# *BIOCHEMICAL ASSAYS AND SIMPLE SEQUENCE REPEAT MARKERS CHARACTERIZATION OF LOCAL AND IMPROVED NIGERIAN COWPEA [Vigna unguiculata (L.) WALP] VARIETIES*

**NWACHUKWU EMMANUEL CHIKA**

**FS 2019 71**

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By

**NWACHUKWU EMMANUEL CHIKA**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

**June 2019**

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**DEDICATION**

In memory of my elder brother, Anthony Chibuzo Nwachukwu who passed on while I was still studying.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

**BIOCHEMICAL ASSAYS AND SIMPLE SEQUENCE REPEAT MARKERS CHARACTERIZATION OF LOCAL AND IMPROVED NIGERIAN COWPEA [*Vigna unguiculata* (L.) WALP] VARIETIES**

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**June 2019**

**Chairman : Associate Professor Rosimah Nulit, PhD**
**Faculty : Science**

Cowpea (*Vigna unguiculata* L. Walp) is an important food and fodder crop in the semi-arid tropics of Africa. In Nigeria, it serves as a principal source of energy, protein, vitamins and mineral nutrients for the people in the region. The aim of this study was to characterize cowpea accessions from Nigeria in order to generate information that could be used to design appropriate breeding and conservation strategies. In this study, five local cowpea varieties from local Abuja market and five improved varieties from International Institute of Tropical Agriculture (IITA) and National Centre for Genetic Resources and Biotechnology (NAGRAB), Nigeria, were screened to ascertain the genetic variation using biochemical analysis (antioxidant, anti-nutritional, mineral element and amino acid) and PCR-based molecular marker; Simple Sequence Repeats (SSR).

In the first objective, the protein content was determined using Kjeldahl method, while spectrophotometer was used to measure the antioxidant (antioxidant activities, phenolics and flavonoids) and anti-nutritional values (phytate, alkaloid and tannin). The mineral analyses of (Calcium, Iron and Zinc) were carried out using Optimal Emission Spectroscopy 2000DV and the amino acid analysis was performed using High Performance Liquid Chromatography (HPLC). There were significant differences for Nutritional, Antioxidants, Anti-nutritional, Amino acids and Mineral content among 10 local and improved cowpea accessions from Nigeria. The seed protein content ranged from 22.61% to 27.92% indicating a significant variation among the cowpea genotypes. Highest protein content was recorded in improved variety NG/SA/0661 (27.92%) as compared to other varieties. The highest moisture contents were found in sweet honey (10.52%) and Sampea 10 (10.52%). Big white (4.28%) showed the highest seed ash content. There was a slight variation in the antioxidant properties ranging from (1.70-3.42mg/TAE/g) in phenolic and (1.67-

2.31mg/QE/g) in flavonoids. The highest total phenolic content (TPC) recorded among local and improved varieties was in the genotypes Big brown (3.43mg/TAE/0.1g) and Sampea 10 (3.132mg/TAE/0.1g). The results obtained showed the highest total flavonoid contents was found in the Butter beans (2.2QE/g) and NG/AO/035 (2.31QE/g) varieties. The anti-nutritional results clearly showed that cowpea seeds contain more tannin (1.92-5.72mg/g) than phytate (0.84-1.94mg/g) and alkaloid (0.24-2.54mg/g). The highest Phytate content was found in Big white (1.94mg/g) and NG/SA/0661 (1.89mg/g). The highest alkaloid was noted in Big white variety at (2.54mg/g) and the highest tannin was recorded in Small white (5.72mg/g). The total amino acid ranged from 13.43g/100g-16.20g/100g. The highest total amino acid was recorded in NG/AO/035 ((16.20g/100g) and Sampea 9 (15.77g/100g). NG/SA/0661 proved the highest total essential amino acid (48.21%), highest total basic amino acid (19.39%), and highest total neutral amino acid (54.17%). The highest total non-essential amino acid and the highest total acid amino acid was recorded in Sampea 11 at (57.85%) and (32.05%) respectively. The mineral element content varied from (45.61±1.22mg/g-14.79±0.24mg/g) in Ca, (2.29±0.06-0.94±0.02mg/g) in Fe and (2.1±0.02-0.96±0.02mg/g) in Zn. The highest calcium content was found in the genotype Big brown (45.61mg/g) and Big white (2.29mg/g) recorded the highest iron content. The highest zinc content was noted in Big white genotype at (2.1mg/g).

In the last objective, a total five local and five improved varieties were genotyped using 19 SSR primers. A wide genetic variation was observed with the allele sizes ranging from 160 to 300bp. The average number of alleles was two per locus. The polymorphic information content (PIC) ranged from 0.32 to 0.59 (average of 0.46). The analysis of molecular variance showed percentages of molecular variance of 76% within population and 24% among population. The study established the existence of considerable genetic variation among Nigerian cowpea varieties. The genetic variation and relationships observed in this research provide insights for cowpea conservation and utilization in Nigeria. The variation in the protein and mineral content found among cowpea accessions could also be exploited for selection of cowpea improvement in Nigeria.

**Keywords;** Cowpea; SSR Marker; Nutritional; Anti-nutritional and Amino acid

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falfasah

**PENYARINGAN KE ATAS VARIETI TEMPAN DAN VARIETI LEBIH BAIK COWPEA [*Vigna unguiculata* (L.) WALP] MENGGUNAKAN BIOKIMIA ASSAYS DAN PENANDA JUJUKAN RINGKAS**

Oleh

**NWACHUKWU EMMANUEL CHIKA**

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Cowpea (*Vigna unguiculata* L. Walp) merupakan sumber utama makanan dan ternakan di Afrika. Di Nigeria, ia berfungsi sebagai sumber utama tenaga, protein, vitamin dan mineral untuk penduduk di rantau ini. Kajian ini dilakukan bertujuan untuk mengenalpasti dan memilih varieti cowpea dari Nigeria yang sesuai digunakan untuk tujuan pembiakbakaan/penghibridan dan pemuliharaan. Dalam kajian ini, lima varieti cowpea tempatan dari pasar Abuja tempatan dan lima varieti yang bertambah baik dari Institut Pertanian Tropika Antarabangsa (IITA) dan Pusat Kebangsaan Sumber dan Bioteknologi Genetik (NAGRAB), Nigeria, telah diperiksa untuk menentukan variasi genetik yang menggunakan analisis biokimia antioksidan, anti-pemakanan, unsur mineral dan asid amino) dan penanda molekul berasaskan PCR; Ulangan Jujukan Ringkas (SSR).

Dalam objektif pertama, kandungan protein ditentukan dengan menggunakan kaedah Kjeldahl, manakala anti-oksidan (aktiviti anti-oksidan, kandungan fenolik dan flavonoid) dan nilai anti-pemakanan (phytate, alkaloid dan tanin) diukur menggunakan spektrofotometer. Analisis mineral iaitu kalsium, besi dan zink dilakukan menggunakan Spektroskopi Pelepasan optimum 2000DV dan analisis asid amino dilakukan dengan menggunakan 'High Performance Chromatography Liquid' (HPLC). Terdapat perbezaan yang ketara ($p<0.05$) untuk kandungan nutrisi, anti-oksidan, anti-nutrisi, asid amino dan kandungan mineral di kalangan 10 varieti cowpea. Kandungan protein adalah 22.61 - 27.92 % di mana menunjukkan variasi ketara antara genotip cowpea. Kandungan protin tertinggi didapati pada varieti NG/SA/0661 (27.92 %) berbanding dengan varieti yang lain. Kandungan kelembapan yang tinggi pada sweet horny (10.52 %) dan Sampea 10 (10.52 %). Big white menunjukan kandungan abu yang paling tinggi (4. 28%).Manakala, profil kandungan fenolik dan flavonoid masing-masing menunjukkan perbezaan yang sedikit (1.70-3.42

mg/TAE /g) dan (1.67-2.31 mg /QE /g). Kandungan jumlah fenolik adalah paling tinggi pada Big brown (3.43 mg/TAE/0.1g) dan Sampea 10 (3.132 mg/TAE/0.1g). Manakala, jumlah kandungan flavonoid adapalah paling tinggi pada Butter beans (2.2 QE/g) and NG/AO/035 (2.31 QE/g). Analisis anti-nutrisi jelas menunjukkan perbezaan yang ketara ($p<0.05$) di mana cowpea mengandungi lebih banyak tanin (1.92-5.72 mg/g) daripada phytate (0.84-1.94 mg/ g) dan alkaloid (0.24-2.54 mg/g). Big white dan NG/SA/0661 menunjukkan kandungan Phytate tertinggi ((1.94 mg/g) dan (1.89 mg/g)). Kandungan alkaloid juga paling tinggi pada Big white (2.54 mg/g) walaubagaimanapun kandungan tannin paling tinggi dalam Small white (5.72 mg/g). Jumlah asid amino adalah 13.43-16.20 g/100g. The highest total amino acid was recorded in NG/AO/35 ((16.20g/100g) and Sampea 9 (15.77g/100g) mengandungi amino asid yang paling tinggi 16.20 g/100g) dan 15.77 g/100g. NG/SA/0661 menunjukkan kandungan yang tertinggi dalam asid amino perlu (48.21 %), asid amino basik (19.39%), dan asid amino neutral (54.17%). Walaubagaimanapun, kandungan asid amino tidak perlu di dapati pada Sampea 11 at (57.85%) Kandungan unsur mineral adalah sangat berbeza ($p<0.05$) kandungan Ca jauh lebih tinggi (45.61 ± 1.22 -14.79 ± 0.24 mg/g) berbanding dengan Fe (2.29 ± 0.06-0.94 ± 0.02 mg/g) dan Zn (2.1 ± 0.02-0.96 ± 0.02 mg/g). Kandungan kalsium paling tinggi pada Big brown (45.61mg/g). Big white menunjukkan kandungan Fe (2.29 mg/g) dan Zink tertinggi (2.1 mg/g).

Dalam objektif terakhir, lima jenis tempatan dan lima jenis yang lebih baik telah genotip menggunakan 19 primer SSR. Keputusan menunjukkan terdapat variasi genetik yang ketara di mana saiz alel antara 160 hingga 300bp didapati. Bilangan purata alel adalah dua setiap lokus. Kandungan maklumat polimorfik (PIC) antara 0.32 hingga 0.59 dengan nilai purata 0.46. Analisis varians molekul menunjukkan peratus varians molekul adalah 76% dalam populasi dan 24% di kalangan penduduk. Kajian ini mewujudkan kewujudan variasi genetik yang agak besar di kalangan 10 genotip cowpea Nigeria. Variasi genetik dan hubungan yang didapati dalam kajian ini berpotensi untuk digunakan pemuliharaan dan penghasilan hybrid baru cowpea di Nigeria. Variasi kandungan protein dan mineral yang terdapat di kalangan 10 varieti cowpea juga boleh digunakan untuk penambahbaikan varieti cowpea di Nigeria.

**Kata kunci;** Cowpea; Penanda SSR; Pemakanan; Asid pemakanan dan asid amino

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I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of Nwachukwu, Emmanuel Chika on his thesis entitled "Biochemical Assays and Simple Sequence Repeats (SSR) Markers Characterization of Five Local and Five Improved Nigerian Cowpea (*Vigna unguiculata* (L.) Walp) Varieties" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the (insert the name of relevant degree).

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| | |
|---|---|
| AFLPs | Amplified fragment length polymorphisms |
| bp | base pairs |
| cM | centiMorgans |
| cm | Centimeter |
| CTAB | Cetyltrimethyl-ammoniumbromide |
| dATP | Deoxyadenosinetriphosphate |
| dNTPs | Deoxynucleotidetriphosphates |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediamine tetra-acetic acid |
| EtBr | Ethidium bromide |
| EtOH | Ethanol |
| FAO | Food and Agriculture Organization of the United Nations |
| g | Gram |
| H | Hour(s) |
| IITA | International Institute of Tropical Agriculture |
| Kl | Potassium iodide |
| M | Molar |
| MAS | Marker-Assisted Selection |
| min | Minute(s) |
| mm | Millimeters |
| PCR | Polymerase chain reaction |
| QTL | Quantitative trait loci |
| RAPD | Random amplified polymorphic DNA |
| RFLP | Restriction fragment length polymorphism |
| RNAse | Ribonuclease |

| | |
|---|---|
| s | Second(s) |
| SNPs | Single nucleotide polymorphisms |
| SSRs | Simple sequence repeats or microsatellite |
| STRs | Short tandem repeats |
| TBE | Tris-Borate-EDTA buffer |
| TE | Tris-EDTA |
| (TE) | Buffer Tris 2-amino-2-(hydroxymethyl)-propane-1,3-diol |
| w/v | Weight/volume |
| v/v | Volume/volume |
| U | Unit |
| % | Percentage |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Cowpea (*Vigna unguiculata)* is a well-known leguminous crop in Africa and is popularly called 'beans' in Nigeria and 'niebe' in the Francophone countries according to African Centre for Biodiversity (2015). Cowpea is one of the ancient crops known to mankind, with its centre of origin in Africa. Cowpea is a drought tolerant plant and it performs well in a wide variety of soils. As a leguminous plant, the leaves and root systems replenish nitrogen-depleted soils thus increasing soil fertility. Cowpea is cultivated across Nigeria but the largest cowpea production comes from the Northern Nigeria. Cowpea production has been increasing at an average rate of 5% annually, with 3.5% annual growth in area and 1.5 % growth in yields (Umar, 2010)

Over the years cowpea has increasingly become a source of food across various socio-economic strata (Abizari et al., 2013). Cowpea is not only a source of food, but used also as animal feed and it is therefore essential to animal breeders. As a legume, there is a symbiotic relationship between cowpea and soil bacterium (*Rhizobium*) in root nodules that fixes atmospheric nitrogen.

The greatest production of cowpea comes from West Africa and an increasingly lucrative regional cowpea seed market has emerged due to high demographic growth and urbanization. Cowpea global production is about 14.5 million hectares, with over 6.5 million metric tons and African continent produced 95 % of the world production on a surface area of 11 million hectres (IITA, 2015). Nigeria is the largest producer of cowpea in the world with an annual average production of 2.7 million metric tons over the last decade unfortunately, Nigeria has been unable to meet the domestic demand and it is also the largest importer of cowpea in the region followed by Ghana, Togo, Cote d'Ivoire and Mauritania (Fatokun et al., 2012; FAO, 2010; Ishiyaku et al., 2010).

## 1.2 Problem Statement and Justification of Study

Despite the high demand for cowpea in Nigeria due to the rich protein content, the need to evaluate the nutritional composition remains a concern. Calcium, iron and zinc deficiencies are among the most common and widespread micronutrient deficiency that affects more than half of the human population (Umar, 2010). A critical study by FAO (2010) showed that mineral deficiencies are the primary cause of stunted growth and underweight children in Nigeria. Only few proximate analysis of some selected cowpea accessions in Nigeria were carried out by Animasaun et al. (2015) however, there was no study on the biochemical components such as anti-oxidants, mineral, amino acids and anti-nutritional compounds. There is a lot of work to be done to improve the nutritional values of cowpea. The cowpea nutritional and mineral

evaluation is of paramount important in the identification of genotype with high biochemical content.

In Africa, most stable crops have low nutritional content due to biotic and abiotic stresses resulting to stunting, underweight, and thereby leads to illness and death among children (FAO, 2011). Good and improved varieties are the best options to combat these problems. One of the major challenges of genetically modified cowpea in Nigeria is the economic importance where farmers and anti-gmo have argued the loss/erosion of local varieties however, this study will provide the best Nigeria accession base on the relevant nutritional and general biochemical composition that could be used for the development of genetically modified cowpea which will be resistance to maruca.

The conventional techniques of improving crop involve the introduction, selection, hybridization, and back cross. Although, consistent progress has been made, but in recent times these methods are found to be inadequate in developing varieties with desirable qualities as these methods are often influenced by various degrees of plant-endogenous and environmental factors and, thus, are not reliable tools for genetic diversity assessment. Hence, there is a need to develop an additional genetic method and also to utilize the already existing techniques by adopting novel breeding systems. Keeping this in view, the present study was undertaken to evaluate the pattern and the existence of genetic variability and relatedness among local and improved varieties of cowpea cultivars.

## 1.3 Limitations of the Present Study

Until date, there is reluctant use of improved cowpea varieties in Nigeria. Although there has not been any clear data on quantity of improved cowpea seeds in Nigeria. Kamara et al. (2011) reported that farmers in North-eastern Nigeria have continued to plant predominately local varieties despite the development of improved varieties with better yield. Therefore, proper verification in the biochemical and genetic divergence between local and improved cowpea varieties would help to clear the doubts and encourage the local farmer on the need to integrate the improved varieties.

## 1.4 Significance of the Study

Cowpea is a protein rich crop for economically poor rural class in Africa**, i**t represents a major source of protein that is far more affordable than meat or dairy products in Africa Hence ‘poor man’s meat’ (African centre for biodiversity, 2015). Cowpea is an essential food security crop as it provides the earliest food available in the ‘hungry season’ before cereals mature. Cowpea is an important crop in several regions of Nigeria. The demand for different varieties of beans are expected to remain strong and increase as consumers go for healthy alternatives in their diet and there are greater numbers of Nigerians with a culinary tradition of consuming beans.

It is proven that the DNA markers have many advantages over the morphological and biochemical markers. The DNA-PCR based marker that uses arbitrary primers, such as simple sequence repeats (SSR) have been widely used for investigating genetic relatedness and diversity in plant population and cultivars. It offers simple, efficient and economic means for cultivar identification and diversity analysis. In many plant species, polymorphism at DNA level is the basic information that is necessary for a variety of recombinant DNA exercises.

## 1.5 Objectives of the Study

In this study, the main objective was to screen and identify cowpea varieties with good seed quality for future cowpea breeding program. The specific objectives were to;

1. To evaluate the anti-oxidant, nutritional and anti-nutritional values of 5 local and 5 improved Nigerian cowpea varieties.
2. To determine the level of amino acids and minerals levels of 5 local and 5 improved Nigerian cowpea varieties.
3. To assess genetic variation in 5 local and 5 improved Nigerian cowpea varieties using SSR markers

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