.1.



a **b**

Figure 2.1: Morphological appearance of a fully grown cowpea plant with green pods, dry pods and flower (a) and seeds (b) (Source: IITA Research Station Ibadan, Nigeria, 2000)

2.4 CULTURAL PRACTICES

Cowpea is a grain legume cultivated in tropical and subtropical regions of the savanna. Its high level of protein content (23-35%) makes it important and its capability to fix

atmospheric nitrogen to ammonia in the soil allows it to germinate well on poor soils and increase its fertility (Steele, 1972). It is cultivated for its seed, pods and/or leaves, which are eaten in the fresh form as green vegetables. Its dry grain is also cooked as snacks and main meal dishes. Most of the edible parts of the plant are nutritious, making it an extremely valuable protein source to vegetarians and people who cannot afford animal protein. The remaining parts of the cowpea plant without the pod is also used as a nutritious fodder for livestock.

Intercropping system of cultivation with cereal crops such as millet and sorghum is supported by cowpea. It is mainly grown in mixtures with other crops and a great diversity of crop mixtures has been reported (Perrin and Phillips, 1978; Henrient *et al.*, 1997; Mortimore *et al.*, 1997). The major reasons why farmers intercrop cowpea are for its flexibility, profit optimization, decrease in risks, soil conservation, weed control management and nutritional benefits (Shetty *et al.*, 1995).

An increase in demand for cowpea in urban settlements has been observed. This has brought about a shift from intercropping to sole cropping of cowpeas in order to enhance the total production output of the crop (Thiaw *et al.*, 1993). The international Institute of Tropical Agriculture (IITA) has improved cowpea varieties as well as and also enhance cropping systems to increase total productivity, with minimum use of purchased inputs (Singh, 1993; Singh and Ajeigbe, 2000).

Its different uses, nutritive values and good storage qualities have enhanced the integration of cowpeas into the farming system in the West African region (Eaglesham *et al.*, 1992). Notwithstanding, a very large percentage of the world's cowpeas are cultivated in areas with low rainfall as well as several other yield-reducing factors (Watanabe *et al.*, 1997). Good cowpea cultural practices that can bring about improved performance include, an

appropriate seeding date, effective method and rate of seeding and the use of varieties with high yields and weed control.

During cultivation, cowpea seeds are placed about 5 cm deep into the soil. Both determinate and indeterminate varieties are cultivated in about 45-50 cm and 75 cm in rows respectively with about 10cm intervals. In some of the provinces of South Africa, planting times are November to early December for hay and mid-December to mid-January for seed. (http://agriculture.kzntl.gov.za/portal/AgricPublications/TechnicalInformation/CowpeasProductioninKZN/tabid/206/Default.aspx).

2.5 SOIL REQUIREMENT AND MANAGEMENT PRACTICES

Cowpeas have the ability to withstand higher soil acidity than maize and have been found to produce good seed yields as much as 1 ton per hectare at over 70% acid saturation. An average of a pH of 5.5 to 6.5 is needed for its optimum yield on a well-drained sandy loams or sandy soils (Davis *et al.*, 1991). Phosphorus as a major soil element is valuable for seed set and recommended to be applied at 40 kg/ha, while nitrogen is added to boost its performance at 20 kg/ha until the rhizobia become active. Whereas rhizobia that are able to inoculate cowpea nodules are present in most of the soils in South Africa, it is recommended to inoculate the seed before planting to ensure effective nitrogen fixation. Cowpeas usually supply an average of 20-30 kg of nitrogen per hectare; application of potassium can be avoided while the soil level is above 80 mg/L. (http://agriculture.kzntl.gov.za/portal/Agricpublications/Agricupdates).

2.6 COWPEA PRODUCTION CONSTRAINTS

In the developing world where soil fertility is low, rainfall is inadequate, and most of the cowpea is cultivated without the use of fertilizers and plant protection strategies (i.e., pesticides or herbicides), several kinds of biotic and abiotic constraints hinder growth and yield (Singh 2005; Timko *et al.* 2007a).While cowpea is inherently more drought-tolerant than other crops; inadequate water supply is still among one of the most important abiotic constraints to its growth and yield (Timko *et al.* 2007a).

Cowpea is vulnerable to a wide range of bacterial, fungal and viral diseases and diverse types of insect pests (Singh 2005; Timko *et al.* 2007b). The main insect pests of cowpea are aphids (*Aphis craccivora*), thrips (*Megalurothrips sjostedti*) and Maruca pod borer (*Maruca vitrata*). Nematodes are significant constraints in some areas (Roberts *et al.* 1996, 1997) and parasitic weeds such as *Striga gesnerioides* and *Alectra vogeliii* also constitute huge hindrances to cowpea production in Africa (Timko *et al.*, 2007b). Striga infection in cowpea is more destructive in regions with sandy soils, low fertility and little rainfall. Both parasites are difficult to control because they produce a large number of seed and up to 75% of the crop damage is done before they emerge from the ground.

2.7. ECONOMIC AND AGRONOMIC IMPORTANCE

Cowpea seed is the most important part of the cowpea plant for human consumption. The seeds are most usually harvested and dried for storage and consumption at a later time, either after cooking whole or after being milled into a flour product and used in various recipes (Nielsen *et al*., 1997; Ahenkora *et al*., 1998). Therefore, cowpea plays an important role as a

major source of dietary protein which serves as a nutritional supplement for low-protein cereal and tuber crops eaten by millions of people in the developing world.

Apart from human consumption, cowpea leaves and stem (stover) are also an important source of high-quality hay for livestock feed (Tarawali *et al*., 1997, 2002). Cowpea is also a valuable component of farming systems in regions where soil fertility is low, especially nitrogen and phosphorus. This is due to its ability to fix atmospheric nitrogen to the soil at a higher rate when in symbiotic relationship with mycorrhizae (Elawad and Hall 1987). It also has the ability to withstand an extensive range of soil pH as opposed to other leguminous plants (Fery, 1990). Cowpea is also an efficient main component in crop rotation system owning of its ability to replenish soil fertility for subsequent cereal crops (Carsky *et al*., 2002; Tarawali *et al*., 2002; Sanginga *et al*., 2003). In addition, early maturing cowpea varieties with the ability to produce seeds within 55 days after planting usually serve as a source of food for farmers prior to the maturity of other crops (Hall *et al*., 2003).

In comparison with other legumes, cowpea is known to possess a good and better adaptation quality to withstand severe temperatures and tolerate drought stress (Hall *et al.* 2002; Hall 2004). Until now, few other legume crop species are known to be capable of producing significant quantities of grain under very harsh conditions.

Several advantages exist for breeders to develop cowpea varieties with resistance to diverse kinds of abiotic factors (e.g., drought, low soil fertility, high salinity), resistance to various diseases, pests and parasites as well as different types of agronomic traits (e.g., plant growth habit, flowering times, maturity dates, numbers of seeds per pod) with definite adaptation to agro-ecological production zones and crop product utilizations (i.e., dual-purpose grain and hay production).

2.8 BREEDING FOR IMPROVED NUTRITIONAL QUALITY

The Harvest Plus initiative in 2003 sponsored by Bill and Melinda Gates Foundation (http://www.gatesfoundation.org/default.htm) and other organizations have developed a dynamic breeding program to facilitate improved cowpea varieties with high protein content as well as other micronutrients. Since its inception, enormous achievements have been recorded. About 2,000 genotypes (cultivars and breeding lines) have been discovered showing high significant genetic variability in protein and micronutrient contents in other parts of the world. Typical values are as follows: protein 21% - 30.7%; calcium 545 ppm - 1,300ppm; iron 48ppm -79ppm; zinc 23ppm - 48ppm; and potassium 12,750ppm - 16,150ppm. Among the genotypes investigated, IT97K-1042-3, IT99K-216-48-1, and IT97K-556-4 revealed a good level of all attributes, whereas IT97K-131-2 and IT86D-724 had the lowest concentration of most of the qualities. These data suggested that cowpea already have fairly high levels of these micronutrients compared to other crops, and there is also a good opportunity to further improve the nutritional attributes of new cowpea varieties. Protein isolates from cowpea grains have good functional properties, including solubility and emulsifying and foaming activities (Rangel *et al*., 2004), and could be substitutes for soy protein isolates for persons (especially infants) with soy protein allergies.

2.9. CHARACTERIZATION OF VIGNA UNGUICULATA WALP (L)

To sustain biodiversity and improve molecular breeding programs, characterization of accessions is of great importance (Nkongolo, 2003). The study further revealed that detailed analysis of traits ranging from morphological to molecular and biochemical is important in investigating genetic diversity (Shafique *et al.*, 2011). Unto this end, characterization data

available in many cowpea databases are derived from morphological characters. This explains why Nkongolo (2003) remarked that better understanding of the genetic relatedness among accessions is imperative for better characterization and identification of gene flow in plant's population. So, it is important to maximize the use of available genetic resources (Zannou *et al*., 2008).

2.9.1 MORPHOLOGICAL AND ISOZYME MARKERS

Many times, investigation of genetic diversity in plant types are majorly as a result of variations in morphological characters and qualitative traits (Schut *et al*., 1997). Also, several agriculturally important characters in crop species are quantitatively inherited (Irzykowska *et al*., 2004).

Isozymes markers verify the level of relatedness between plant varieties (Brown, 1979). Various studies including genetic relationship and diversity, taxonomy as well as populations has also been investigated using isozymes markers in cowpea improvement programs. Evolutionary analysis of 150 wild genotypes of *Vigna unguiculata* was studied using 20 isozymes and 35 morphological markers (Pasquet, 2000). The result suggested *Vigna unguiculata var. spontanea* as the most probable ancestor of cultivated cowpea and North-West Africa as the centre of origin for *Vigna unguiculata*. In another similar experiment, the same author analyzed 191 cultivated *Vigna unguiculata* species and discovered a low level of isozyme polymorphisms (Pasquet, 1999). Furthermore, a number of experiments to study diversity among cultivated cowpea germplasm using isozyme markers have revealed very low genetic diversity (D'Urzo *et al*,. 1990; Penella *et al*., 1993 Vaillacourt *et al*., 1993).

2.9.2. MOLECULAR MARKERS

Functional and genetic variations in plant crops are easily detected with DNA markers. Molecular markers as a result of differences in DNA sequences between individuals majorly detect higher level of polymorphisms than morphological and biochemical markers (Tanksley *et al*., 1989). They are not influenced by environmental factors, unaffected by the plant growing conditions and are easily measured at various stages of the plant growth (Mohan *et al*., 1997).

Polymorphism in the nucleotide sequence is often enough for it to function as a molecular marker in mapping. These polymorphisms are elucidated by various molecular techniques such as restriction fragment length polymorphism (RFLP) (Federici *et al*., 1998; Desplanque *et al.*, 1999), amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995), microsatellite or simple sequence repeat polymorphism (SSRP) (Dje *et al*., 1999), random amplified polymorphic DNA (RAPD) (Rodriquez *et al*., 1999), cleavage amplified polymorphic sequence (CAPS) (Jarvis *et al*., 1994), and single strand conformation polymorphism (SSCP). These various makers have been employed in the construction genetic maps for several other crop species on a single segregating population as well as fingerprinting of genotypes and plant clones.

Diversity studies on cultivated cowpea genotypes using isozyme and other biochemical markers have revealed very low level of variation in genetic make-up (D'Urzo *et al*., 1990; and Panella *et al*., 1993). In addition, there was no difference between cultivar group s*esquidalis* and the cultivar group *V. unguiculata* (Vaillancourt *et al*., 1993). Amplified Fragment Length Polymorphisms (AFLPs) have evolved as a unique and efficient method for DNA fingerprinting and genome mapping (Zabeau and Vos, 1993).

The DNA amplification fingerprinting (DAF) technique is a simple and efficient method for producing molecular markers that are highly polymorphic and relatively low-priced. (Winter *et al*., 2000; Benko-Iseppon *et al*., 2003; Rakshit *et al*., 2003; Simon *et al*., 2007).

2.10. GENETIC MAPS

Several attempts have been made to construct a detailed genetic map of cowpea (Fatokun *et al*., 1992; Fatokun *et al*,. 1993; Menancio-Hautea *et al*., 1993; Menendez *et al*., 1997; Liu *et al*., 1999; Ubi *et al*., 2000). The most complete and up-to-date genetic map version was developed by Quedraogo *et al*. (2002) using a recombinant inbred population derived from a cross between IT84S-2049 and 524B (Menendez *et al*., 1997). IT84S-2049 is an advanced breeding line produced at IITA in Nigeria with high tolerance capability for diverse disease and pest resistance.

The map is comprised of a total of 441 markers from which 432 were assigned to one of 11 linkage groups (LGs) spanning a total of 2,670 cM, with an average distance of *ca* 6 cM between markers. The markers comprise 242 AFLPs and 18 disease or pest-resistance-related markers developed by Quedraogo *et al*., (2002) integrated with 133 RAPD, 39 RFLP, and 25 AFLP markers from the map of Menendez *et al*., (1997). Genes responsible for various phenotypic and biochemical characters have been located within these marker loci on the map.

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CHAPTER THREE

MORPHOLOGICAL AND MOLECULAR EVALUATION OF EIGHT COWPEA [*Vigna unguiculata (*L) Walp] GENOTYPES BY DAF- PCR TECHNIQUE IN THE FARMING SYSTEMS OF EASTERN CAPE, SOUTH AFRICA.

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CHAPTER THREE

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER THREE

MORPHOLOGICAL AND MOLECULAR EVALUATION OF EIGHT COWPEA [*Vigna unguiculata (*L) Walp] GENOTYPES BY DAF- PCR TECHNIQUE IN THE FARMING SYSTEMS OF EASTERN CAPE, SOUTH AFRICA

ABSTRACT

Characterization studies on the genetic diversity among cultivated cowpea (*Vigna unguiculata* L.) varieties are valuable tools to optimize the use of available genetic resources by farmers, local communities, researchers and breeders. Eight cowpea (*Vigna unguiculata (L.)* genotypes (Vegetable cowpea, Ivory grey, Okhalweni, Fahari, Fahari dark, 97K-1069-8, IT93K-73h, and 129-3) grown in South Africa were subjected to molecular and morphologic characterization. DNA amplification fingerprinting (DAF) markers based on nine random markers were used to evaluate the genetic diversity among the eight genotypes. A total of 43 bands were generated which are all polymorphic with the resulting data matrix including a total of 344 characters. Neighbour joining (NJ) method was employed to generate the dendrogram which clustered the genotypes into two groups at an agglomerate coefficient of 0.30 irrespective of their geographical origins. The dendrogram generated based on the fifteen morphological traits scored among the genotypes showed significant differences among them. This study reveals the presence of important genetic variability among the genotypes assessed in the Eastern Cape Province of South Africa using DAF-PCR techniques and could serve as a baseline study for breeding programs on cowpea in the Eastern Cape Province.

Keywords: Cowpea, *Vigna unguiculata*, Genotypes, Neighbour joining, Morphological characterization, DAF-PCR Technique.

3.1 INTRODUCTION

Cowpea (*Vigna unguiculata*) is a leguminous species used as forage and vegetable crops mainly in the tropics (Steele, 1972). Its leaves, green pods and grain are eaten as a dietary source of protein and a vital nutrient for healthy growth in humans and livestock (Tarawali *et al.*, 1997, 2002). Its seeds and leaves contain about 23% and 27 – 34% proteins respectively.

Effective research studies on the cultivation and management of cowpea production in South Africa have been left unattended to in the last 30 years owning to poor financial commitment by the government as well as loss of interest of researchers to work on the advancement of the crop (Asiwe, 2009). The author also noted lack of knowledge of good agronomic practices, absence of good seeds for planting and discouraging poor marginal returns to farmers are factors which further aggravate the limitations to cowpea production in some of the provinces across the country including Kwazulu-Natal, Limpopo and Mpumalanga.

Meanwhile, there is currently insufficient data on genetic diversity of landraces especially in South Africa. Genetic diversity in the available gene pool is fundamental for all plant improvement programs. It is important to reduce crop susceptibility to abiotic and biotic stresses, enhance long-term selection gain in genetic improvement, and increase rational use of genetic resources (Martin *et al.*, 1991; Messmer *et al.*, 1993; Barrett and Kidwell, 1998).

Evaluation studies on the genetic diversity in cowpea genotypes would increase the development of culti-groups for targeted production constraints by generating an index of parental lines to be used in breeding programs. Many investigation of genetic diversity in

crop species are based on differences in morphological characters (Schut *et al*., 1997). However, evaluation of genetic similarities using morphological markers among germplasm is lengthy and entails costly methods (Cooke, 1984).

Molecular markers are often used to characterize genetic diversity within or between populations or groups of individuals because of their ability to typically detect high levels of polymorphism (Kosman and Leonard 2005). Other researchers (Weising *et al*., 1995; Karp *et al*., 1996; Kumar, 1999) have also demonstrated that DNA markers were until now the most promising technique used to differentiate among genotypes at species and subspecies level.

Polymorphisms in the nucleotide sequence are enough for them to function as a molecular marker in mapping. These polymorphisms are elucidated by various molecular techniques such as restriction fragment length polymorphism (RFLP) (Beckman and Soller 1983) , amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995), microsatellite or simple sequence repeat polymorphism (SSRP) (Becker and Heun 1994; Nkongolo, 2003), random amplified polymorphic DNA (RAPD) (Williams *et al*., 1993), cleavage amplified polymorphic sequence (CAPS) (Jarvis *et al*., 1994) and DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al*., 1991).

DNA amplification fingerprinting (DAF) technique is a simple and efficient method for producing molecular markers that are highly polymorphic, relatively low-priced and can distinguished closely related cultivars (Caetano-Anolles 1998; Winter *et al*., 2000; Benko-Iseppon *et al*., 2003).

This study aimed to distinguish between eight cowpea genotypes grown in the Eastern Cape Province, South Africa using some morphological traits and most importantly, to elucidate the degree of genetic relatedness among these landraces using DAF-PCR techniques

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIALS

Eight genotypes of cowpea (*Vigna unguiculata* (L).) namely Vegetable cowpea, Ivory grey, Okhalweni, Fahari, Fahari dark, IT93K-73h, 97K-1069-8 and 129-3 grown in South Africa were used in this study. These genotypes were obtained from the Agricultural Research Council, Vegetable and Ornamental Plant Institute, Pretoria, South Africa and the origin of each genotype is as shown in Table 3.1. Data on the agronomical and morphological characters were collected from observation of the selected materials and recorded accordingly. Fifteen qualitative and quantitative traits were measured using the International Board for Plant Genetic Resources (IBPGR) cowpea descriptors.

Table 3.1: Names and origin of the eight cowpea genotypes used.

Genotypes	Origin
Vegetable cowpea	South Africa
Ivory grey	South African Cultivar
Okhalweni	Land race from KwaZulu Natal, South Africa
Fahari	Land race from AVRDC Arusha Tanzania
Fahari dark	Land race from AVRDC Arusha Tanzania
97K-1069-8	IITA, Nigeria
IT93K-73h	IITA, Nigeria
129-3	IITA, Nigeria.

3.2.2 DNA ISOLATION

Cowpea seeds were sown in pots in the glasshouse of the University of Fort Hare with 5kg soil capacity containing a mixture of two parts of soil and one part manure (goat) as previously described by Spiaggia *et al*., (2009) . Six seeds of each accession were grown in pots and leaf samples were collected at 14 days after planting from all the cultivars for DNA isolation and analysis.

Total DNA was isolated from each genotype using a modification of the method described by Yerramsetty *et al*., (2008). Two grams of plant tissue were harvested for DNA isolation from leaf tissues. The leaf tissue was frozen in liquid nitrogen and ground in a mortar and pestle to a fine powder. Genomic DNA was extracted using the DNeasy plant mini-extraction kit (Qiagen Inc, Valencia CA) as per directions provided by the supplier. DNA quality was further assessed by 5× TBE 1.8% agarose gel electrophoresis to ensure that extracts showed no signs of DNA degradation. The gel was visualized using the UVP BioDoc-It™ System 2UV Trans illuminator.

3.2.3 DNA QUANTIFICATION

Concentration of extracted DNA was determined following the method described by Wang *et al*., (2010) using a Quant-iT^{TM} DNA Assays Kit in a Qubit$^{®}$ fluorometer (Invitrogen) following the manufacturer's instructions.

3.2.4 DNA AMPLIFICATION

DNA amplification fingerprinting (DAF) was carried out following a modification of the method described by Caetano-Anolles *et al*., (1991). PCR was carried out using random primers listed in Table 3.2. Each 25 µl PCR reaction contained 12.5 µl master mix (2×) (0.05

units/µl *Taq* DNA polymerase in reaction buffer; 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP), 40 pmol oligonucleotide primer and 1 µg of template DNA. The DNA was first denatured for 2 minutes at 95 ^{o}C followed by 40 cycles of 15 sec denaturation at 95 ^{o}C, 1 min annealing at 35 ^{o}C and 2 min elongation at 72 ^{o}C, with a final elongation for 2 min. The amplified products were separated on 1.8% TBE agarose gels stained with ethidium bromide and viewed under a UV Transilluminator.

3.2.5 FRAGMENT AND PHYLOGENETIC ANALYSIS

The analyses of the amplification products were done manually with consideration of the number of fragments and repeatability of the reaction following the procedures described by Roodt *et al.,* (2002). Each lane of amplified product was checked manually and scored for presence (+) or absence (-) of fragments.

Pair wise comparison of banding patterns was evaluated using the PAST program (Hammer *et al*., 2001). The data were analyzed to generate Jacquard's similarity coefficients. These similarity coefficients were used to construct a dendrogram using neighbor-joining analysis by MEGA version 5.0 program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2011).

Data obtained from the morphological analysis was subjected to cluster analysis using the PAST (Paleontological Statistics) package. In the process of hierarchical clustering, the unweighted pair group method of arithmetic average (UPGMA) was employed using the Euclidean similarity distance coefficient within the PAST package (Hammer *et al*., 2001).

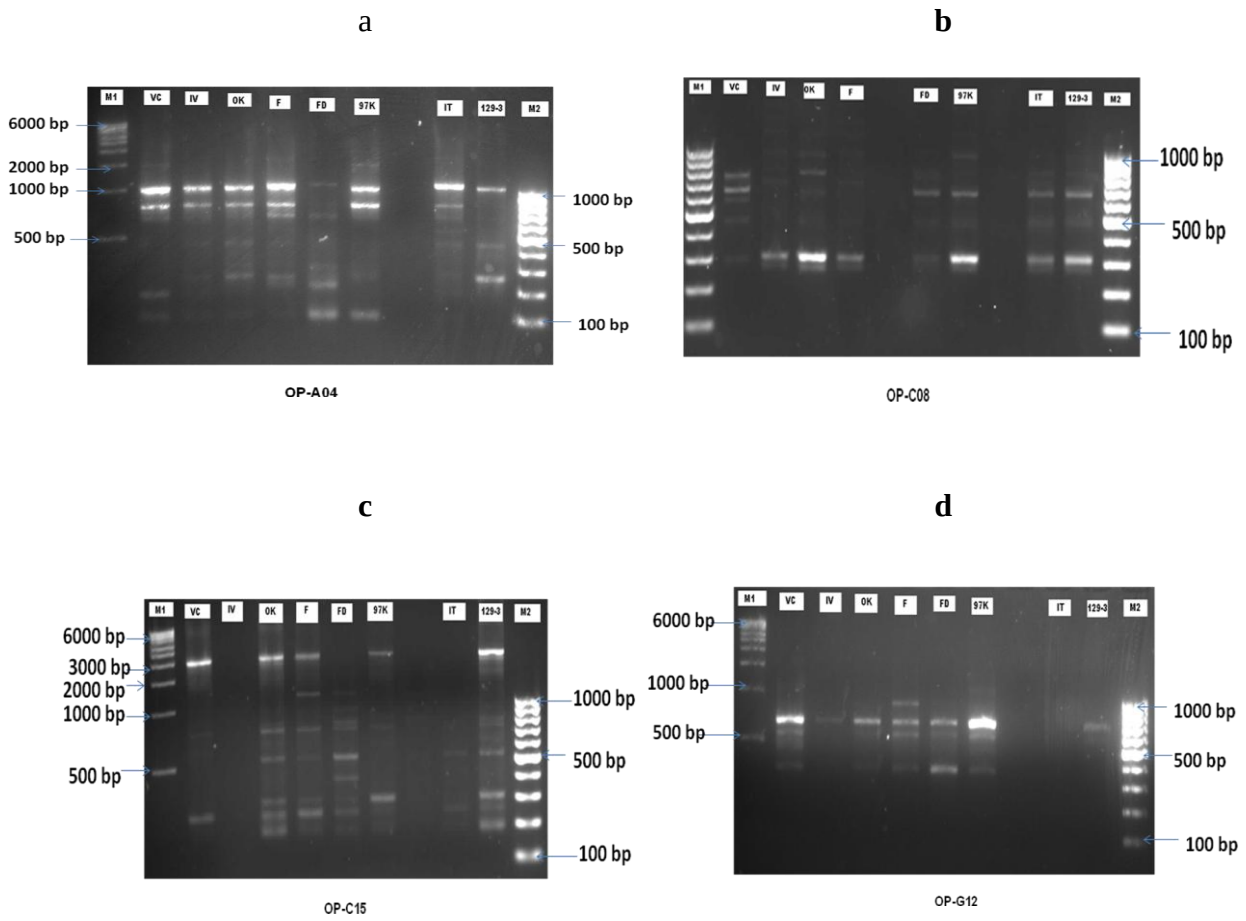
3.3 RESULTS

The results of the DAF-PCR of the eight genotypes using the seven selected primers are presented in Figure 3.1. The most informative primers considering the total number of bands and the total number of polymorphisms are OP-G06 (11 bands, 11 polymorphic), and OP-C15 (8 bands, 8 polymorphic). Both primers OP-A04 and OP-G10 have 6 polymorphic bands each, Primers OP-G05 and OP-G10 detects 5 and 4 polymorphic bands respectively. The primer OP-C08 revealed the lowest number of amplicons, generating only 3 bands which are polymorphic. Table 3.2 summarizes the number of generated and polymorphic bands. A total of 43 bands were generated which are all polymorphic. On average, the primers generated a total of 6.1 polymorphic bands. The resulting data-matrix included 43 analyzed bands with a total of 344 characters as shown in Table 3.3.

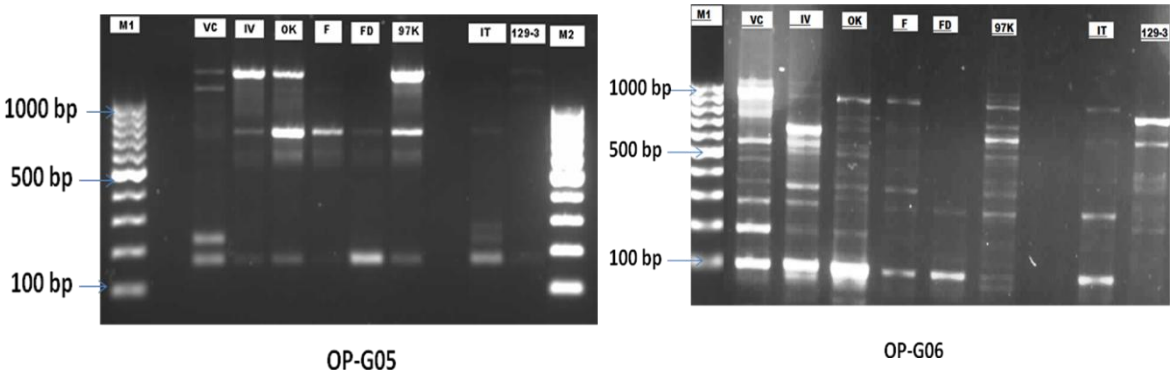
Neighbour joining analysis was used to generate the dendrogram as presented in Figure 3.2. All the seven primers screened for this study basically clustered the eight genotypes into two major branches irrespective of the source of the materials.

Considering the consensus tree generated by the combined data matrix, the eight genotypes were divided into two major clusters (A and B) at an agglomerate coefficient of 0.30 (similarity level) and each had two sub-clusters (I and II) (Table 3.4 and Figure 3.2). Cluster A, sub-cluster I, consisted of two sub-groups (**a** & **b**), with group **a** comprising of three genotypes (Fahari dark, IT93K-73h and Ivory grey) and group **b** of one genotype (129-3). Sub-cluster II had only one genotype (Fahari). Cluster B, sub-cluster I had one genotype (97K-1069-8) and sub-cluster II consisted of two genotypes (Vegetable cowpea and Okhalweni). The strongest relationship was scored between Fahari dark and Fahari genotypes while Vegetable cowpea and Okhalweni were shown to be the most genetically distant genotypes as shown in the topology tree in Figure 3.2.

The dendrogram constructed on the basis of the data generated from the qualitative and quantitative traits divided the eight accessions into two major clusters (I and II) as shown in Figure 3.3 and Table 3.5. Cluster I comprised of two accessions (97K-1069-8 and IT93K-73h). Sub-cluster A of cluster II had four groups **a, b, c**, and **d**. Group **a** comprised of two accessions (Ivory grey and 129-3) while group **b, c,** and **d** had one accession each (Fahari, Okhalweni and Fahari dark respectively). Sub-cluster B of cluster II had only one accession (Vegetable cowpea).



e **f**



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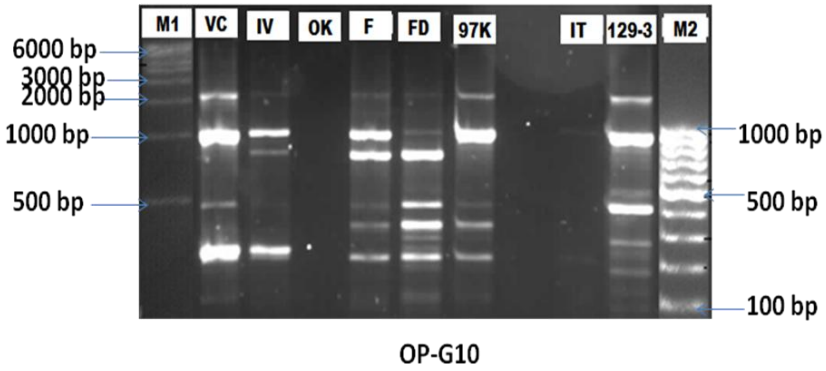

Figure 3.1 : Agarose gel (1.8%) in TAE buffer stained with ethidium bromide showing DAF-PCR polymorphism of DNA for eight cowpea genotypes (Lane 2-9, Vegetable cowpea, Ivory grey, Okhalweni, Fahari, Fahari dark, 97K-1069-8, IT93K-73h and 129-3 respectively) using 7 random primers [(**a**) OP-A04, (**b**) OP-C08, (**c**) OP-G12, (**d**) OP-C15, (**e**) OP-G05, (**f**) OP-G06, (**g**) OP-G10)]. M1 and M2 refer to 1-kb and 100 base pairs DNA ladders respectively.

Table 3.2: Proportion of generated bands and the number of polymorphic bands during DAF reactions

Primer	Sequence (5'→3')	Total no of bands	No of polymorphisms
OP-A04	AATCGGGCTG	6	6
OP-C08	TGGACCGGTG	3	3
OP-C15	GACGGATCAG	8	8
OP-G05	CTGAGACGGA	5	5
OP-G06	GTGCCTAACC	11	11
OP-G10	AGGGCCGTCT	6	6
OP-G12	GAGCTCACGA	4	4
Average no of bands generated per primer		6.1	6.1

Table 3.3: Banding patterns of DAF-PCR for eight genotypes of cowpea (*Vigna unguiculata* L.)

COWPEA GENOTYPES									
Primers	**Number of Bands**	**VC**	**IV**	**OK**	**F**	**FD**	**97K**	**IT**	**129-3**
OP-G06	1	+	+	+	+	+	+	+	-
	2	+	+	+	+	+	+	+	-
	3	+	+	+	-	-	-	-	-
	4	+	+	-	-	-	+	+	-
	5	-	+	+	+	-	+	-	-
	6	+	+	-	-	-	+	-	+
	7	-	+	-	-	-	+	-	-
	8	+	-	-	-	-	+	-	+
	9	+	-	+	+	-	+	+	-
	10	+	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	+
OP-C15	1	-	-	+	-	-	+	+	-
	2	+	-	+	+	-	+	+	-
	3	-	-	+	+	+	-	-	-
	4	-	-	+	+	+	-	-	+
	5	-	-	-	-	+	-	-	-
	6	-	-	+	-	-	+	-	+
	7	+	-	+	+	-	-	-	-
	8	-	+	-	-	-	-	-	+
OP-G10	1	+	+	-	+	+	+	-	-
	2	-	-	-	+	+	+	-	-
	3	+	-	-	+	+	+	-	+
	4	-	+	-	+	-	+	-	+
	5	+	+	-	+	-	+	-	+
	6	+	-	-	-	-	+	-	+
OP-A04	1	-	+	+	+	+	-	-	+
	2	+	+	+	+	+	+	-	-
	3	+	+	+	+	+	+	-	-
	4	+	+	+	+	+	+	-	+
	5	+	-	-	-	-	-	-	-
	6	-	-	-	-	+	+	-	-
OP-G05	1	+	-	+	-	+	+	+	-
	2	+	-	-	-	-	-	+	-
	3	-	+	+	+	+	+	+	-
	4	+	-	-	-	-	-	-	-
	5	+	+	+	-	-	+	-	+
OP-G12	1	-	-	-		-	+	-	-
	2	+	-	-	+	+	+	+	+
	3	-	-	-	+	+	+	-	+
	4	-	-	-	+	+	+	+	+
OP-C08	1	+	+	+	-	+	+	+	+
	2	-	-	-	-	+	+	+	+
	3	-	+	-	-	-	-	+	-

VC=vegetable cowpea, IV=Ivory grey, OK=Okhalweni, F=Fahari, FD=Fahari dark, 97K=97K-1069-8, IT=IT93K-73h, and 129-3 respectively.

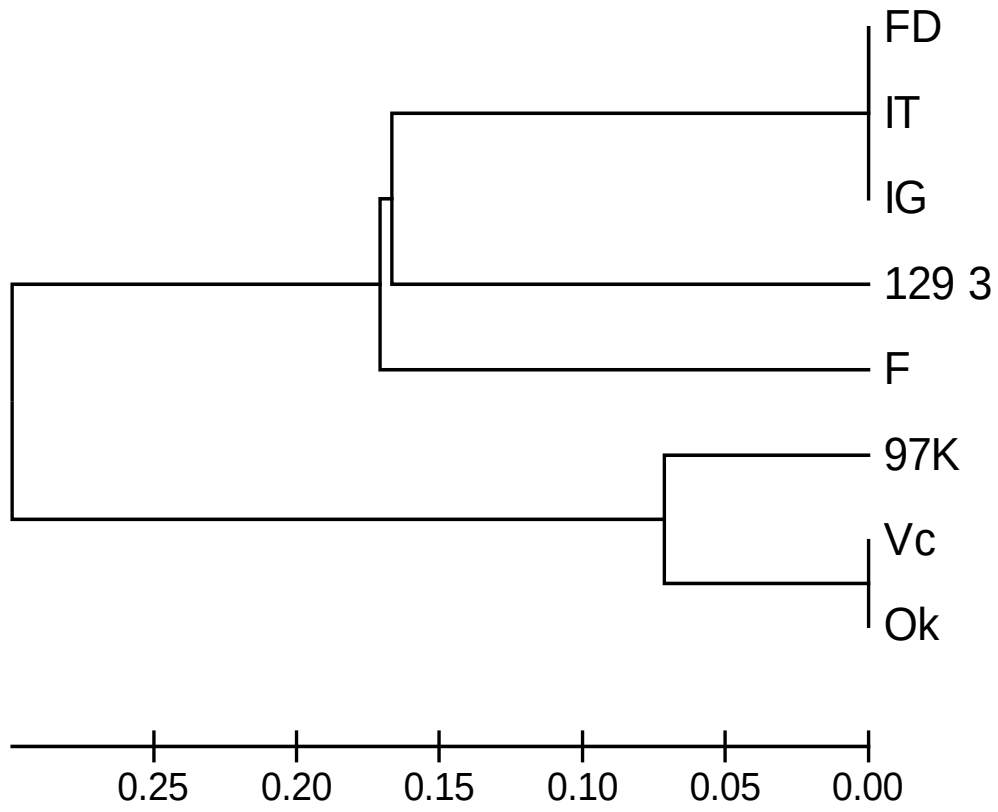

Figure 3.2: **Dendrogram demonstrating the relationship among eight (*Vigna unguiculata L.*) genotypes based on a compiled data set showing a linerized trees using neigbour joining method.**

Table 3.4: Cluster distribution of the eight genotypes based on DAF-PCR

Clusters	No. of accessions	Name of accessions
Cluster A		
I	4	**a**-(Fahari dark, IT93K-73h & Ivory grey). **b**-(129-3)
II	1	Fahari
Cluster B		
I	1	97K=97K-1069-8
II	2	Vegetable cowpea Okhalweni

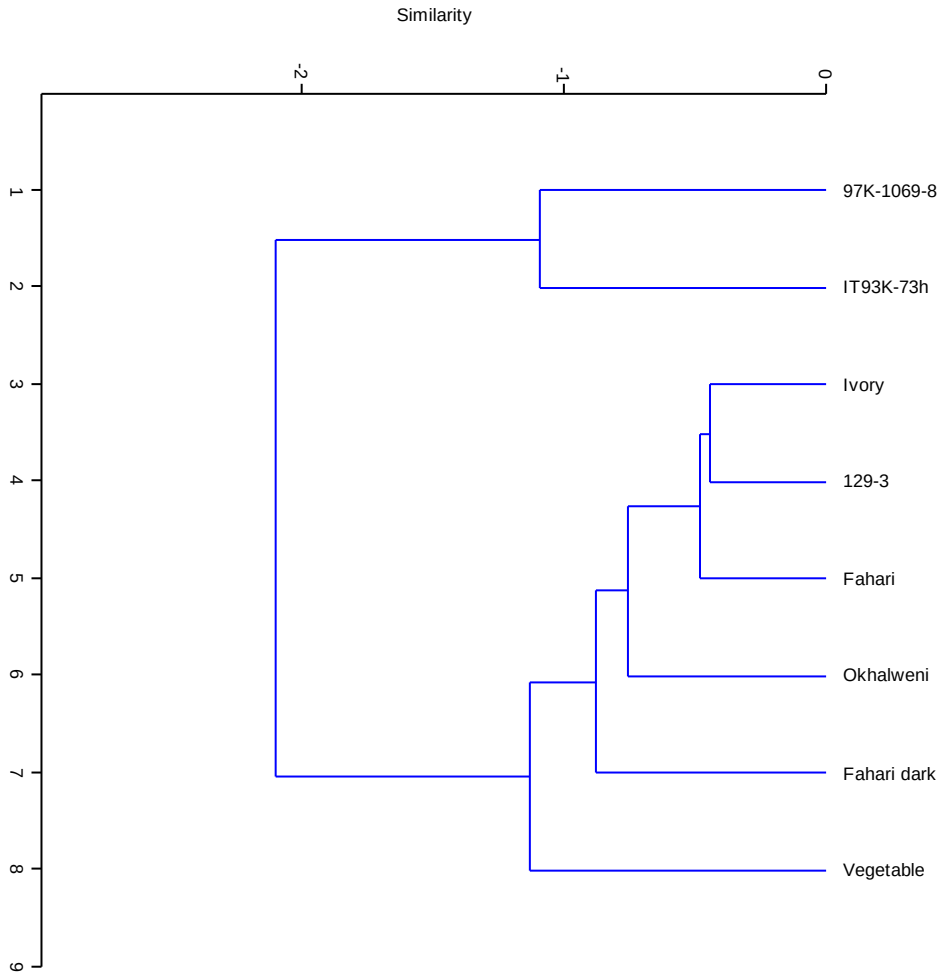

Figure 3.3: Dendrogram of the studied accessions based on 15 qualitative and quantitative traits using PAST program.

Table 3.5: Cluster distribution of eight cowpea genotypes based on 15 qualitative and quantitative traits using morphological descriptors

Clusters	No. Of accessions	Name of accessions
Cluster I	2	97K-1069-8 & IT93K-73h
Cluster II		
A	5	**a** (Ivory grey, 129-3), **b** (Fahari) **c** (Okhalweni) **d** (Fahari dark)
B	1	Vegetable cowpea

3.4 DISCUSSION

DAF analysis was employed to evaluate genetic diversity in eight cowpea accessions. All the accessions studied are from the same cultivar, *unguiculata*. Significant genetic diversity was detected in the cowpea germplasm investigated confirming previous evidence presented by Simon *et al*., (2007) and Spiaggia *et al*., (2009). In spite of the low number of genotypes used, DAF methodology was very efficient in generating molecular markers for this first evaluation. In comparison to this study, the genetic diversity detected by Spiaggia *et al.*, (2009) was higher probably because of the higher number of accessions (30 compared to 8) used. However, pair-wise genetic distance between individuals (or groups of individuals) rather than allele frequencies are important in phylogenetic studies (Ochieng *et al*., 2007).

Simon *et al*., (2007) used a higher number of primers (26) selected from a total of 262, the average number of polymorphism per primer was 13.6 as against 6.1 in this study. Thus, considering the number of accessions screened in this study as well as the number of primers (7) used with respect to the average number of polymorphic bands per primer (6.1), DAF methodology was found to be more efficient in this study as compared to those reported by Simon *et al.*, (2007) and Spiaggia *et al*., (2009).

The reports of Pasquest (1993; 1999; 2000) using isoenzyme polymorphisms to analyze wild and cultivated accessions of *V. unguiculata* revealed only a low level of polymorphism between cultivated accessions. The degree of variability between the geographical clusters was also low. In this study, only 7 DAF markers were sufficient to distinguish all the genotype analyzed, however most of the genotypes in the molecular study were not grouped according to their geographical origin. The same conclusion was reached by Zannou *et al*., (2008) in which the classification of accessions into different groups is independent of collection zones, agro-ecozones and market place. For instance, the genotypes

in sub-cluster I (Fahari dark, IT93K-73h and Ivory grey) in the major cluster A were from Tanzania, Nigeria and South Africa respectively. Accessions of morphologically different characters including shape of seeds, seed coat colour, eye colour are very close according to the dendrogram constructed based on the presence or absence of amplified DNA fragments of a particular size. This lack of correlation between morphological traits and other genetic markers such as isozymes markers has been previously reported in cowpea and other crops (Doebley 1989; Zannou *et al*., 2008).

This result also supports the report by Zannou *et al*., (2008) that during the process of domestication, changes in a few genes can lead to marked phenotypic differences and cowpea accessions retained some parts of their genetic components during the process of domestication as self-pollinated crops. The same observation was made in this study for Fahari dark, IT93K-73h and ivory grey genotypes in which despite the differences in their seed shape, eye colour and seed coat colour, they were closely clustered together as shown in Figure 3.2.

Several earlier studies on cowpeas using morphological traits such as plant pigmentation, growth habit, root shape, leaf area, pod length, seed shapes, eye colour or pigmentation, grain quality, and yield were all found to be sufficient to distinguish genetic variability, and have led to a better classification of cowpea genotypes (Apte *et al*., 1987; Emebiri and Obisesan 1991; Fery and Dukes 1994; Ogunbodede, 1988; Uguru and Uzo 1991; Roquid and Patnaik 1990; Nkouannessi, 2005). Characterization using flower size and style length on cowpea cultivars was also reported by Emebiri (1989) to be highly heritable. As previously documented, this study also confirms that agro-morphological traits (quantitative and qualitative) are still very important tools for cowpea genetic diversity studies. For example, some of the morphological traits used in this study, such as seed coat colour, eye

colour, growth habit, terminal leaflet shape and seed shape had the largest grouping of the accessions and were found to be very efficient in distinguishing the accessions.

The eight accessions used for the agro-morphological study clustered according to their geographical origin, for instance, 97K-1069-8 and IT93K-73h in the major cluster I were from Nigeria. Also, it was observed that all accessions from Tanzania and South Africa were clustered together in cluster II as shown in Table 3.3. This confirms the conclusion by Nkouannessi (2005) that a very high level of similarity was revealed between many accessions from the same region for most of the characters studied.

Conclusively, this study reveals the presence of important genetic variability among the cowpea genotypes grown in the Eastern Cape Province of South Africa. This information can be used to plan for future improvement programmes of the crop.

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CHAPTER FOUR

PROTEIN VARIATION IN COWPEA GENOTYPES (*VIGNA UNGUICULATA* L. WALP) GROWN IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA AS AFFECTED BY MINERALISED GOAT MANURE

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CHAPTER FOUR

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER FOUR

PROTEIN VARIATION IN COWPEA GENOTYPES (*VIGNA UNGUICULATA* L. WALP) GROWN IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA AS AFFECTED BY MINERALISED GOAT MANURE

ABSTRACT

Nutrient availability of cultivated soils in the Eastern Cape region of South Africa is generally low, and recognizing that manure application has been one of the most effective methods to improve soil fertility and crop yield in tropical African countries, we assessed the effect of manure application on the leaf crude protein content of six cowpea genotypes (Vegetable cowpea, Ivory grey, Okhalweni, Fahari, Fahari dark, and 97 K-1069-8) in a greenhouse experiment. Fresh leaves were collected from each cowpea plant 21 days after planting for protein extraction and quantification. The results showed that Fahari had the highest concentration of crude protein (46.51 mg/mg dry leaf) while Vegetable cowpea (24.41 mg/mg dry leaf) had the lowest in the absence of manure application. However, upon application of manure (goat), Fahari dark had the highest crude protein concentration (53.53 mg/mg dry leaf) while Vegetable cowpea had the lowest (29.08 mg/mg dry leaf). Fahari, 97K-1069-8, Ivory grey and Okhalweni contained 51.79, 49.03, 44.83 and 38.33 (mg/mg dry leaf) crude protein concentrations respectively. This study demonstrated that genotypes as well as manure application significantly influence cowpea yields in terms of its leaf crude protein content.

Keyword: Cowpea, genotypes, crude protein, manure**.**

4.1 INTRODUCTION

Grain legumes have been described as one of the most important crops in many countries providing about one-quarter of the world's dietary protein. They are an essential source of protein for about 700 million people (Nagl *et al*., 1997), particularly in the developing countries of South America, Africa and Asia, where plants provide 83% of total protein in the average diet (Mahe *et al*., 1994).

Cowpea is a grain legume cultivated in the savannah areas of the tropics and sub-tropics. The crude protein from the seed and leaves range between 22 to 30% and between 13 to 17% in the haulms on a dry weight basis with high digestibility value and low fibre level (Tarawali *et al*., 1997; Bressani, 1985; Nielsel *et al*., 1997). Cowpea seed pods and leaves are consumed in fresh form as green vegetables in some African countries (Ghaly *et al*., 2010) while the rest of the cowpea plant after the pods have been harvested serve as a nutritious fodder for livestock (Abebe *et al*., 2005). Its nutritive value makes it an important protein source to vegetarians and people who cannot afford animal protein.

Poor soil fertility has been reported as one of the main limiting factors to efficient crop production in arid areas owning to low organic matter, high temperature and low rain fall (Abebe *et al*., 2005). Manure application has been recognised as one of the most effective methods to improve soil fertility and crop yield in tropical African countries (Kihanda *et al*., 1998); and serves as a source of all essential nutrients for improved crop production and soil sustainability.

Most of the soils in former homelands of South Africa have very low fertility status (Laker, 1976). Mandiringana *et al*., (2005) reported that nutrient availability of cultivated soils in the Eastern Cape region of South Africa is generally low due to low soil organic matter content

and low geological reserves of phosphorus (P), potassium (K) and calcium (Ca). Generally, nutrient supplies for crop production by small-scale farmers in the Eastern Cape Province depend on livestock manure because of poor access to inorganic fertilizers (Mandiringana *et al*., 2005; Mnkeni and Mkile, 2006). Although cowpea is known to be a low management crop that can grow in poor soils, its yield could be improved by application of manure (Abebe *et al*., 2005).

Ghaly *et al*., (2010) reported that nutrient deficiencies such as sulphur, phosphorus, potassium, and magnesium are known to affect plant growth and may influence its protein content leading to a decrease in protein yield. Abebe *et al*., (2005) also argued that manure from confined animal feeding is very valuable for crop production and soil sustainability as a source of essential nutrients and provides an excellent source of organic matter when added to soil.

Protein deficiency has been reported as one of the main sources of nutritional problems in the developing world (FAO, 1997). According to Ghaly *et al*., (2010), two debilitating diseases; Marasmus and kwashiorkor, occur in children who suffer protein malnutrition. About one billion people are still reported to be suffering from protein deficiency and malnutrition worldwide (WHO/ FAO, 2007), therefore breeding high yielding varieties of cowpea with quality traits, such as high protein content, will not only contribute to food security and alleviate poverty but could also contribute to the alleviation of protein deficiencies.

Previous reports showed that in most of the developing countries, the cost of animal protein is too high and is unaffordable to most families (Ghaly *et al*., 2010). Hence, an urgent need arises to increase current agricultural practices in marginal lands in order to bring a lasting solution to the menace of protein deficiency and world food shortage.

It has also been reported that the leaves of cowpea are served as food in some of the African countries spanning across Nigeria, Zaire, Zimbabwe and Zambia where they are cooked fresh together with immature pods or may be dried and conserved for later use, due to their high nutritive value (Ghaly *et al*., 2010). Protein synthesis through the process of photosynthesis remains the only non-depletable protein source which can also supply various essential amino acids as well as serve as a source for nitrogen in diet for the production of non-essential amino acids (Ghaly *et al*., 2010).

The report of Ghaly *et al*., (2010) also revealed that leafy vegetable protein is about half the vegetable protein present in the human diet and probably amounts to more of the world total protein than do fish. In addition, only a very minute percentage (8-20%) of plant protein from animal's consumption can be recovered as protein supplement for human nutrition. Thus, more efficient ways of utilizing plant protein must be found. Interestingly, several studies have revealed nutritional differences among genotypes of different species of plant (Nielson *et al*., 1993; Hall *et al*., 2003).

South Africa recently acquired selected genotypes of cowpea for agronomic and nutritional characterization (Rhandzu, 2007). A study to evaluate the performance of this species under different microclimates of the country is needed for the eventual introduction of these genotypes into the farming systems of the country. Several previous reports on the protein content of cowpea were carried out on the seed (Sharawy *et al*., 2002; Tshovhote *et al*., 2003; Kokiladevi *et al*., 2005) whereas the percentage crude protein in cowpea leaves is higher than the amount found in the seed (Tarawali *et al.*, 1997, 2002). Evaluating the protein content is the first step at improving the protein quality of any crop. However, there is currently no evaluation study on the leaf protein content and the most appropriate time to introduce accessions of cowpea into the farming systems of the Eastern Cape Province of South Africa. This study therefore aimed at assessing the nutritional value of the leaf crude protein content

of six cowpea genotypes as affected by manure application and also to determine the feasibility of using them as a protein supplement in the Eastern Cape Province of South Africa.

4.2 MATERIALS AND METHOD

4.2.1 PLANT MATERIALS

Six genotypes of cowpea (*Vigna unguiculata* (L).) namely Vegetable cowpea, Ivory grey, Okhalweni, Fahari, Fahari dark, 97K-1069-8 and were used in this study. These genotypes were obtained from the Agricultural Research Council, Vegetable and Ornamental Plant Institute, Pretoria, South Africa and their origin are as shown in Table 4.1. The six accessions were planted in two separate groups in plastic pots in a green house at the University of Fort Hare, South Africa. Dried goat manure was applied to one group while the other group was raised without manure application. The plants were cultivated in pots with 5 kg soil capacity containing a mixture of two parts of soil and one part manure. Fresh leaves were collected from each cowpea plant 21 days after planting and ground in liquid nitrogen.

Table 4.1: Names and origin of the eight cowpea genotypes used.

Genotypes	Origins
Vegetable cowpea	South Africa
Ivory grey	South African Cultivar
Okhalweni	Land race from KwaZulu Natal, South Africa
Fahari	Land race from AVRDC Arusha Tanzania
Fahari dark	Land race from AVRDC Arusha Tanzania
97K-1069-8	IITA, Nigeria

4.2.2 PHYSICOCHEMICAL ANALYSIS OF THE SOIL AND MANURE USED

The soil and goat manure used for this study were collected from the University of Fort Hare farm. The samples were obtained from a 0 to 20 cm depth using a spade and were air-dried and pass through a 2 mm sieve for characterization, at the Department of Soil Science, University of Fort Hare. Organic carbon content was determined by the Walkely-Black procedure as described by Nelson and Sommers, (1996). Total Ca, Na, K and Mg in both the soil and manure were estimated following wet digestion with Sulphuric acid and hydrogen peroxide (Okalebo *et al*., 2002). The total nitrogen and phosphorus were determined calometrically as described by Okalebo *et al*., (2002).

4.2.3 EXTRACTION OF CRUDE PROTEIN

The extraction of protein from cowpea leaves was carried out following the method of Mirkov *et al*., (1994). One hundred mg of finely ground leaf flour was extracted in the extraction buffer (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 1% 2-mercapto-ethanol, 0.1% Triton- X-100, 2 mM phenylmethylsulphonyl fluoride [PMSF] [1 ml/ml sample]) by homogenization followed by incubation at 4^0C for 1 hour. It was then centrifuged at 15, 000 rpm for 15 min at 4^0C. The supernatant was collected and stored frozen in aliquots.

4.2.4 QUANTIFICATION OF LEAVE CRUDE PROTEIN

The protein content in the extract was quantified using a Pierce® BCA Protein Assay Kit following the manufacturer's instructions (www.piercenet.com). A protein standard was prepared using Bovine serum albumin (BSA) and absorbance was measured at 562 nm using a microtitre plate reader, an analytical & diagnostic product manufactured by Biotek Company USA, from which a standard curve for BSA was constructed ranging from 25 mg/ml to 2000 mg/ml. The mean concentration of the leaf samples was determined in duplicate by interpolation from the BSA standard cuve. Data were analyzed using analysis of variance (ANOVA) in the SPSS version 13 at $P \leq 0.05$.

4.3 RESULTS AND DISCUSSION

The result of the physicochemical properties of the soil and manure used is presented in Table 4.2. Previous reports have shown that macronutrients available in the soil should be in the range of N (0.1 to 0.5%), P (0.08 to 0.5%), and K (1.5 to 3.0%) in order to produce a

good yield (Dutta, 2005). The total soil nitrogen was 0.05% which is below the range of most cultivated top soils (0.06 – 0.5%). Also, the concentration of P and K were not sufficient according to Ryan et al., (1996). Exchangeable Ca in soils should range between 12 to 75% of CEC while the exchangeable Mg in soils ought to range between 4 to 20% of CEC (Eckert and McLean, 1981). Also, the Ca and Mg concentrations in this study were very much below average confirming the report of Mandiringana et al., (2005) that soils in the Eastern Cape Province contained extremely low macronutrients. The C: N ratio of the soil is far higher than that present in the manure thereby accounting for the low rate of mineralization seen in the chemical properties of the soil than the manure. Meanwhile, the optimum macronutrients content of the goat manure used is higher than that present in the soil as presented in Table 4.2.

Table 4.2: Some chemical and physical characteristics of the soil and manure used

Parameters	**Soil (sandy loamy)**	**Manure (goat)**	**Mixture**
Organic carbon (%)	2.23 ± 0.23	7.36 ± 0.32	6.40 ± 0.35
Total Nitrogen (%)	0.05 ± 0.02	0.45 ± 0.06	0.71 ± 1.26
Phosphorus (%)	0.67 ± 0.76	2.04 ± 0.55	1.09 ± 0.06
Potassium (ppm)	0.80 ± 0.07	9.63 ± 0.83	0.76 ± 0.12
Magnesium (ppm)	0.03 ± 0.02	2.19 ± 0.15	0.09 ± 0.05
Sodium (ppm)	0.35 ± 0.07	0.55 ± 0.06	0.26 ± 0.06
Calcium (ppm)	0.54 ± 0.04	9.15 ± 0.80	0.47 ± 0.02
C :N	45:1	16:1	9:1

Mean of Three replicates.

The results for the protein studies as presented in Table 4.3 showed that Fahari had the highest concentration of crude protein content (46.51 mg/mg dry leaf) while Vegetable cowpea (24.41 mg/mg dry leaf) had the lowest crude protein content without the influence of manure application. Ivory grey, Okhalweni, Fahari dark and 97K-1069-8 yielded significantly different ($p \leq 0.05$) protein concentrations of 42.72, 37.70, 39.85 and 38.51 mg/mg dry leaf respectively. However, upon the application of manure (goat), Fahari dark had the highest crude protein concentration (53.53 mg/mg dry leaf) while Vegetable cowpea had the lowest value (29.08 mg/mg dry leaf). Fahari, 97K-1069-8, Ivory grey and Okhalweni contained 51.79, 49.03, 44.83 and 38.33 mg/mg dry leaf crude protein content respectively.

Table 4.3: Leaf crude protein concentration of six cowpea genotypes showing the effect of manure application.

	Crude protein concentration (mg/mg dry leaf)	
Cowpea genotypes	Manure application	Without manure application
Vegetable cowpea	29.08 ± 0.91	24.41 ± 4.96
Ivory grey	44.83 ± 21.4	42.72 ± 1.03
Okhalweni	38.33 ± 17.4	37.70 ± 1.42
Fahari	51.79 ± 3.25	46.51 ± 4.40
Fahari dark	53.53 ± 18.76	39.85 ± 1.08
97K-1069-8	49.03 ± 5.45	38.51 ± 0.81

F- LSD 1.3 (P ≤ 0.05)

The present study found significant differences ($p \leq 0.05$) among the six different cowpea germplasms for leaf crude protein yield when tested on goat manure mixed with sandy loamy soil as well as untreated soil. The differences were probably due to genetic variations among genotypes, environmental factors and climatic conditions (Ali-khan and Youngs, 1973).

The result of the total protein content in this study is much higher than those obtained by Ghaly et al., (2010) probably due to the geographical location where the plants were cultivated and season, the optimum harvest age for protein extraction (70 days compared to 21 days) as well as the influence of manure application. These same conclusions were suggested in previous work of Ghaly et al., (2010).

Also, Abebe et al., (2005) reported that the release of essential nutrients upon the decomposition of air dried manure by microbes mainly contributes to the availability of nutrients in soils and plant as well as improvement in the protein content of cowpea. Plants grown on manure treated soils showed higher content of protein than those without manure treatment. Interestingly, as documented in a previous study of Abebe et al., (2005), application of dried goat manure influenced an increase in the crude protein concentration as presented in Table 4.3. For example, Fahari dark contained 53.53 mg/mg dry leaf of crude protein as compared to 39.85 mg/mg dry leaf when cultivated without manure application. The same variations in crude protein contents were discovered for all the studied genotypes. The higher content of protein was due to high nitrogen supplied as a result of manure treatment (Abebe et al., 2005). However, the influence of manure application was minimal on the crude protein concentrations in Ivory grey (44.83 mg/mg dry leaf against 42.72 mg/mg dry leaf) and Okhalweni genotypes (38.33mg/mg dry leaf against 37.70 mg/mg dry leaf) respectively; an indication that cowpea genotypes react differently to the application of plant nutrients.

This study also agreed with the report of Abebe et al., (2005) that differences in protein level that is shown within species may be as a result of variations in genotypes as well as agronomic practices. This probably explained the variations observed in the crude protein concentrations among the studied genotypes. For instance, Fahari variety has 46.51 (mg/mg dry leaf) crude proteins while vegetable cowpea contained 24.41 (mg/mg dry leaf) crude proteins without the influence of manure treatment. The same scenario was discovered for all the studied genotypes as shown in Table 4.3. Fahari and Fahari dark out-performed all the other genotypes with crude protein concentrations of 46.51 and 53.53 (mg/mg dry leaf) respectively. Thus, these two genotypes could be better alternative sources for cheap and affordable plant proteins for vegetarians as well as to the marginal income bracket communities of the Eastern Cape Province, South Africa. Conclusively, this study demonstrated that genotypes as well as manure application significantly influence cowpea yields in terms of its leaf crude protein content.

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CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

CHAPTER FIVE

TABLE OF CONTENTS

CHAPTER FIVE

GENERAL DISCUSSION

Several earlier studies on cowpeas using morphological traits such as plant pigmentation, plant habit, root traits, leaf traits, pod traits, seed traits, grain quality, and yield were all found to be of great significance to distinguish genetic variability, and have led to a better classification of cowpea genotypes.(Apte *et al*., 1987; Emebiri and Obisesan, 1991; Fery and Dukes, 1994; Ogunbodede, 1988; Uguru and Uzo, 1991; Fawole, 1988; Drabo *et al.*, 1985; Roquid and Patnaik, 1990, Nkouannessi., 2005). Also, characterization using flower size and style length on cowpea cultivars was also reported by Emebiri (1989) to be highly heritable. This study also found that agro-morphological traits (quantitative and qualitative) are still very important tools for cowpea genetic diversity studies. For example, some of the morphological traits used in this study, such as seed coat colour, eye colour, growth habit, terminal leaflet shape and seed shape had the largest grouping of the accessions and were found to be very efficient in discriminating the accessions as presented in Appendix II**.** More so, the eight accessions used for the agro-morphological study clustered according to their geographical origin. For instance, 97K-1069-8 and IT93K-73h in the major cluster I were from Nigeria. Similarly, it was observed that all accessions from Tanzania and South Africa were clustered together in cluster II as shown in Figure 3.3. This finding agrees with the conclusion by Nkouannessi, (2005) that a very high level of similarity was revealed between many accessions from the same region for most of the characters studied. However, investigation of genetic diversity of crop species based on differences in morphological and agronomical traits alone is not wholesome enough for gaining complete understanding of the extent of genetic variation and distribution of crop species as a result of strong environmental effects. Therefore, there arises the need to assess the level of genetic diversity among crop

species using molecular markers (Tanksley *et al*., 1989; Liu and Furnier, 1993; Lin and Binns, 1994)

Molecular markers are often used to characterize genetic diversity within or between populations or groups of individuals because of their ability to typically detect high levels of polymorphism. DAF, a varied form of RAPDs is efficient in allowing multiple loci to be analysed for each individual in a single gel run. In analysing banding patterns of molecular markers, the data typically are coded as (0, 1)-vectors; 1 indicating the presence and 0 indicating the absence of a band at a specific position in the gel (Kosman and Leonard, 2005).

Caetano-Anolles (1998) reported that when genetic diversity and phylogeny are determined, the taxonomical level of analysis becomes relevant and techniques such as DNA amplification fingerprinting can be useful for distinguishing between closely related organisms below the species level such as cultivars, accessions and lines.

In spite of the low number of genotypes used in this study, DAF methodology was very efficient in generating molecular markers for this first evaluation. This result confirms previous evidences presented by Simon *et al* (2007) and Spiaggia *et al* (2009). With respect to this study, the genetic diversity detected by Spiaggia *et al.* (2009) was higher probably because of the higher number of accessions (30 compared to 8) used. However, pair wise genetic distance between individuals (or groups of individuals) rather than allele frequencies are important in Phylogenetic studies (Ochieng *et al.*, 2007). Thus, DAF methodology was found to be more efficient in this study as compared to both the work of Simon *et al* (2007) and Spiaggia *et al.* (2009).

Generally, most of the genotypes in the molecular study were not grouped according to their geographical origin. The same conclusion was reached by Zannou *et al.* (2008) in which the classification of accessions into different groups is independent of collection zones, agro-

ecozones and market place. For instance, as shown in Table 3.4, all the genotypes in sub-cluster I (Fahari dark, IT93K-73h and ivory grey) in the major cluster A were from Tanzania, Nigeria and South Africa respectively. Interestingly, accessions of morphologically different characters (97K-1069-8, Vegetable cowpea & Okhalweni) including seed shapes, seed coat colour, eye colour, growth pattern etc., were clustered very closely according to the dendrogram constructed based on the presence or absence of amplified DNA fragments of a particular size. This lack of correlation between morphological traits and other genetic markers such as isozymes markers has been reported in cowpea and other crops (Doebley, 1989). This study also corroborates the conclusion by Zannou *et al.* (2008) that during the process of domestication, changes in a few genes can lead to marked phenotypic differences and cowpea accessions retained some parts of their genetic components during the process of domestication as self-pollinated crops. The same observation was reported for Fahari dark, IT93K-73h and Ivory grey genotypes in which despite the differences in their seed shape, eye colour and seed coat colour, they were closely clustered together as shown in Figure 3.2.

The percentage level of essential amino acids found in leaf protein has been seen to compare adequately with those in animal proteins (Byers, 2006). Leaf protein has also been found to provide essential amino acids (tryptophan, methionine, histamine, lysine, valine, leucine, isoleucine, and phenylalanine) (Lugg and Weller, 1944; Ghaly *et al.*, 2010). The work of Parrish and Kroger (1974) as well as Kinsella (1970) cited by Ghaly *et al.*, (2010) revealed that the majority of leaf proteins contained maximum biological value and compares favourably with soybean, sunflower seed and cotton seed meals. The actual digestibility of leaf protein has also been reported to compare well with optimum quality animal and vegetable proteins (Pirie, 1975).

The total leaf protein content obtained in this study was much higher than those obtained by Ghaly *et al.*, (2010) probably due to the geographical location where the plants were

cultivated and season, the optimum harvest age for protein extraction (70 days compared to 20 days) as well as the influence of manure application. These same conclusions were suggested in previous work by Ghaly *et al.* (2010). It has been reported that the leaves of cowpea are served as food in some of the African countries where they are cooked fresh together with its green pods or dried and conserved for later use, due to their high nutritive value (Ghaly et al., 2010). Therefore, as presented in Table 4.3, Fahari dark and Fahari genotypes with high protein contents could be used as alternative sources of protein for vegetarians as well as in developing countries like South Africa.

Kinsella (1970) reported that nutrient deficiencies such as sulphur, phosphorus, potassium, and magnesium known to affect plant growth may influence its protein content and decrease in protein yields. Manure from confined animal feeding such as goats has been reported to be very valuable for crop production, soil sustainability as well as a source of essential nutrients (Abebe *et al.*, 2005) which provides an excellent source of organic matter when added to soil meanwhile cowpea plants are known to respond very well to fertilizer application. Abebe *et al.* (2005) also reported that the release of essential nutrients upon the decomposition of air dried manure by microbes contributes to the availability of nutrients in soils and plants with the improvement in the protein content of cowpea. The application of dried goat manure was shown in this study to influence the protein concentration in leaves (Table 4.2). For example, Fahari dark contained 53.53 mg/mg dry leaf of crude protein after manure application as compared to 39.85 mg/mg dry leaf when cultivated without manure. The same variations in crude protein were also discovered for all the studied genotypes. Thus, this study supports the report by Abebe *et al.*, (2005) that nutrient availability of cowpea was improved by the application of manure and that difference in protein level that is shown within species may be as a result of variations in genotypic and environmental factors, as well as agronomic practices (Ali-khan and Youngs, 1973).

Summarily, a range of observations were made in these analyses of genetic diversity of cowpea using molecular, morphologic and agronomic markers. Overall, a relatively high level of similarity was observed among the accessions for the molecular characterization using DNA amplification fingerprinting (DAF), especially those from different countries (Fahari dark, IT93K-73h & Ivory grey) although some of the genotypes from the same countries also clustered together in the same main branch (Fahari dark & Fahari, IT93K-73h & 129-3, Okhalweni & Vegetable cowpea). This indicates better possibilities for genetic improvement of the crop through selection and cross breeding. However, high level of similarity was revealed between many accessions from the same origin for most of the characters studied for the morphologic and agronomic evaluation.

Thus, the use of material from different geographical origins in any cross breeding programme with the aim of developing suitable varieties with specific characters is therefore recommended. This would prevent the use of materials with similar genetic backgrounds. For instance, the use of Fahari (from Tanzania) in a breeding programme aiming to improve Vegetable cowpea (from South Africa) for leave protein yield would have a better chance of success than the use of Fahari dark from the same origin.

Therefore in conclusion, both Fahari and Fahari dark are recommended for cultivation by farmers in the Eastern Cape Province because of their high protein content which makes cowpea leaves a better source of dietary protein for both humans and livestock. Vegetarians as well as under resourced populations may use cowpea as a cheaper and affordable source of protein as well as an alternative for animal proteins. This study also revealed that application of goat manure also improves protein yield of cowpea.

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APPENDICES

Appendix I

MORPHOLOGICAL AND AGRONOMIC CHARACTERIZATION

Morphological traits of cowpea plants can be grouped as either quantitative or qualitative. Data on the agronomical and morphological characters were collected from 10 randomly selected plants and their means were recorded for all observations. Fifteen qualitative and quantitative traits were measured using the international board for plant genetic resources (IBPGR) cowpea descriptors**.**

QUALITATIVE AND QUANTITATIVE TRAITS EVALUATION METHOD

The qualitative and quantitative traits were evaluated using various scoring scales.

A- Growth pattern: 1= Determinate (apical bud of main stem reproductive), 2= Indeterminate

B- Growth habit: 1= Acute erect (branches form acute angles with main stem), 2= Erect (branching angles less acute than above), 3= Semi-erect (branches perpendicular to main stem, but not touch ground), 4= Intermediate (most lower branches touch the ground), 5= Semi-prostrate (main stem reaches 20 0r more centimeters), 6= Prostrate (plants flat on ground; branches spread several meters), 7= Climbing.

C- Twining tendency: 0 = none, 3 = Slight, 5 = Intermediate, 7 = Pronounced

D- Plant pigmentation (recorded for stem, branches, petioles and peduncles in the 6th week after planting): 0 = none, 1= Very slight, 3 = Moderate at the base and tips of petioles, 5 = Intermediate, 7 = Extensive, 9 = Solid

E- Terminal leaflet shape (recorded for the terminal leaflet of a young, mature leaf in the 6th week after planting): 1 = Globose, 2 = Sub-globose, 3 = Sub-hastate, 4 = Hastate.

F- Seed shape: 1 = Kidney, 2 = Ovoid, 3 = Crowder, 4 = Globose, 5 = Rhomboid,

G- Seed coat colour (Recorded at maturity): 1 White, 2 Cream, 3 Brown, 4 Red, 5 Purple, 6 Black, 99 other (i.e. 'yellow' or 'blue', specify in the descriptor Notes)

H- Eye colour: 0= Eye absent (white, cream), 1= Brown splash or grey, 2= Tan brown, 3= Red, 4= Green, 5= Blue to black, 6= Blue to black spots or mottle, 7= Speckled(even distribution of fine speckling), 8= Mottled(dark brown pigment typically absent around hilum), 9=Mottled and speckled (victor), 10= Other.

I- Testa texture: 1 = Smooth, 3 = Smooth to rough, 5 = Rough (fine reticulation), 7 = Rough to wrinkled 9 = Wrinkled (coarse folds on the testa).

J- Leaf colour (intensity of green colour): 3 = Pale green, 5 = Intermediate green, 7 = Dark green

K- Leaf texture: 1= Cariaceous, 2= Intermediate, 3= Membranous

L- Leaf marking (presence/absence of V mark on leaflets): 0 = absent, 1 = Present.

M- Flower colour: 1=White, 2=Violet, 3=Mauve-pink, 4=other.

N-Days of germination: days of germination were recorded for all the accessions.

O- Pod colour: 1= Pale tan or straw, 2= Dark tan, 3= Dark brown, 4= Black or dark purple, 5= other

DATA ANALYSIS

The data was subjected to cluster analysis using PAST (Paleontological Statistics) package. In the process of hierarchical clustering, the unweighted pair group method of arithmetic average (UPGMA) (Sokal and Michener, 1958), was employed using the Euclidean similarity distance coefficient within the PAST package (Hammer *et al*., 2001).

Appendix II: Scores of 15 qualitative and quantitative traits of Cowpea.

GENOTYPES	QUALITATIVE AND QUANTITATIVE TRAITS														
	SS	**TT**	**SCC**	**EC**	**GP**	**GH**	**TT**	**PP**	**TLS**	**LC**	**LM**	**LT**	**FC**	**PC**	**DG**
Vegetable cowpea	2	1	3	1	1	3	3	0	3	7	1	1	2	1	6
Ivory grey	2	1	3	1	1	2	0	0	3	5	1	1	1	1	6
Okhalweni	1	1	3	2	1	3	0	0	3	7	1	1	1	1	6
Fahari	1	1	3	2	1	1	0	0	3	5	1	1	1	1	7
Fahari dark	1	1	4	3	2	1	0	0	4	5	1	1	1	1	8
97K-1069-8	1	1	3	2	2	3	0	0	3	7	1	1	2	1	13
IT93K-73h	1	1	1	2	1	1	0	0	3	5	1	1	1	1	15
129-3	1	1	2	1	1	1	0	0	3	5	1	1	1	1	6

SS= Seed Shape, **TT**= Testa Texture, **SCC**= Seed Coat Colour, **EC**= Eye Colour, **GP**= Growth Pattern, **GH**= Growth Habit, **TT**= Twinning Tendency, **PP**= Plant Pigmentation, **TLS**= Terminal Leaflet Shape, **LC**= Leaf Colour, **LM**= Leaf Marking, **LT**= Leaf Texture, **FC**= Flower Colour, **PC**= Pod Colour, **DG**= Days of Germination.

Appendix III: Univariate statistics of qualitative and quantitative traits extracted from PAST program

	Quantitative and qualitative traits													
Univariate statistics	**SS**	**TT**	**SCC**	**EC**	**GP**	**GH**	**TT**	**PP**	**TLS**	**LC**	**LM**	**LT**	**FC**	**PC**	**DG**
Min	1	1	1	1	1	1	0	0	3	5	1	1	6	1	1
Max	2	1	4	3	2	3	3	0	4	7	1	1	15	2	1
Mean	1.25	1	2.75	1.75	1.25	1.875	0.375	0	3.125	5.75	1	1	8.375	1.25	1
SEM	0.16366	0	0.31339	0.25	0.16366	0.35038	0.375	0	0.125	0.36596	0	0	1.26685	0.16366	0
Variance	0.21429	0	0.78571	0.5	0.21429	0.98214	1.125	0	0.125	1.07143	0	0	12.8393	0.21429	0
Stand. dev	0.46291	0	0.88641	0.70711	0.46291	0.99103	1.06066	0	0.35355	1.0351	0	0	3.58319	0.46291	0
Median	1	1	3	2	1	1.5	0	0	3	5	1	1	6.5	1	1
Skewness	0.94511	0	-0.673	0.26517	0.94511	0.20468	1.85616	0	1.85616	0.42267	0	0	0.90605	0.94511	0
Kurtosis	-1.2135	0	-0.5386	-1.2969	-1.2135	-2.0734	1.70313	0	1.70313	-2.0302	0	0	-1.0931	-1.2135	0

SS= Seed Shape, **TT**= Testa Texture, **SCC**= Seed Coat Colour, **EC**= Eye Colour, **GP**= Growth Pattern, **GH**= Growth Habit, **TT**= Twinning Tendency, **PP**= Plant Pigmentation, **TLS**= Terminal Leaflet Shape, **LC**= Leaf Colour, **LM**= Leaf Marking, **LT**= Leaf Texture, **FC**= Flower Colour, **PC**= Pod Colour, **DG**= Days of Germination.

Appendix IV: Principal components for qualitative and quantitative traits extracted from PAST program

Components	Eigen value	Percentage
1	13.3005	73.6
2	2.60211	14.399
3	1.19384	6.6062
4	0.620664	3.4345
5	0.226524	1.2535
6	0.0939243	0.51974
7	0.0338754	0.18745
8	1.4519E-15	8.0342E-15
9	8.93634E-17	4.945E-16
10	4.49344E-17	2.4865E-16
11	0	0
12	0	0
13	-2.09805E-17	-1.161E-16
14	-2.68176E-16	-1.484E-15
15	-1.25996E-15	-6.9721E-15

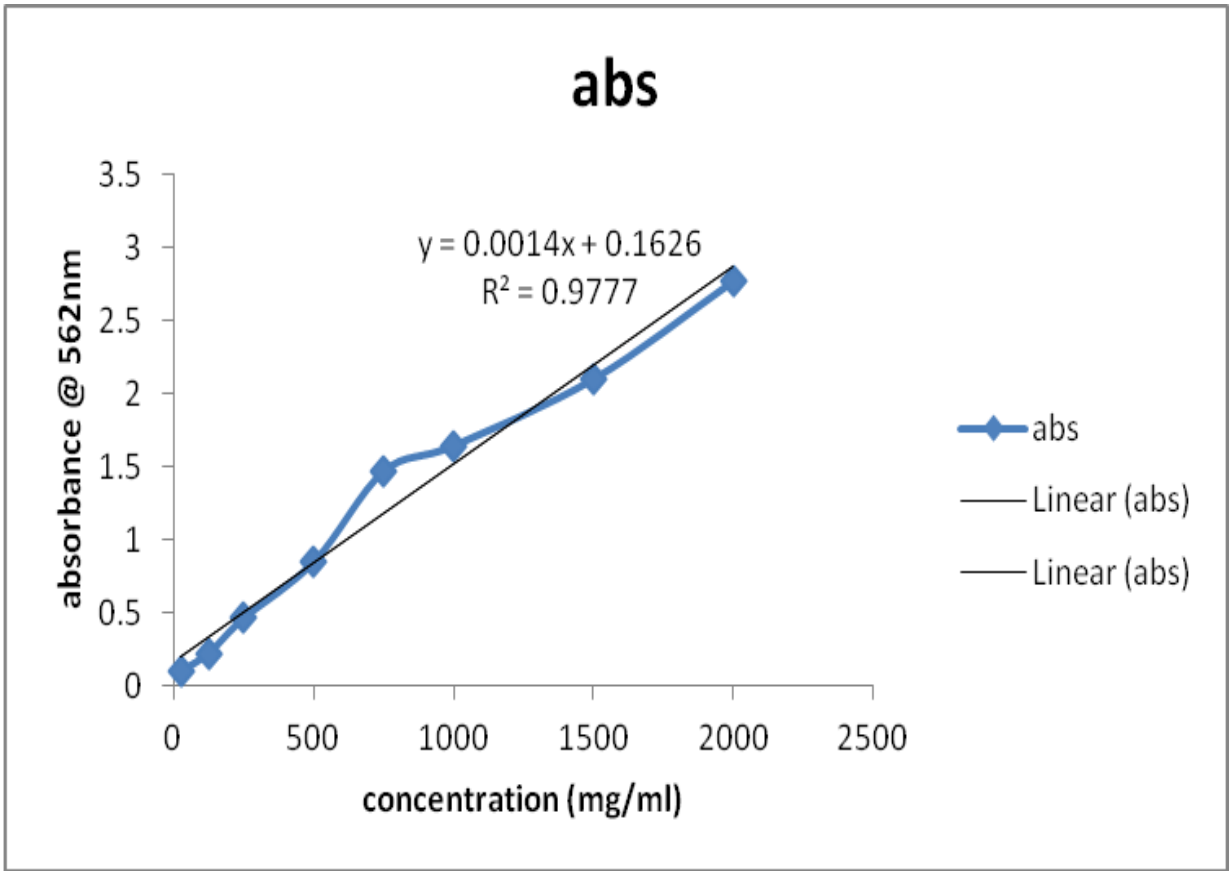

Figure V: standard curve for Bovine Serum Albumin (BSA)

Appendix VI: C**owpea grown in a pot in the green house of the University of Fort Hare, Alice, South Africa.**