GENETIC ANALYSIS OF TRAITS RELATED TO BIOLOGICAL NITROGEN FIXATION IN COWPEA [*Vigna unguiculata* (L.) WALP] UNDER LOW SOIL PHOSPHORUS.

BY

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DECLARATION

I hereby declare that except for references to works of other researchers, which have been duly cited, this work is my original research and that neither part nor whole has been presented elsewhere for the award of a degree.

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ABSTRACT

Studies were undertaken to obtain information on the genetics of traits related to biological nitrogen fixation in cowpea, in soils deficient in phosphorus prevailing in the humid forest zone (HFZ) of Cameroon. In this zone, farmers are not usually consulted in the development of new varieties in cowpea breeding programmes. Participatory rural appraisal (PRA) technique through focus group discussions and questionnaire administration was used to assess farmers' knowledge on the role of grain legumes in improving soil fertility in cropping systems; and to identify farmers' preferred cowpea traits in five sites in the HFZ of Cameroon. Ninety percent of the farmers were of the view that legume root nodules were disease causing agents, which they referred to as soil "cysts". This means productivity on farmers field may be declining due to lack of knowledge. Farmers grew four varieties of cowpea, the white seed coated being the dominant (75%) and preferred variety. They also mostly grew and preferred the erect and early maturing cowpea type and were ready to adopt cowpea for soil fertility improvement if residual effects of legumes is demonstrated on-farm. Three cowpea varieties (Dsch MMBr, Vya niebe and 58-77) were used as trap crops to estimate the population of indigenous *Bradyrhizobia spp* in two study sites. The results of the Most Probable Number (MPN) counts indicated that the total *Bradyrhizobia* population in Nkoemvone soil was between 1.0×10^5 and 5.8×10^5 cells per gram of soil sample while in Nkometou, it was between 5.8×10^3 and 1.0×10^4. These levels of *Bradyrhizobia* populations observed in the two sites were adequate to give satisfactory nodulation and nitrogen fixation. This suggests that cowpea production in the HFZ of Cameroon may not require inoculation. Morphological characterization of 50 cowpea genotypes including 10 Cameroon landraces was carried out in three environments: low phosphorus (LP; 0 mg P kg^{-1} soil), high phosphorus (HP; 30 mg P kg^{-1} soil) and high phosphorus and nitrogen (high P and N; 30 mg P and 90 mg N kg^{-1} soil). Data collection involved 20 morphological traits comprising

phenological, nitrogen fixation and yield related traits. Thirteen out of the 20 traits were selected by the principal component analysis and explained 55.3% of the variation. Correlation studies indicated N-fixed and grain yield were positively correlated only under low P conditions, while shoot N was highly and positively correlated with N-fixed in all three environments. Shoot N was considered the best nitrogen fixation related trait and was used in place of N-fixed in subsequent studies. A study was undertaken to determine gene effects controlling nitrogen fixation related traits through generation mean analysis (GMA). The parents included a high N_2-fixing genotype, 58-77 and a low N_2-fixing type Lori-niebe. Data collected included nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), and shoot nitrogen (shoot N) content. All the traits showed significant additive and/or additive x additive gene effects, while additive x dominance effect was significant for SDW and shoot N, suggesting that early generation selection for improved nitrogen fixation would be effective only for NN and NDW. QTL were mapped using 372 polymorphic EST-SNPs on 88 F_{11} recombinant inbred lines (RILs) from 58-77 (high N_2-fixing cowpea) and Yacine (poor N_2-fixing cowpea) cross. The traits under study included: NN, NDW, SDW, number of pods per plant (Npod), and weight of hundred seeds (HUN_SDW) phenotyped in eight environments. Win Cartographer identified a total of 31 QTL for NDW, 12 QTL for HUN_SDW, 26 QTL for NN, 32 QTL for SDW, and 14 QTL for Npod across the eight environments. QTLnetwork identified a total of 13 main QTL for all the traits across the eight environments. Fifteen pairs of epistatic QTL were also identified for all the traits. The results demonstrated the importance of epistasis as a genetic basis of the quantitaive traits studied. All the traits revealed epistatatic gene effects with more that 90% of QTL found with significant additive x additive effects. Therefore, in marker assisted selection, markers linked to epistatic QTL should also be taken into consideration. QTL x environment interaction

effects were detected and might indicate that gene expression could be greatly influenced by environments.

DEDICATION

I dedicate this piece of work to my late Mum Joana Bezah Fonji, my Dad Mr Michael Bezah Fonji, my son Kriss – Samuel Nkoumki and husband Remy Nkoumki. Your affection, guidance and support enabled me to move this far.

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LIST OF ABBREVIATIONS

% Ndfa	Percentage of nitrogen derived from atmosphere
^{14}N	Light nitrogen Isotope
^{15}N	Heavy nitrogen Isotope
^{15}NE	Enriched nitrogen isotope
1D	One dimensional
2D	Two dimensional
[a]	Additive main effect
[aa]	Epistatic main effect
[aae]	Epistasis x Environment interaction effect
ABA	Abscisic acid
ADP	Adenosine diphosphate
[ae]	Additive x Environment interaction effect
AFLP	Amplified Fragment Length Polymorphisms
AGRISTAT	Annuaire Statistiques Agricoles du Secteur Agricole
Al	Aluminum
ANOVA	Analysis of variance
APE	Agronomic phosphorus use efficiency
ATP	Adenosine triphosphate
BC_1P_1	Backcross to parent one
$BC_1 P_2$	Backcross to parent two
BNF	Biological Nitrogen fixation
C_2H_2	Acetylene

CIM	Composite interval mapping
cM	CentiMorgans
CO_2	Carbon dioxide
CV	Coefficient of variation
Dflw	Days to 50% flowering
DM	Dry matter
DNA	Deoxyribonucleic acid
ECEC	Exchangeable Cation Exchange Capacity
ECV	Environmental Coefficient of Variations
ENV	Environment
E-QTL	Epistatic quantitative trait loci
F_1	First filial generation
F_2	Second filial generation
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization statistics
FGD	Focus group discussion
FHP1	Nkometou high phophorus field experiment
FHP2	Nkoemvone high Phosphorus field experiment
FLP1	Nkometou low phosphorus field experiment
FLP2	Nkoemvone low Phosphorus field experiment
FN	Number of functional nodules
GCV	Genotypic Coefficient of Variation
GECV	Genotype by Environment Interaction Coefficient of Variation

GLM	General linear model
GM	Grand mean
GMA	Generation mean analysis
GMF	Gravimetric moisture fraction
h^2_b	Broad sense heritability
h^2_n	Narrow sense heritability
$H_2PO_4^-$	Dihydrogen phosphate
$H_2PO_4^-$	Dihygroden orthophosphate anion
HFZ	Humid forest zone
HPO_4^{2-}	Hydrogen orthophosphate anion
HUN_SDW	Weight of hundred dry seeds
IITA	International Institute of Tropical Agriculture
IRAD	Institute of Agricultural Research for Development
ISRA	Senegalese Agricultural Research Institute
ISSR	Inter simple sequence repeat
LCS	Leaf Colour Score
LOD	Logarithm of odds
LUS	Four land use system
MIM	Multiple interval mapping
MLG	Molecular linkage groups
MPN	Most probable number
MPS	Marker pair selection
M-QTL	Main quantitative trait loci

NDW	Nodule dry weight
NDW	Nodule dry weight
N-fixed	Nitrogen fixed
NFN	Number of non-functional nodules
NGOs	Non-governmental organization
NI	Nodulation index
NN	Nodule number
Npod	Number of pods per plant
NSC	Nodule score
Nsize	Nodule size
P_1	Parent one
P_2	Parent two
PC	Principal component
PCR	Polymerase chain reaction
PCV	Phenotypic Coefficient of Variation
PER	Phosphorus efficiency ratio
Pf	Fertilizer P
*P*fix	Relative to atmospheric N_2
pH	Hydrogen ion potential
PHP1	Nkometou high phophorus pot experiment
PHP2	Nkoemvone high Phosphorus pot experiment
Pi	Inorganic Phosphate
PLP1	Nkometou low phosphorus pot experiment

PLP2	Nkoemvone low Phosphorus pot experiment
PPUE	Physiological P use efficiency
PRA	Participatory rural appraisal
PUE	Phosphorus use efficiency
PUpE	Phosphorus uptake efficiency
PUtE	Phosphorus utilization efficiency
QE	QTL-by Environment interactions
QTL	Quantitative trait loci
R^2	Coefficient of determination
RAPD	Random Amplified Polymorphic DNA
RDW	Root dry weight
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant inbred line
SAS	Statistical analysis software
Sdpod	Number of seeds per pot
SDW	Shoot dry weight
SE	standard errors
SFW	Shoot fresh weight
Shoot N	Shoot nitrogen content
Shoot P	Shoot phosphorus content
SMA	Single-marker analysis
SNF	Symbiotic nitrogen fixation
SNP	Single nucleotide polymorphism

SPSS	Statistical Package for Social Scientist
SSR	Simple sequence repeats
Stdev	Standard deviation
TSP	Triple super phosphate
UCR	University of California, Riverside
USA	United states of America
V_G	Genotypic variance
V_{GE}	Genotype by environment interaction variance
V_P	Phenotypic variance
YLDplt	Grain yield per plant
$\delta^{15}N$	Sample natural abundance expressed as parts per thousand relative to atmospheric nitrogen
$\delta^{15}N$ leg	Legume natural abundance expressed as parts per thousand relative to atmospheric nitrogen

CHAPTER ONE

GENERAL INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is the most important grain legume crop grown in sub-Saharan Africa (Badiane *et al.*, 2012). The value of cowpea lies in its major role in human nutrition as a protein source and also because cowpea hay is critical for feeding animals during the dry season in many parts of Africa. Cowpea is a valuable source of income for farmers and traders in West and Central Africa (Timko *et al*., 2007; Diouf, 2011). Moreover, its nitrogen-fixing ability is extremely valuable when used in crop rotation with cereal crops (Timko *et al*., 2007).

According to the Food and Agriculture Organization (FAOSTAT, 2013), the top six cowpea producing countries in Africa are Nigeria, Niger, Burkina Faso, United Republic of Tanzania, Mali and Cameroon. In Cameroon, the annual production is estimated at 165,350 tons (FAOSTAT, 2013) with an average yield of 300-500 kg/ha on farmers' fields (Ecocrop, 2009; AGRISTAT, 2012) while cowpea has a potential yield of 7000 kg/ha in the USA (Ehlers and Hall, 1997; Tignegre, 2010). Breeding efforts in Cameroon have resulted in the development of cowpea varieties with grain yields of more than one ton/ha (TVX3236 with 1,667 kg/ha) and resistance to insects (Lori-niebe and CRSP niebe). Four of the recently developed cowpea varieties, VYA niebe, Lori-niebe, CRSP niebe and BR1 have been widely adopted and appreciated by farmers (Ta'ama, 1986).

The soils in the humid forest zone (HFZ) of Cameroon where most of the cowpea is grown are generally Oxisols and Ultisols (US taxonomy), equivalent to Ferralsols and Acrisols (FAO taxonomy). These are strongly weathered and acidic soils. Farmers in this zone practise low-input cropping systems and usually do not apply fertilizers to their crops due to the cost and

unavailability of fertilizer needed for meaningful management of these poor and high phosporus (P)-fixing soils (Jemo *et al*., 2006b).

The productivity of cowpea in Cameroon, like the rest of sub-Saharan Africa, is reduced by low access to inputs, heat stress, striga infestation, drought stress, insect pests, pathogens, and low soil fertility levels especially low nitrogen (N) and P. However, in the humid forest zone (HFZ) of Cameroon, the acidic nature of the soils, that leads to low inherent levels of plant-available P, of basic cations and high levels of exchangeable aluminum (Menzies and Gillman, 1997) is considered a major production constraint. The P-content of these soils (2.5 -5 mg kg–1) is far below the critical level (10.8 mg kg–1) of available soil P for grain legumes (Adeoye and Agboola, 1985; Aune and Lai, 1995).

Farmers use indigenous soil fertility management practices including the integration of grain legumes in their cropping systems. Despite the importance of farmers' perception of the assessment of soil fertility management in many localities, no such study has been conducted in the HFZ of Cameroon.

The symbiosis between *rhizobium* and legume contributes a considerable amount of N in tropical soils. For example, studies indicate cowpea fixed 61-171 kg N/ha in the savanna zone of Ghana (Belane and Dakora, 2009). Symbiotic nitrogen fixation (SNF) by legumes in heavily weathered acid soils is limited. This is because phosphorus (P) is generally deficient, limiting nodulation (Waluyo *et al.,* 2004). Phosphorus deficiency linked to soil acidity is of great importance in the HFZ of Cameroon, as acid soils cover more than 80% of arable lands (The, 2000). In this region, cowpea is usually intercropped with cereals and other crops to exploit its ability to fix N and contribute to the N economy. However, the cowpea genotypes that have been evaluated in Cameroon have lower N (36-49 kg/ha) fixing capacities (Jemo *et al.,* 2006a) compared to 66-120

kg N/ha in the humid regions of western Nigeria (Awosaike *et al.,* 1990). Due to this problem, there is the need to develop new low P tolerant cowpea varieties with high N_2-fixing capacities in Cameroon. In order to achieve this, careful selection of parental material from different groups is necessary. The involvement of farmers is also critical to ensure that their preferred traits are catered for in the varieties developed. This is addressed through participatory plant breeding, participatory variety selection (Gibson *et al.*, 2007; Gasura *et al.*, 2008).

Cowpea and soybean are increasingly being used to improve the low input cropping systems of the HFZ of southern Cameroon (Wendt and Atemkeng, 2004). However, the nitrogen-fixing potential of these cowpea varieties is not known and their inclusion in intercropping systems may lead to high competition for soil nutrients with cereal crops thus leading to low yields in both crops (Jemo *et al.,* 2006b). According to Jemo *et al*. (2006b) only P-efficient soybean and cowpea genotypes increased subsequent maize yields in the HFZ of Cameroon and concluded that the potential positive rotational effect of cowpea on the acid, highly P-sorbing soils of this zone depends on breeding and using P-efficient genotypes. Determining the nitrogen fixation potentials of cowpea varieties grown by farmers is of importance in the HFZ of Cameroon where intercropping with cowpea is a common practice. Legumes including cowpea can mitigate soil phosphorus (P) deficiency by changing the soil pH of the root zone and nitrogen (N) deficiency through symbiotic biological N_2 fixation (Giller *et al.*, 1997; Alkama *et al.,* 2009; Betencourt *et al.*, 2012, Latati *et al*., 2014). Identification of Quantitative Trait Loci (QTLs) for nitrogen fixation related traits in cowpea as found in common bean (*Phaseolus vulgaris* L**.)** and soybean [*Glycine max* (L.) Merrill] (Tsai *et al.,* 1998; Nicolas *et al.,* 2006) will facilitate the development of new low P-tolerant varieties with high nodulation and biological nitrogen fixation.

The objectives of the study were to:

a) Elicit farmers' perceptions on the role of legumes in soil fertility and cowpea trait preferences in cropping systems.
b) Characterize local and introduced cowpea genotypes for biological nitrogen fixation morphological traits under low P condition.
c) Determine the inheritance of genes controlling N fixation traits under low P in cowpea.
d) Analyse QTL for epistasis and phosphorus environment effects in biological nitrogen fixation and yield related traits in cowpea.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin, domestication and distribution of Cowpea [*Vigna unguiculata* (L.) Walp.]

Cowpea (*Vigna unguiculata*) is one of the most ancient human food sources and has probably been used since Neolithic times (Summerfield *et al*., 1974). The origin of cowpea is a controversial issue and this is due to lack of archaeological evidence. There are views supporting Africa, Asia, and South America as origins (Johnson, 1970; Summerfield *et al*., 1974; Tindall, 1983; Coetzee, 1995). One of the views suggests that cowpea was introduced from Africa to the Indian sub-continent approximately 2000 to 3500 years ago (Allen, 1983). Another view is the fact that the transversal region of the Republic of South Africa was the centre of speciation of *V. unguiculata*, due to the presence of most primitive wild varieties (Padulosi and Ng, 1997). Presently, cowpea is grown throughout the tropic and subtropic areas around the whole world. According to Ng (1995), during the process of evolution of *V. unguiculata*, there was change of growth habit, from perennial to annual growth habit and from predominantly outbreeding to inbreeding, while cultivated cowpea (subsp. *unguiculata*) evolved through domestication and selection of the annual wild cowpea (var. *dekindtiana*).

Annual wild cowpea is widely distributed throughout sub-Sahara Africa.This may imply that the species could have been brought under cultivation in any part of the region. However, the center of maximum diversity of cultivated cowpea is found in West Africa, in an area encompassing the savannah regions of Nigeria, Southern Niger, part of Burkina Faso, Northern Benin, Togo, and the Northwestern part of Cameroon (Ng and Marechal, 1985). Carbon dating of cowpea (or wild cowpea remains from the Kintampo rock shelter in central Ghana) has been carried out (Flight, 1976), and is the oldest archaeological evidence of cowpea found in Africa. The controversies

related to the origin of cowpea suggest that more research needs to be done and advanced archaeological technology developed. Recently, SNP makers were used by Huynh et al. (2013) to examine the gene pool structure of African wild annual cowpea *V. unguiculata subsp. dekindtiana* from both East and West Africa and to determine their relatedness to African wild cowpeas and non-African domesticated cowpeas. The results distributed the genetic materials into two gene pools (Figure 2.1).

Figure 2. 1 Worldwide distribution and gene pool structure of cowpea landraces.
Relative proportions of blue and red colors for each symbol represent the likelihood of an accession assigned to gene pools 1 and 2, respectively. (Source: Huynh *et al.* 2013).

2.2 Cowpea production in Africa and the world

Cowpea is grown in about 14.5 million hectares globally, with over 6.5 million metric tons (Fatokun *et al.*, 2012). A total of 5,914,552 tonnes of dry cowpea grains was produced in Africa in 2012 (FAO, 2013). The six top producing countries in Africa were Nigeria, Burkina Faso,

United republic of Tanzania, Mali, Cameroon and Niger. Production was higher in all of these countries compared to the nuited states of America (Figure 2.2).

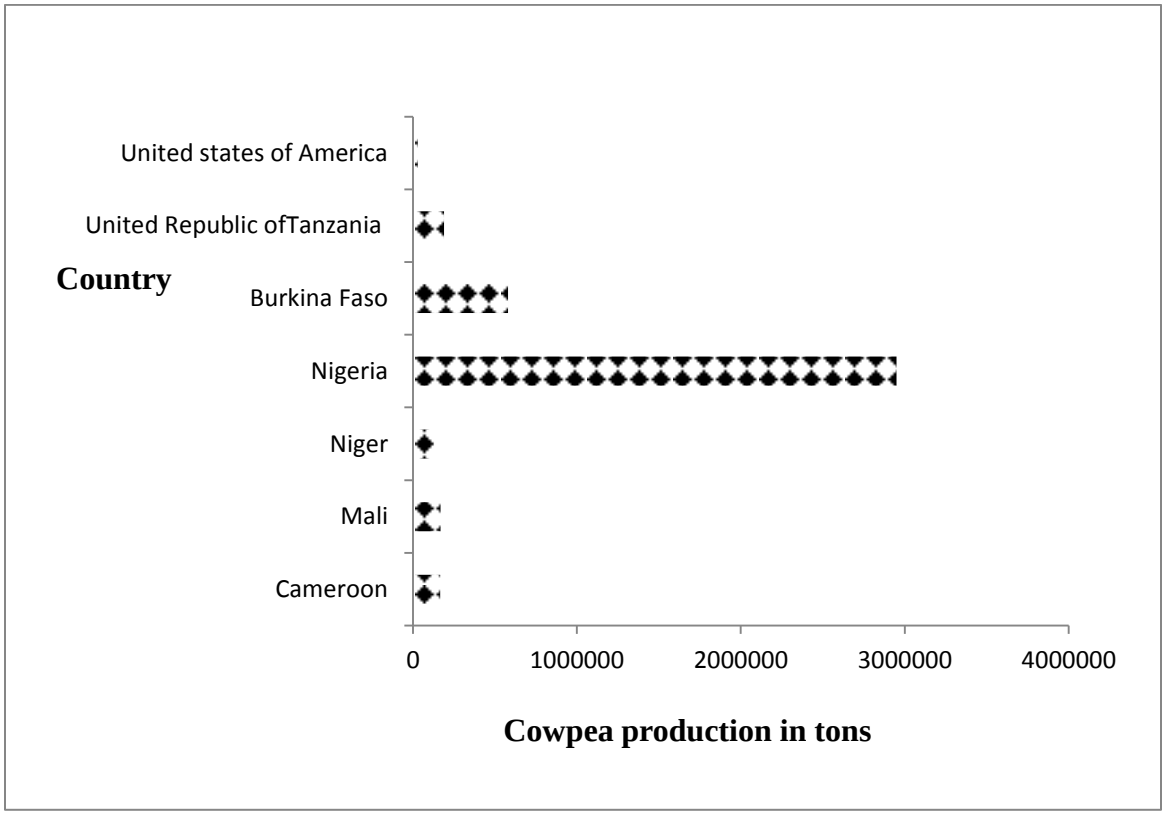


Figure 2. 2 Cowpea production in Africa

Source: FAOSTAT, 2013. Accessed 22nd November 2015.

2.3 Uses of Cowpea

Cowpea is the favorite legume in many parts of Africa. The mature seeds are cooked with spices and palm oil, to produce a thick soup of beans, which accompanies the basic food cassava, yam, or plantain. In West Africa, the seeds are husked then transformed into flour and mixed with onions and spices for the production of pastes that can be fried, " akara" or steamed into bean cake, "moinmoin" . Small quantities of flour of cowpea are transformed into biscuits, composite flour and food for babies in Senegal, Ghana and Benin (Madamba *et al.*, 2006). The leaves, as

well as the seeds and immature pods of cowpea are consumed as vegetables. The roots are sometimes consumed, e.g. in Ethiopia and the Sudan. (Madamba *et al.,* 2006). The dry seeds have been used as a substitute for coffee (Nkouannessi, 2005, Madamba *et al*., 2006, Valenzuela and Smith, 2002). Several medicinal uses of the cowpea have also been reported (Madamba *et al.,* 2006). In Cameroon, cowpea is grown traditionally for its leaves, pods and seeds. It is the third most important grain legume after peanuts and common bean. Cowpea occupies an important place in the diets of both the rural and urban populations in Cameroon (Nkamleu *et al*., 2001). In animal nutrition, cowpea is used as forage plant or pasture (Taffouo *et al*., 2008).

2.4 Characteristics of soils in humid forest zones

2.4.1 Chemical properties of humid forest soils

The Humid Forest zone of Cameroon is dominated by acid and aluminum (Al) toxic soils with P deficiency as an additional limiting factor to plant growth and nitrogen fixation (Waluyo et al. 2004). Phosphorus deficiency results from high fixation by Al and Iron (Fe) oxides and hydroxides (Selles *et al*., 1995). About 20 to 80% of the total phosphorus is retained in the organic matter and the rest exists as inorganic compounds of calcium (Ca), Al and Fe (Sanchez, 1976 cited by Ronnie, 1986). Depending on the conditions of the soil, the concentrations of Al and Fe can increase. Thus the proportion of soluble calcium phosphates will decline because they have been transformed into Al and Iron phosphate (Sanchez, 1976 cited by Ronnie, 1986). The negative effect of soil acidification is the tendency of phosphorus to become unavailable to plants. As aluminum and iron are released during the acidification process, they become more accessible in the cation exchange sites in solution or simply on exposed surfaces. These two ions react spontaneously with phosphate to form compounds relatively insoluble leading to phosphate fixation (Harter, 2007).

2.4.2 Effect of soil acidity on P availability

Soil acidity is determined by the concentration of hydrogen ions (H^+) active in soil solution and is influenced by edaphic, climatic and biological factors. The acidity of a soil is defined by its hydrogen ion potential (pH). Low P (Mullen *et al.*, 1988) and low Ca (Munns, 1970), are soil properties associated with acidic soils. Their deficiencies deleteriously affect the nodulation process independently of soil pH. Rhizobial strains with relatively higher capacity to nodulate their hosts under low P (Leung and Bottomley, 1987) or in acidic soils (Howieson, 1995) have been isolated. These strains are essential for good symbiosis under such adverse conditions that prevail in many tropical soils farmed by subsistence farmers who are unable to invest substantial funds required to alleviate soil acidity. In general, slow-growing bradyrhizobia (cowpea type) are more acid-tolerant than fast-growing rhizobia (Cooper *et al.*, 1985).

2.4.3 Effect of Aluminum toxicity on P availability

In the earth's crust, Al is the third most common element. When soil pH decreases to 5.0 or below, toxic forms of Al become solubilized into the soil solution, inhibiting root growth and function, altering nutrient availability in the rhizosphere, affecting nutrient uptake and translocation by plants (Roy *et al.*, 1988; Taylor, 1988; Matsumoto, 2000) and thus reducing crop yields (Kochian *et al*., 2005). What is more important is the fact that Al increases P fixation by precipitation of Al–P complexes thus enhancing low P availability (Foy, 1984; Macklon *et al.*, 1994). Exchangeable Al concentrations above 1cmol (+) kg^{-1} in soils are often toxic to plants (Andersson, 1988; Ritchie, 1995). Since Al increases P fixation and P unavailability limits nitrogen fixation, these soils need the application of lime to neutralize the effect of toxic aluminum (Kamprath, 1970).

2.5 Role of Nitrogen and Phosphorus in nodulation and plant growth

Nitrogen (N) is essential for the normal growth of plants. All vital biological processes are related to the existence of functional plasma, of which N is a basic constituent (proteins, nucleic acids). Nitrogen is also a basic constituent of many other compounds of primary physiological importance to plant metabolism, such as chlorophyll, nucleotides, proteins, alkaloids, enzymes, hormones and vitamins (Marschner, 1995). In plants, nitrogen promotes growth and the absorption of other mineral elements (Boubie, 2002) by enhancing the use of carbohydrates, and stimulating the development and activity of roots. Plants absorb nitrogen in the form of nitrate (NO^{3-}) and ammonium (NH^{4+}). However, legumes in symbiosis as well as some free living organisms can acquire nitrogen directly from the atmostphere. The relative importance of each of these forms depends on the plant species and environmental conditions (Layzell, 1990; Hageman 1984, cited by Boubie, 2002). Nitrogen deficiency may exert pronounced adverse effects on crop development and yield. Growth is stunted, and leaves become chlorotic because lack of N limits the synthesis of proteins and chlorophyll. Lack of chlorophyll inhibits the capacity of the plant to assimilate CO_2 and synthesize carbohydrates, leading to poor and premature flowering and fructification, with shortening of the growth cycle.

Plants acquire P from the rhizosphere solution as phosphate (Pi), primarily in the form of $H_2PO_4^-$ (Vance *et al.*, 2003; Hammond *et al*., 2004; White and Hammond, 2008). P is a key component of molecules such as nucleic acids, phospholipids, Adenosine Triphosphate (ATP) and therefore plants cannot grow without a sufficient supply of this nutrient. The inorganic phosphate (Pi) is involved in the regulation of metabolic pathways (Theodorou and Plaxton, 1993; Schachtman *et al.*, 1998). After nitrogen, phosphorus is the second most important macroelement used by the plant for its growth. One of the main roles of phosphorus in living organisms is the transfer of

energy through the plant. Phosphorus is essential for the production of seeds, promotes root growth, and promotes early maturity of the plant. The process of nitrogen fixation requires P in sufficient quantities. Among all the nutrients, deficiency in phosphorus (P) has a large impact on the legumes which generally rely on Nitrogen fixation for their N nutrition. Effective nodulation of legumes requires a balanced availability of phosphorus and other nutrients such as K, S, Fe, Bo and Mo (Ronnie, 1986). Nodulation and N_2 fixation are delayed or inhibited by a high nitrogen content in the soil (Franco, 1977 cited by Ronnie, 1986).

2.6 Managemant of nitrogen and Phosphorus in Agricultural systems

2.6.1 Management of Nitrogen

Nitrogen as an element is needed by plants in relatively higher quantities compared to other soil nutrients. Its endogenous application to crops often results in improved yields. Although most species obtain N only from the soil, many legumes and certain other plants can acquire N from the atmosphere. In crop plants, N is acquired from the soil through mineralization of soil organic matter and from exterior resources, both organic and inorganic (FAO, 1994). In order to achieve optimal yield, the N supply must be available according to the needs of the plant, matching its pattern and total amount. The use of N in excess encourages development of the aerial plant organs with relatively poor root development. It also expands the development pattern, prolongs the maturity period, and often reduces the quality of the harvestable products (FAO, 1994). Research has shown that losses from soil due to N uptake, leaching, erosion, denitrification, etc., are obviously replenished to different extents by biological N_2 fixation processes (FAO, 1994).

Intensive farming methods through the use of high yielding varieties, chemical fertilizers and pesticides, irrigation and mechanization (the Green Revolution) has been responsible for

significant increases in grain production in some developing countries during the past three decades. In 1974, the oil crisis, shortage of mineral fertilizers and their high prices led to the global initiation of several research programmes on biological N_2 fixation in legumes. Since then, biological N_2 fixation is being regarded to be a practical, affordable substitute or supporting remedy to the improved use of industrially manufactured N fertilizers (Roy and Hardarson, 2001; Hardarson and Broughton, 2003).

2.6.2 Management of phosphorus

Plants require phosphorus to grow and P unlike others is an element that cannot be substituted and is therefore vital for food production (Steen, 1998). Nowadays, P is mostly obtained from mined rock phosphate and is often combined in mineral fertilizers with sulphuric acid, nitrogen, and potassium. Moreover, the fertilizer industry has recognized that the quality of reserves is declining and the cost of extraction, processing and shipping is increasing (EcoSanRes, 2003). The financial costs involved in the use of P fertilizers, will increase in the future as a result of unsustainable production of P fertilizers from commercially viable, but non-renewable, finite reserves of phosphate rock, which at current rates of use could be exhausted in the next 100–400 years (Johnston, 2008). Secondly, unstable energy prices, which will have an impact on the mining, transport, and spreading of phosphate rocks and fertilizers (Helsel, 1992), and thirdly, potential introduction of financial instruments associated with meeting climate change, and other soil management targets (Hammond *et al.*, 2009). Matching increased food demand with rapid population growth in the 20th century, guano and later rock phosphate were applied extensively to food crops (Smil, 2000). Given that these P sources may get exhausted with time, there is thus the need to explore alternative means of sustaining phosphorus availability for crop production.

One of the alternatives is to breed for P-efficient crop varieties that can be cultivated in low P soils.

2.7 The process of Biological Nitrogen Fixation (BNF)

Nitrogen is an essential element contained in many biomolecules necessary to sustain life (Smil, 2004). This element is abundantly available in earth's atmosphere in the form of dinitrogen (N_2) gas, yet most organisms are unable to metabolize N_2 because it is relatively inert (Jia and Quadrelli, 2014). Biological N_2 fixation (BNF) is the process whereby bacteria use the enzyme nitrogenase to convert atmospheric N_2 into ammonia (NH_3), a form of nitrogen (N) that can then be incorporated into organic components, e.g. protein and nucleic acids, of the bacteria and associated plants. This bacteria–legume association is a type of symbiosis that evolved some 60 million years ago and is an archetypal example of a mono specific association (Bonfante, 2003; Hirsch, 2004). The N fixation process requires a lot of energy (adenosine triphosphate or ATP) to break the triple bond that exists between N atoms in N_2: N_2 + 8H+ 16 MgATP → (nitrogenase) → $2NH_3$ + 2H+ 16 Mg ADP + 16 Pi. Sixteen molecules of ATP are hydrolyzed for each molecule of N_2 reduced (Hoffman *et al.*, 2014). The huge amount of energy required for the nitrogen fixation process shows the importance of phosphorus (a component of ATP) in the process. Therefore, P efficient genotypes are favourable in low P conditions when selecting for enhanced nitrogen fixation. Morevover, reduced dependence on N fertilizer and attention to farming practices that favor the more economically viable and environmentally prudent N_2 fixation will benefit both agriculture and the environment.

Symbiotic N_2 fixation is achieved by bacteria inside the cells of *de novo* formed organs, the nodules, which usually develop on roots of various leguminous plants. This process results from the complex interaction (Udvardi and Poole, 2013; Oldroyd *et al*., 2011) between the host plant

and rhizobia (used as a colloquial reference to *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium*). The nodulation process in the rhizobia-legume symbiosis is very complex and involves distinct parts. First, plants secret root exudates containing flavonoids that induce *nod* genes in rhizobia to mediate the nodulation process. Secondly, the product of the regulatory rhizobial *nod* gene induced transcription of rhizobial structural *nod*, *nol* and *noc* genes which code for extracellular lipochitooligosaccharides, protein Nod O, and other extracellular Nod factors (Vlassak and Vanderleyen, 1997). Lipo-chitooligosaccharide nodulation factors produced by rhizobia determine the host range. Thirdly, endogenous plant hormones, as well as factors produced by rhizobia, are important mediators of nodule initiation (Van Rhijn and Vanderleyen, 1995; Vlassak and Vanderleyen, 1997).

The process of legume BNF is complex and affected by environmental conditions such as soil N concentration, plant physiological growth stage, water content, root zone pH, plant nutrient status including carbon and nitrogen substrates in roots, genetic variability in N fixation capacity and energy for fixation. Plant nutrients availability such as phosphorus (P) and potassium (K) that control nodule growth and nitrogenase activity affect BNF directly or indirectly (Havelka *et al*., 1982). When plant-available mineral N in the soil is limiting but water and other nutrients are plentiful, the rate of N_2 fixation tends to reach the maximum. Plants have an effective feedback mechanism on N_2 fixation whereby rates decline progressively with increasing availability of mineral N. Since most farmers' fields are dominated by cereals that utilise large quantities of soil mineral N, high rates of N_2 fixation are commonly achieved. The literature reveals that soil mineral N in the root zone inhibits legume nodulation (Abdel Wahab *et al.,* 1996; Herridge *et al*., 1984), nodule establishment (Imsande, 1986) and nitrogenase activity (Purcell and Sinclair, 1990; Eaglesham, 1989) since less energy is required for legumes to take up N from soil than fix

N biologically from the atmosphere (Cannell and Thornley, 2000; Phillips, 1980; Wood, 1996). It has been reported that a certain concentration of mineral N in the root zone, defined as "starter N", stimulates nodule establishment and N_2 fixation compared to non-mineral N in some circumstances. The physiological growth stages of plants affect the rate of legume BNF. Low rates of BNF are experienced in the early growth stages while nodules are establishing (Lawrie and Wheeler, 1973) and a maximum value is reached between early flowering and early seed-filling, depending on the species and growing conditions (Lawn and Brun, 1974; Klucas, 1974; Nelson *et al*., 1984; Jensen, 1987). From this point, N_2 fixation decreases dramatically or even ceases during seed-filling (Herridge and Pate, 1977; Beverly and Jarrell, 1984; Sinclair *et al.*, 1987). This is explained by the fact that carbon supply becomes limited and nodule senesce (Lawrie and Wheeler, 1973) as a result of the strong demand for seed dry matter accumulation (Herridge and Pate, 1977; Voisin *et al.*, 2003, 2007). Therefore, for the same species, the amount of N fixed may vary depending on the stage at which the plants were sampled.

2.8 Estimation of N_2 fixation.

Many techniques are available for measuring legume BNF in the field as well as in controlled environments (Herridge *et al*., 2008; Carlsson and Huss-Danell, 2008). The implementation of most of these methods involves destructive sampling of plants. In addition, the original soil structure is also disturbed. The only exception amongst the destructive methods is the acetylene (C_2H_2) reduction assay (Carlsson and Huss-Danell, 2008). The rate of potential N fixation at a single plant level can be estimated either by plant N demand and uptake (Bouniols *et al.*, 1991; Cabelguenne *et al*., 1999), nodule biomass (Boote *et al*., 2002; Wu and McGechan, 1999), root biomass (Thornley *et al*., 1995; Soussana *et al*., 2002) or aboveground biomass (Sinclair, 1986;

Robertson *et al.*, 2002). The choice of methods and traits to be measured depends on several factors and the objective of the study.

Ideally, the objectives of the study should determine the appropriate measurement method. N_2 fixation may be quantified in order (a) to determine if a particular plant has the capability to fix N_2, (b) to determine if management practices affect N_2 fixation, (c) to determine the amount of N-fixed by a field crop or pasture and (d) to determine the importance of N_2 fixation to the functioning of an ecosystem. Generally, if the objective is simply to assess if a particular legume is fixing N_2, then the presence of effective root nodules will provide practical insight. On the other hand, in cases where the main objective is to compare relative differences between genotypes, species or treatments, rather than to estimate absolute amounts of N-fixed, techniques that enable ranking of different crop varieties or treatments should be used (Unkovich *et al.*, 2008).

In measuring the amount of N fixed, accuracy and precision are very important. However, it is possible to know the true value of N_2 fixation only under defined conditions when the organism in question either fixes no N_2 or is totally dependent upon N_2 fixation for growth. Thus, plants grown with high mineral N supply and without an association with N_2-fixing bacteria must take up all N from the soil and have no (0%) contribution from N_2 fixation. On the other hand, plants growing with their competent N_2-fixing bacteria in the absence of any other plant-available sources of N will derive all N (100%) from N_2 fixation, except for a very small amount in the sown seed. These conditions should be borne in mind when applying some of the most acceptable methods to quantify N fixation (Unkovich *et al.*, 2008).

The N balance method , N difference, N yield, N isotope (^{15}N), acetylene (C_2H_2) reduction and xylem solute (ureide) methods have all been used to quantify N_2 fixation in selection and

breeding programs (Unkovich *et al*., 2008).. Although there is no single best method for measuring legume N_2 fixation, the limitation of each method determines it's appropriateness for large-scale plant selection and breeding. The use of the C_2H_2 reduction assay is arguably the most contentious. It was the method of choice in the early 1980s and was supplanted by the ^{15}N techniques during the latter half of the 1980s and throughout the 1990s. Consistently, legume dry matter and N yield have served as measures of N_2 fixation. Generally, the methodologies fall into three broad approaches (Unkovich *et al*., 2008). The first methodologies estimate N_2 fixation as the net increase in total N of a plant–soil system (N balance method). The second aims to separate plant N into the fraction taken up from the soil and the fraction derived from the N_2 fixation (N difference, ^{15}N natural abundance, ^{15}N isotope dilution and ureide methods). The third measures the activity of nitrogenase, the enzyme responsible for N_2 fixation (acetylene reduction and hydrogen evolution methods).

2.8.1 Nitrogen balance method

The Principles behind the method is that if all possible external inputs, except N_2 fixation, and outflows of N can be accounted for and incremental changes in soil N quantified, a net positive N balance in the system under study may be attributed to N_2 fixation. This method has the advantage of being simple (Unkovich et al., 2008) However, failure to include estimates of N losses through natural processes will result in an underestimation of N_2 fixation inputs. Since this method relies on many independent and unrelated measurements, each made with a differing degree of accuracy, the confidence in the final estimate of N_2 fixation can be low. Consequently, the use of N balance tends to be restricted to long-term experiments where land use is dominated by the N_2-fixing association of interest. Thus, it may be appropriate to perennial species in agroforestry and/or pasture systems than to annual crops.

2.8.2 Nitrogen difference method

The N difference compares total N of the N_2-fixing species with that of neighboring non N_2-fixing species, with the difference between the two measures assumed to be due to N_2 fixation. Here the assumption is that the N accumulated by the non N_2-fixing control is derived only from soil N, and its N content represents the amount of soil mineral N available for plant growth secondly, the N_2-fixing plants use the same amount of soil mineral N as the non N_2-fixing control. This method is a simple, low-cost method that can be applied when facilities for only dry matter determinations and total N analyses are available. The limitations come from the fact that the method requires a non N_2-fixing control to be included in the experimental design. Errors may occur in accurately quantifying total N accumulated by the N_2-fixing plants and control plants. Considering the limitations, the technique is likely to be most reliable under conditions of low plant available N and where large differences in N yield between the N_2-fixing plants and non N_2-fixing control exist (Unkovich et al., 2008).

2.8.3 Nitrogen yield method

The N yield is the simplest estimate of N_2 fixation. N fixed is quantified by measuring the amount of N in the legume biomass. This method assumes that the legume will derive most of its N from N_2 fixation. In almost all field-grown plants, values obtained will overestimate N_2 fixation because the contribution of soil N to plant growth is ignored (Unkovich et al., 2008). This method is appropriate in zero or low N soils, for assessing plant genotypes because differences in N_2 fixation are either fully expressed or largely expressed without the complication of soil N inputs. In moderate to high N soils this method is not reliable as the contribution of soil N to plant growth may effectively mask the differences in N_2 fixation. Bliss et al., (1991) reported that selection of breeding lines based on plant and/or seed N was most

effective and most cost-effective compared with other methods to quantify N_2 fixation. In their studies, these parameters were always highly correlated with N_2 fixation, determined using ^{15}N (St Clair et al., 1988). The only condition was that the plants were grown in a low N soil and that the plants were effectively nodulated.

2.8.4 ^{15}N isotope techniques

There are two main stable isotopes of N, ^{14}N and ^{15}N with ^{14}N naturally more abundant than ^{15}N. In absolute terms, the isotopic abundance of ^{15}N is usually expressed as a percentage of the total N (atom% ^{15}N). The isotope ^{15}N occurs in atmospheric N_2 at a constant abundance of 0.3663 atom% (Mariotti, 1983). The ^{15}N enriched and natural ^{15}N abundance methods are the reference methods for assessing legume N_2 fixation (Chalk, 1985; Danso *et al*., 1993). Their main advantage is that they provide time averaged estimates of N-fixed, integrated for the period of plant growth to the time of harvest. However, given the high cost of instrumentation to measure ^{15}N plus the expense of the ^{15}N-labelled materials their use is limited in breeding programs. These methods assume that the legume and non-fixing reference plants utilise soil mineral-N with the same isotopic composition (Unkovitch and Pate, 2000). This may not always occur and is a major weakness of the method (Witty *et al*., 1988; Unkovitch and Pate, 2000). Therefore, the choice of non- fixing reference plant is of utmost importance.

The principles behind all ^{15}N-isotope technique is that if the ^{15}N concentration in atmospheric N_2 differs significantly from that of plant-available soil N, and these values are known, it is possible to calculate N_2 fixation on the basis of ^{15}N analyses of the putative N_2-fixing plant and a non N_2-fixing plant. The assumptions here are that, firstly, either there is no discrimination or identical discrimination between ^{14}N and ^{15}N during the uptake and metabolism of plant-available soil N

and N_2, or the discrimination can be accounted for. Secondly, any variability in the ^{15}N composition of the air and soil is small compared to the difference between them.

^{15}N-based methodologies are considered as standards but their use is limited because of the high cost of a mass spectrometer, the technical skills required to accurately determine the isotopic composition of plant (and sometimes soil) samples and the expense of analyses (Unkovich *et al.*, 2008).

2.8.4 .1 ^{15} N enrichment method

With this method, the roots of intact or detached plants are placed in a chamber with an atmosphere enriched in ^{15} N. The amount of ^{15}N in the plant at the end of the incubation period is a direct measure of the rate of N_2 fixation. The assumptions of the method: exposure to ^{15} N is sufficiently long to allow adequate equilibration and gaseous exchange with nodules, and for measurable amounts of ^{15}N to be fixed; and rates of N_2 fixation under assay conditions are related to rates of N_2 fixation achieved in situ. This method has the advantage of being a direct measure of N_2 fixation. However, its application involves the use of high cost of ^{15} N and the instruments used to quantify ^{15}N. There may also be difficulties in keeping incubation systems completely sealed while maintaining adequate environmental conditions inside the chamber, e.g. temperature, oxygen level. Therefore, the ^{15} N enrichment method is generally used for short-term laboratory incubations and is much less suitable for field use (Unkovich *et al.,* 2008).

2.8.4.2 Natural ^{15} N_2 abundance method

The principles and limitations of the natural ^{15}N abundance method are quite different from the ^{15}N enrichment method. The method uses the natural enrichment of the soil mineral-N, necessitating measurement of much lower ^{15}N values. An isotope ratio mass spectrometer capable of accurately measuring differences of about 0.00004 atom% ^{15}N is needed and great

care is essential in sample preparation. The accuracy of the method will depend ultimately on the levels and uniformity of the ^{15}N in the soil. Levels of delta ^{15}N ($\delta^{15}N$) >6.0% are preferable, although values as low as 2% might still be useful, depending on the level of N fix (Unkovitch *et al.*, 1994). Because the $\delta^{15}N$ can be relatively constant with time and depth (Bergersen *et al*., 1990), the major limitation of ^{15}N enrichment, i.e., choice of appropriate non-fixing reference plant, is less critical.

None of the methods to measure BNF, used singly can be considered the best. All current methodologies have limitations, thus, measuring the exact amount of N-fixed remains a challenge. The ideal is therefore, the use of several different methods simultaneously, particularly if they are complementary. Under favorable conditions, the amount of N-fixed by legumes is primarily influenced by plant growth and dry matter production. Legumes generally fix 15–25 kg shoot N for every ton (t) of shoot dry matter accumulated, the average being about 20 kg shoot N per t shoot dry matter. In most cases just the total plant or shoot N is measured. Since below-ground N represents 25–50% of total plant N, the general rule becomes 30 kg total N-fixed per ton of shoot dry matter for most legumes (Unkovitch *et al.*, 2008).Therefore, the use of nitrogen fixation related traits that correlate strongly with nitrogen fixation and can easily be measured will enhance the breeding process for enhanced nitrogen fixation in crop legumes.

2.9 Breeding and Selection for BNF related traits

Crop legumes fix N_2 between 0–400 kg N/ha (Unkovich and Pate 2000). If adequate numbers of highly effective rhizobia are present in the soil, the amount of N_2 that the crop fixes can be accounted for by the growth of the legume, i.e. legume's N demand, and the proportion of legume N derived from N_2 fixation (*P*fix), the remaining legume N being derived from soil nitrate.

Several breeding methods have been used to improve N_2 fixation levels in legumes. Some of the methods include recurrent selection for enhanced nitrogen fixation (Barron *et al*., 1999); backcross-inbred methods for population development (Bliss, 1993); bidirectional selection for specific nodule enzymes (Degenhart *et al*., 1992). Through these methods breeders have succeeded in raising levels of N_2 fixation. Generally, some strategies for increasing legume N_2 fixation through breeding involve: 1) Enhancing symbiotic nitrate tolerance, the ability of the legume to nodulate and fix N_2 in the presence of soil nitrate and optimising legume nodulation through specific nodulation traits (e.g. mass and duration) (Bernal, 1993), 2) Depending on the circumstances, for promiscuous or selective nodulation and by selecting rhizobia for specific environmental niches (Bliss, 1993) e.g. acid tolerant strains for acidic soils (Howieson and Ewing, 1986; Hungria and Vargas, 2000).

According to Bliss (1991) effective selection for increased N_2 fixation in grain legumes depends on many factors among which is the choice of traits as selection criteria that can be measured precisely and economically, while providing for discrimination between superior and inferior selection units. Field trials should be carried out without application of large amounts of N fertilizer to facilitate selection and simulate farming conditions where low purchased inputs are common. Coale *et al*. (1995) selected common bean varieties for superior N_2 fixation using low N soil conditions and only seed yield and plant biomass N as selection criteria. They included the latter trait to distinguish between superior N_2 fixation levels and high harvest N index. Both Elisondo et al. (1999) and Hungria and Bohrer (2000) pointed to losses in N_2-fixing ability in breeding populations evaluated using seed yield or seed N alone as selection criteria. Quantitatively inherited traits such as nodulation are greatly influenced by the environment and this is a serious constraint for BNF improvement (Bliss, 1985; McFerson, 1983). The other trait

used for BNF evaluation, nodule biomass seems also to be affected by the nutritional status of the plant, as a compensatory effect of increased nodule size may be due to a surplus of C-sources from shoot, or nutrients from the soil.

Cultivar variation in traits associated with N_2 fixation has been demonstrated in many legumes. However, not all of these cultivar differences would be of value in a breeding programme. Nodule number and mass are usually inversely related, with an ineffectively nodulated cultivar having many small nodules (Nutman, 1967). Nodule mass per plant is a more useful indicator of symbiotic potential. Nodulation and N_2 fixation in legumes are generally thought to be quantitatively inherited traits (Bliss, 1993). Therefore, traits used in the parental selection process need not necessarily be ones that can also be applied at a field scale for progeny selection.

Breeding for improved N_2 fixation has, to a large extent, not been successful Rose (2000). Thus, breeders are encouraged to conduct their entire programmes in low N soils to benefit from constant selection pressure for high N_2 fixation and against defective nodulation and N_2 fixation. Growing plants in low N soils and measuring dry matter and N yields is far simpler and has the added advantage that the effects of the nodulation traits on accumulation of fixed N_2 by the plant are integrated in yield. Provided that genetic variation existed in the breeding populations, then high N_2-fixing lines would be produced as a normal outcome (Herridge and Rose, 2000). Given the importance of P in the process of nitrogen fixation and the high cost and unavailability of P fertilizers, research on cowpea breeding has to target low P soils. Under low P conditions, cowpea varieties that are P efficient with high N_2-fixing capacity can easily be selected for use by resource poor farmers.

2.10 Nodulation and inoculation in grain legumes

For effective nodulation and nitrogen fixation, inoculation may not be always necessary. Legumes that require specific rhizobial strains to form an effective symbiosis are those that most often need inoculation. They may need to be inoculated only when they are grown in regions outside their centres of diversity, or where they have not traditionally been grown or have not been grown for a number of years (Brockwell *et al*., 1995). Soybean and ground nuts respond better to inoculation than cowpea. In a study to compare soybean and ground nuts with cowpea, nodulation, N uptake dry matter yield and BNF in soybean and groundnut were significantly higher in the control than in other treatments as compared to cowpea (Aliyu *et al.*, 2013). This could be due to competition between indigenous population and the inoculants which may culminate in antagonism. Cowpea is a very promiscuous legume host (Ahmad *et al*., 1981; Ranga Rao *et al.*, 1985) and Bradyrhizobium strains with which it can form effective nodules are normally present. Thus, some tropical legumes including cowpea have rarely been found to respond to inoculation unless they are grown in a soil where the conditions are not conducive for the survival of rhizobia (Giller, 2001). Introduction of new strains can be difficult and is often ineffective in soils with high ($>10^3$ Rhizobium bacteria g/m soil) naturalized rhizobial population (Thies *et al.*, 1991; Brockwell *et al.*, 1995). Generally in most soils, inoculant strains are disadvantaged. These strains have a limited gene pool for adapting to local conditions as opposed to the well adapted indigenous strains which can adapt to changes in soil conditions (Vlassak and Vanderleyden, 1997). It is therefore very important for inoculant rhizobia to have strong nodulation effectiveness for the host being grown as well as good adaptation to the relevant soil conditions.

2.11 Quantitative trait loci mapping in grain legume improvement

Several important traits in agriculture including yield and nitrogen fixation are controlled by many genes. Regions within the genome that contain genes associated with a particular quantitative trait (Collard *et al*., 2003) are known as quantitative trait loci (QTL).

In QTL mapping, mapping population are phenotypically evaluated (data collected for each trait) and genotyped. Based on individual DNA markers, the mapping population is split into two or three classes for dominant or co-dominant markers respectively. For each class, means and variances are calculated for each trait. After statistical analysis, if a significant difference exists between the trait means of individuals within each marker class, it suggests a relationship between the marker and the trait. That particular marker is then considered to be linked to a QTL (Young, 1996).

2.11.1 Genetic markers

Genetic markers are differences between individual organisms or species. There are three major types of genetic markers: (1) morphological (also 'classical' or 'visible') markers which themselves are phenotypic traits or characters;(2) biochemical markers, which include allelic variants of enzymes called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA (Jones *et al*., 1997;Winter & Kahl, 1995). The major disadvantages of morphological and biochemical markers are that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant (Winter & Kahl, 1995). However, despite these limitations, plant breeders consider morphological and biochemical markers very useful (Eagles *et al.,* 2001; Weeden *et al*., 1994).

Since DNA based molecular markers were developed, the limitations associated with other types of markers like isozymes and morphological markers were overcome (Hillis *et al*., 1996;

Melchinger, 1999; Semagn e*t al*., 2006a). Molecular markers occur in great abundance, can be identified at any stage of plant development, and are highly polymorphic and well distributed throughout the entire genome (Tanksley, 1983; Bohn *et al*., 1999).

The development and use of biochemical-based analytical techniques and molecular marker technologies, such as restriction fragment length polymorphisms (RFLPs),random amplified polymorphic DNAs (RAPDs) , amplified fragment length polymorphisms (AFLPs), and microsatellites or simple sequence repeats (SSRs), have greatly facilitated the analysis of the structure of plant genomes and their evolution, including relationships among the *Legumioseae* , cowpea inclusive (Choi *et al.*, 2004; Yan *et al*., 2004; Gepts *et al*., 2005). Using RFLP analysis, Fatokun *et al.* (1993a) analyzed 18 *Vigna* species including five of the subgenus *Ceratotropis* to determine the taxonomic relationship between the subgenus *Ceratotropis* and other subgenera. The results supported the taxonomic separation of the Asian and Africa genera as proposed by Maréchal *et al.* (1978) and underscored the previously held viewpoint that Africa is the likely center of diversity for *Vigna*. In general, the placement of species and subspecies based upon molecular taxonomic procedures by Fatokun *et al.* (1993a) substantiated prior classifications based on classical taxonomic criteria, such as morphological and reproductive traits. Genetic variation in 23 accessions of five species within the subgenus *Ceratotropis* was subsequently reinvestigated by using RAPD analysis by Kaga *et al.* (1996a). The accessions were separated into two main groups differing by approximately 70% at the molecular level. Within each of the main groups, the accessions could be further divided into five subgroups whose composition were in complete agreement with their taxonomic species classifications. Similarly, Sonnante *et al.* (1996) examined isozyme variation between *V. unguiculata* and other species in the subgenus *Vigna* and showed that *V. unguiculata* was more closely related to *V. vexillata*, a member of the

subgenus *Plectotropis*, than to any other species belonging to section *Vigna*. Polymorphisms in 21 different enzyme systems were used by Pasquet (1999) to evaluate the relationship between 199 accessions of wild and cultivated cowpea differing in breeding system and growth characteristic (i.e., annual vs. perennial growth habit). Based on these allozyme data, perennial subspecies of cowpea (spp. *unguiculata* var. *unguiculata*) were shown to form a coherent group closely related to annual forms (ssp. *unguiculata* var. *spontanea*). Later, Ajibade *et al.* (2000) used inter simple sequence repeat (ISSR) DNA polymorphism analysis to study the genetic relationships among 18 *Vigna* species. Cultivated cowpea grouped closely with the wild subspecies of *V. unguiculata*, and the entire species was separated from its most closely allied species *V. triphylla* and *V. reticulata*. ISSR polymorphism analysis split *Vigna* into groupings that differed in their composition from previous classifications. Another marker type, repetitive DNA sequences have been shown to represent a substantial fraction of the nuclear genome of all higher plant species and to account for much of the variation in genomic DNA content observed among species (Flavell *et al.*, 1994).

Ba *et al.* (2004) used RAPD analysis to characterize genetic variation in domesticated cowpea and its wild progenitor, and their relationships. Their data support the single domestication hypothesis and further underscore the gap between wild and domesticated cowpea and the widespread introgression phenomena between wild and domesticated cowpea.

Single nucleotide polymorphism (SNPs) are modern markers and represent DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genome sequence is altered. SNPs can occur in coding (genes) and non-coding regions of the genome but have shown to be more commonly found in the intron regions (Rafalski, 2002a). Depending on their location in the genome and impact on expression of a gene, they are classified into non-coding SNPs, coding

SNPs, exonic SNPs, cDNA SNPs and candidate SNPs (Kahl *et al*., 2004). SNPs in non-coding and coding regions have been extensively studied in a few plant species such as *Arabidopsis*, maize and soybean (Ching *et al*., 2002; Jander *et al*., 2002; Zhu *et al*., 2003).

SNPs are important tools in the development of molecular markers for important genes, traits and biodiversity assessment in crop plants. They are bi-allelic, amenable to automation, mutationally stable and are scored as co-dominant markers making them ideal for genetic analysis and applications (Gupta and Prasad, 2001). SNP markers are increasingly having useful applications in crop improvement. They have a wide variety of applications such as genetic diversity and phylogenetic studies, association mapping, QTL mapping, high-throughput MAS, evolutionary biology and marker density increase (Rafalski, 2002b; Olsen, 2004; Lopez *et al.*, 2005; Rajesh and Muehlbauer, 2008). The current cowpea consensus genetic map was constructed with EST-SNP markers (Lucas *et al*., 2011). Thus, identifcation of markers linked to nitrogen fixation related traits after QTL mapping will be facilitated with the use of these EST-derived SNP markers.

2.11.2 Linkage mapping

Semagn *et al*. (2006b) have described the steps for the construction of a genetic linkage map. Firstly, the mapping population has to be developed and the sampling size determined. The parents for the mapping population should have sufficient variation for the trait of interest at both phenotypic and DNA sequence levels. Generally, a population size of 50-250 individuals are used in mapping, but to have high resolution mapping and a statistical power to detect linkage, a large population is required (Mohan *et al*., 1997); the second step is to decide on the type of molecular marker(s) to use for genotyping the mapping population; next is to screen the parents for marker polymorphism followed by genotyping the mapping population together with the

parents and finally the data is analyzed for linkage. In the linkage analysis, each marker is screened to test conformity to Mendelian segregation patterns, pair wise recombination frequencies are calculated between markers and the existence of linkage tested. Linkage groups are established, map distance estimated and map order determined. In linkage mapping the null hypothesis being tested is the absence of linkage between two markers whose recombination frequency (r) is 50% (H0: r = 0.5). Many statistical computer packages exist that aid in the analytical computation during linkage mapping (Cheema and Dicks, 2009). The most widely used include mapmaker (Lander *et al.*, 1987), GMendel (Echt *et al*., 1992), Linkage (Suiter *et al*., 1983), MapManager QTX (Manly *et al*., 2001) and JoinMap (Van Ooijen, 2006).

Linkage mapping in cowpea has progressed with marker technology to yield informative and increasingly dense genetic maps (Menendez *et al.,* 1997; Ouedraego *et al.,* 2002; Muchero *et al.,* 2009). It all started with an Illumina 1536 Golden Gate single nucleotide polymorphism (SNP) assay that was developed and implemented to map 928 expressed sequence tag (EST)-derived SNPs in cowpea (Muchero *et al*., 2009). This map represented a substantial improvement over a previous but population-specific cowpea map, which utilized 441 amplified fragment length polymorphism (AFLP),restriction fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD) markers (Ouedraego *et al.,* 2002). The 2009 cowpea SNP map contained 645 bins with 928 markers arranged on 11 linkage groups (680 cM) and was constructed by genotyping 632 individuals from six recombinant inbred line (RIL) populations. Recently, a consensus genetic map was constructed for cowpea (Lucas *et al.,* 2011). Both the 2009 and 2011 consensus maps are based entirely on EST-derived SNPs and all populations were genotyped for the same 1536 loci.

2.11.3 Quantitative trait loci linkage and analysis

Yield, drought, quality, nitrogen fixation and some forms of disease resistance are some of the traits of agricultural importance in plants controlled by many genes. These quantitative traits are most often difficult to measure (Paterson, 1996b) and markers offer a potential value in their diagnostics. According to Paterson (1996b), one of the examples of such traits is nematode resistance that requires one to dig up the plant to examine the damage on the roots. This is also true for nodulation traits that require uprooting of the whole plant and scoring or counting nodules.

QTL mapping implores a genetic linkage map and data from the phenotypic evaluation of the mapping population for the trait of interest. Therefore, QTL mapping involves three basic steps: (a) selection of appropriate genetic material (mapping population) and evaluation to generate phenotypic and genotypic data, (b) building a genetic linkage map and (c) detection of QTL and estimation of their number, location, effects and interactions. QTL analysis is based on the principle of detecting an association between trait values (phenotype) and the variants (genotypes) of a marker because genetic markers which tend to be transmitted together with a specific value of the trait are likely to be close to a QTL affecting that trait. Based on the presence and absence of a particular marker locus, the mapping population is partitioned into different genotypic groups (either two or three) and the existence of significant differences between groups for the trait being measured is determined (Tanksley, 1983; Collard *et al*., 2005). Depending on the marker system and type of population used, the observed significant differences between phenotypic means of the groups indicate that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait (Collard *et al*., 2005).

2.11.4 QTL detection methods

In QTL analysis, many methods are involved. They include single-marker analysis, simple interval mapping, composite interval mapping (CIM) (Tanksley, 1983; Liu, 1998) and multiple interval mapping (MIM) (Kao *et al.*, 1999). The Single-marker analysis (SMA) is the simplest method for detecting QTL associated with single markers. It uses the statistical procedures of t-tests, analysis of variance (ANOVA) and linear regression analysis. The coefficient of determination (R^2) obtained by linear regression explains the phenotypic variation arising from QTL linked to a marker. The limitations of SMA are: a) the further a QTL is from a marker, the less likely it will be detected. This is due to crossover events between marker and QTL, b) the magnitude of the effect of any detected QTL is likely to be underestimated, due to recombination between the marker and the QTL. To overcome these challenges, dense markers covering the entire genome (usually at intervals less than 15 cM) can be used (Tanksley, 1993). With the simple and composite interval methods, more than one marker is analyzed at a time. Both methods use linkage maps to analyze the interval between adjacent pairs of linked markers along the linkage group or chromosome simultaneously instead of one marker at a time (Lander and Botstein, 1989). The use of linked markers compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis (Falconer and Mackay, 1996). CIM on the other hand, combines interval mapping and linear regression by including additional markers in the statistical model in addition to adjacent pairs of linked markers for interval mapping (Jansen and Stam, 1994). In QTL mapping, the method used to detect QTL has to be chosen wisely. The interval mapping method is widely used for mapping of QTL in segregating generations derived from crosses between inbred lines. When dealing with linked QTL, CIM is more precise and effective at mapping QTL compared to single point

analysis and interval mapping. The Multiple interval mapping is even more powerful as it uses multiple marker intervals simultaneously to fit multiple putative QTL directly in the model for mapping QTL (Kao *et al*., 1999). The MIM model is based on Cockerham's model for interpreting genetic parameters and the method of maximum likelihood for estimating genetic parameters. MIM has the possibility to estimate and analyze epistasis between QTL, genotypic values of individuals, and heritabilities of quantitative traits (Kao *et al.*, 1999).

QTL analysis is done using various softwares, for example QGene and MapManager QTX/QTL are commonly used computer programmes to perform single marker analysis and simple interval mapping (Nelson, 1997; Manly *et al*., 2001) while QTL Cartographer, MapManager QTX and PLABQTL are used for CIM (Utz and Melchinger, 1999; Basten *et al*., 2001; Manly *et al*., 2001). MapQTL (Van Ooijen, 2004) is software which uses interval mapping and is designed to accommodate population types from cross pollinated crops. QTLnetwork that uses MIM is good at estimating epistasis and QTL by environment effects (Yang *et al*., 2005).

2.11.5 Epistasis and QTL by environment interactions

After QTL detection and classification they can be categorized according to the stability of their effects across environmental conditions. A ‘‘constitutive’’ QTL is consistently detected across most environments, while an ‘‘adaptive’’ QTL is detected only in specific environmental conditions or increases in expression with the level of an environmental factor .The presence and magnitude of these adaptive QTL, therefore, vary greatly between experiments, while the sensitivity of the same trait to environmental conditions can be underscored by stable QTL across a range of environments. The sensitivity to environmental conditions may be due to the responsiveness of regulation (e.g. transcription) of the QTLgene to an environmental cue. Alternatively, differences in response may have an indirect cause (e.g. genotypes with larger root

systems will be less affected by water shortage or nutrient deficit, so genes controlling root development may underpin QTL defined by abscisic acid (ABA) content, stomatal conductance, or biomass accumulation (Reynolds and Tuberosa, 2008).

In order to obtain reproducible data and effectively assess the value of a trait, it is important to consider the environment dependence of QTL expression. This aspect is particularly relevant for stress tolerance traits, which are themselves influenced by the intensity of the stress. In addition to environment-dependent expression, other factors are likely to hamper the utilization of QTL for genetic improvement. A desirable QTL allele discovered in non-elite genetic material may not offer any improvement, because the allele may already be ubiquitous in current varieties. Additionally, the effects of the positive allele may not be transferable to elite backgrounds due to unfavorable epistatic interactions (Podlich *et al.*, 2004).

Understanding how interactions among set of genes affect diverse phenotypes is having a greater impact on agriculture and evolutionary biology. Mapping and characterizing the isolated effects of single quantitative trait locus (QTL) is a first step, but there is also the need to assemble networks of QTL and define non-additive interactions (epistasis) together with a host of potential environmental modulators. In contrast to Mendelian traits controlled by individual genes, the phenotypic variations of complex traits result from the segregation of alleles at multiple quantitative trait loci (QTL) with effects sensitive to genetic and environmental factors (Mackay, 2001). Understanding the genetic architecture of complex traits is a major challenge in the post-genomic era, especially for QTL-by-QTL interactions (epistasis), QTL-by environment (QE) interactions, epistasis-by-environment interactions and more complex higher order interactions. The term epistasis was primarily coined to describe the distortions of Mendelian segregation ratios that were due to one gene masking the effects of another in classical genetic

studies on qualitative variations such as coat color (i.e. the albino allele of tyrosinase masks the phenotypes of other loci such as brown and agouti; Carlborg and Haley, 2004). Intensive work on quantitative variation also provided evidence of epistatic interactions. Strong interactions between QTL have been observed in maize (Lukens and Doebley, 1999) and soybean (Lark *et al.*, 1995). In addition, QTL with minor or no individual effect can also be involved in epistatic interaction, a finding that is well documented for a number of physiological traits in Drosophila melanogaster (Montooth *et al*., 2003). With the advance of molecular marker techniques, fine-scale genetic and physical chromosomal maps of various organisms are now available. Using these maps, methods of mapping QTL in experimental populations have become pervasive if not even high throughput (Haley and Knott, 1992; Zeng, 1994). Methods have been developed for detecting QE interactions (Piepho, 2000) and for detecting epistasis among QTL (Kao *et al*., 1999; Ljungberg *et al*., 2004; Yi *et al*., 2003). Some of these methods are based on simultaneous scans to detect epistatic QTL that do not have individual effects (Ljungberg *et al.,* 2004). However, none of these methods has integrated QE interactions and epistasis into one mapping system. The qtlnetwork is a full-QTL package for modeling the genetic architecture of complex trait, which integrates the effects of multiple QTL, epistasis and QE interactions into one mapping system.

2.12 BNF related QTL mapped on grain legumes

In the legume family, only few genetic studies to detect natural variation and QTL for BNF and related traits have been performed so far. Most of these studies focused on QTL analyses of nodulation traits and shoot dry weight under nitrogen-fixing conditions. Preliminary studies about genomic regions controlling BNF in soybean have been performed (Tanya *et al*., 2005; Nicolas *et al*., 2006; Santos *et al*., 2006). There is also information about other legumes, such as

pea (Bourion *et al*., 2010) and common bean (*Phaseolus vulgaris* L.; Nodri *et al*., 1993; Tsai *et al.*, 1998b). These studies have demonstrated the potential of mapping QTL for BNF traits, but additional information is still required on other species like cowpea. Nodari *et al*., (1993) detected QTL of minor effects associated with nodulation traits (Nodule Number) in common bean using 70 individual plants from an F_2-mapping population. Twelve (12) QTL ($P < 0.05$) contributing between 9–18% of the phenotypic variation were identified. Souza *et al.* (2000) also identified QTL ($P < 0.01$) explaining 4–11% of Nodule Number variation in an F8:9 (recombinant inbred lines, RILs) population of common bean.

Nicolas *et al*. (2006) crossed two Brazilian soybean cultivars and used the F_2 population and 45 SSR to identify two genomic regions associated with nodule number (NN) and nodule dry weight (NDW). A total of 21 SSR loci were mapped with a LOD score of 3.0 and a maximum Haldane distance of 50 cM, and were distributed in nine linkage groups with coverage of 251.2 cM. The contribution of putative QTL was 7.1 and 10%, respectively. The low level of variation of these QTL is consistent with the quantitative nature of BNF and nodulation inheritance in soybean. Six marker loci were located on three molecular linkage groups (MLG): MLG B2 (Satt066), MLG H/J (Satt192), MLG B1 (Satt197, Satt251 and Satt509) and another one including Sat_123 (MLG B1). Shoot dry weight (SDW) and NDW were significantly associated at Satt197, while NDW and NDW/NN were associated at Sat_123 marker loci. Six epistatic interactions among non-linked QTL for SDW were also revealed. The NN and NDW explained up to 15 % of the trait variation.

Using an F_2-derived recombinant inbred line (RIL) population, Tanya *et al*. (2005) found various QTL for NN, NDW, SDW as well as for acetylene reduction activity. Recently, a study in pea identified nine QTL for NN, four for NDW and eight for nodule area in a RIL population derived

from parents which are contrasting for root and nodule traits (Bourion *et al.* 2010). In common bean, Nodari *et al*. (1993) were the first to report on the identification of four QTL for NN in the RIL population of a cross between BAT-93 and JaloEEP558, with the QTL together accounting for 50 % of the phenotypic variation. This same RIL population was used to detect QTL for NN under low and high mineral nitrogen (N) fertilization (Tsai *et al.*, 1998a; Souza *et al*., 2000). In a nut shell, important advances have been achieved by the characterization and mapping of QTL in soybean and other crops. However, genetic studies on BNF in cowpea and other legumes are sparse, despite the need for genetic information on BNF in breeding. There has been little progress toward the development of markers linked to QTL useful in the selection of agronomic characteristics in cowpea. Progress has been faster in other related legumes (such as common bean and soybean) and it is possible that some of this information may be leveraged since there is a significant degree of synteny between these legumes and cowpea genomes.

2.13 Estimation of genetic effects in quantitative trait expression

The genetics of agricultural traits can be studied based on several biometrical tools. These tools depend on different mating designs such as diallel, line x tester, North Carolina design and biparental crosses. Among the tools, generation mean analysis (GMA) has been the most powerful biometrical analysis since it gives additional information about the epistatic interactions. GMA (Mather & Jinks, 1982) is a useful technique that provides the estimation of main genetic effects (additive, dominance and their digenic interactions) involved in the expression of quantitative traits. This analysis estimates the component variance which provides information about the predominant type of gene action for the important characters of crop species. The presence or absence of epistasis can be detected by generation means analysis using the scaling test, which measures epistasis accurately whether it is complimentary or duplicate at

the digenic level (Sharmila *et al.*, 2007). Information is gathered from the means of six generations that is, P_1, P_2, F_1, F_2, BC_1P_1 and BC_1P_2. Following the analysis, an effective breeding strategy can be formulated based on the outcomes. Information on gene effects allows breeders to know how much of the variation in a crop is genetic and to what extent this variation is heritable. This in turn influences the efficiency of selection that mainly depends on additive genetic variance, environmental factors and interaction between genotype and environment.

Symbiotic N_2 fixation depends on the interaction among the host plant, microbial symbiont and environment. Both partners of the association are subject to genetic variation. While considerable effort has been expended on study of the bacterial aspects of the symbiosis, surprisingly little has been expended on the host plant component. Improving the nitrogen-fixing ability of cowpea through breeding requires (a) sufficient genetic variation, (b) an understanding of genetic control of the traits, (c) techniques for measuring the traits, and (d) a breeding strategy to effectively use the variation (Hungria *et al*., 2003b).

Genes are located on chromosomes and represent the basic units of inheritance. Gene action can be defined as the way genes express themselves. Genes can control the expression of characters, individually or in combinations. Mendelian and biometrical genetic approaches are used to study the way genes express themselves. In Mendelian genetics, the dominant gene action refers to the expression of a character that appears in the F_1 generation; but recessive genes are not expressed in the presence of dominance in the F_1, but rather re-appear in the segregating F_2 generation (Welsh, 1981). However, quantitative genetics, splits gene action into additive and dominance variance and epistasis (Robinson *et al*., 1949; Falconer, 1981). Knowledge about the way genes act and interact will determine which breeding system can optimize gene action more efficiently

and will help elucidate the role of breeding systems in the evolution of crop plants (Hallauer and Miranda1988).

In the presence of additive gene action, characters of heterozygotes in the F_2 generations are the intermediate of the two parents, because additive variation is associated with the average effects of the particular alleles (Falconer, 1981). The additive portion, which is reflected in the narrow sense heritability, reflects the degree to which progenies are likely to resemble their parents. Plant breeders implore the knowledge of heritability in making decisions on which genetic improvement is possible through selection. Gene action is non-additive when the additive model cannot adequately explain the variation (Falconer, 1981). The size of dominance relative to the additive variance indicates the degree of dominance (Robinson *et al*., 1949). Therefore, levels of dominance, in relation to the mean of the parents of progenies spreads from partial to overdominance in the progenies.

Falconer, (1981) defines epistatic gene action as variation that cannot be explained on the basis of the additive-dominance model alone. Epistasis occurs when alleles at different loci interact. According to Falconer,(1981) there are three types of epistasis.

1) **Additive x Additive Interaction**: this occurs when two or more genes, each showing additive action individually, interacts with each other causing differential genotypic values as influenced by the genetic content of genes situated at other loci.
2) **Additive x Dominance**: This interaction occurs when two or more loci, one showing additive and the other dominance action, interact.
3) **Dominance x Dominance** interaction occurs when two or more loci, each showing dominance action individually, interact.

Studies indicate that the mode of gene action(Rosario *et al*., 1997) is additive for nodulation traits (number of primary root nodules, number of secondary root nodules, total number of root nodules, nodule score and nodule dry weight) and % Ndfa (percentage of nitrogen derived from atmostphere) in mungbean (*Vigna radiata* (L) Wilczek). If the ability to form a more efficient symbiotic relationship for nitrogen fixation is controlled by genes with additive or additive types of epistatic effects, then conventional plant breeding methods for cowpea could be used to increase biological nitrogen fixation. However, since there are other non-genetic factors that can influence the expression of a trait, the heritability of a trait becomes very important in breeding programmes.

2.14 Heritability and genetic advance of nitrogen fixation related traits

According to Fehr, (1987) heritability is the relative importance of genetic and non-genetic factors in the expression of phenotypic differences among genotypes in a population. There are two basic types of heritability: broad-sense heritability (h^2_b) and narrow-sense heritability (h^2_n; Nyquist, 1991; Holland *et al.,* 2003). The broad sense heritability is the proportion of the phenotypic variance of family means that is due to all genetic effects (Falconer and Mackay, 1996; Holland *et al*., 2003). Broad-sense heritability ($H = h^2_b = \sigma_G^2/ \sigma_P^2$) can be estimated from standard analysis of variances. The narrow sense heritability represents the proportion of phenotypic variance among individuals in a population that is due to heritable genetic effects (Nyquist, 1991, Holland *et al*., 2003). Narrow sense heritability ($h^2n = \sigma A^2/ \sigma_P^2$) can be estimated from variance components or from parent-offspring regression.

The heritability for nitrogen fixation traits is usually low to moderate as environmental and genotype x environment effects are large, especially when measured on a single plant basis (Bliss, 1993). The heritability values range from 0.22 to 0.76 and in many of the studies few

progeny performed better than the superior N_2-fixing parent (Graham *et al.*, 2004). However, this is not surprising if N_2 fixation is quantitatively inherited, and if parental selection is based mainly on nodule mass or overall N_2 fixation. This implies that in order to produce transgressive segregants with markedly superior N_2 fixation, genes contributing to nodule mass, activity and duration, will need to be pyramided, and approaches that parallel those used in yield enhancement will also have to be used. However, in the meantime the priority must be the development of markers with which superior nodulation or N_2 fixation can be dissected. To this point, there has been a surprising dearth of studies on marker-assisted selection for traits associated with nodulation and N_2 fixation in cowpea.

CHAPTER THREE

FARMERS' PERCEPTION ON THE ROLE OF LEGUMES IN SOIL FERTILITY AND COWPEA PREFERENCE IN CROPPING SYSTEMS.

3.1 Introduction

In west and central Africa, cowpea is the second most important grain legume after groundnuts (*Arachis hypogaea*; Sing *et al*., 2000). In Cameroon, cowpea is the third most important food legume after groundnuts and common beans (*Phaseolus vulgaris*) and it is frequently intercropped with cereals (Taffouo *et al*., 2004). Besides its use as food and feed, an important beneficial attribute of this legume is its contribution to the soil nitrogen status through symbiotic nitrogen fixation, thereby enhancing soil fertility. Hence it reduces the need for N-fertilizer application (Martins *et al*., 2003). The importance of grain legumes as green manure in enhancing soil fertility has been demonstrated, but these practices and adoption are still limited in sub-Saharan Africa (Sanginga, 2003). However, grain-legume cereal rotation are more attractive to the farmers both for food and cash (Giller and Wilson, 1991). In the case of the HFZ of Cameroon, cowpea and soybean are increasingly being used in cropping systems (Wendt and Atemkeng 2004). Soil fertility in the HFZ of Cameroon is low due to the old nature of the landscape.

Even though the decline in soil fertility is a major concern for most farmers (Turton *et al*., 1995), their adoption of improved techniques has been limited (Shrestha *et al.*, 2000). This poor adoption may be due to inadequacies in the agricultural extension system, but an important factor may be the different ways that farmers, extension workers and researchers all perceive and assess soil fertility, leading to differences in the problems perceived and solutions required. With an increase in the use of participatory research approaches, it is becoming clear that farmers have a

well-developed ability to perceive differences in the level of fertility on their farms. Thus, there is a strong need to compare the indicators used by farmers with those used by researchers.

Farmers have developed various techniques that can be used to detect the decline in soil fertility, through their long experience of agricultural practices. The soil fertility decline indicators used by farmers includes: change in weed biomass and species; changes in soil color, and thickness; reduced growth and color changes of crops and low crop yields in climatically good seasons (Elias, 2002). Participatory rural appraisal (PRA) techniques have been employed widely to assess farmer's perception on soil quality, soil fertility problems and indigenous soil fertility management practices (Erkossa *et al.*, 2004; Elias, 2002; Desbiez *et al.*, 2004).

Despite, the importance of farmers' perception on soil fertility improvement, no such study has been conducted in the HFZ of Cameroon on the role of legumes in improving soil fertility. Thus, recent and adequate information is lacking on farmers' perception on the role of grain legumes in improving soil fertility in cropping systems, and the farmer preferred cowpea traits.

The objectives of the study were to:

1) assess farmers' perception on soil fertility and associated problems and indigenous soil fertility management practices;

2) assess farmers' knowledge on the role of grain legume in improving soil fertility in cropping systems;

3) identify farmers' preferred cowpea traits in the HFZ of Cameroon.

3.2. Materials and Methods

3.2.1 Study site description

The study was conducted at five sites (Asso'oseng, Nkoemnvone, Nkolfoulou, Nkoemetou II and Nkometou III) in the HFZ of Cameroon. Nkolfoulou (3°56'N, 11°35'E, Nkometou II (4° 08'N, 11° 55'E) and Nkometou III (3°62'N, 11°15'E) are villages in the Center region while Nkoemvone (2°90'N 11°20 'E) and Asso'oseng (02°49'N, 11°08'E) are in the South region of Cameroon (Fig 3.1). The five villages were selected by the convenience sampling method (Saunders *et al*., 1997) as representative of cowpea growing areas of the HFZ of Cameroon, and also representative of soils with contrasting physicochemical properties. The climate of the sites is subtropical, with a bimodal rainfall distribution. The humid seasons are September – November (heaviest rainfall) and April - May, and the dryer seasons are December - March and June – August. Rainfall ranges from 1500 to 1800mm annually (Tiki-Manga and Weise, 1995) in this zone and this high precipitation is responsible for leaching of soil nutrients especially nitrogen. The mean annual temperature is 25^{o}C. The climate gradually changes with elevation. The vegetation of the study site is a humid tropical forest. The population density is less than 10 inhabitants km^{-2} (Kanmegne, 2004)

The major ethnic groups are Ntoumou, Mvae, Beti (Ewondo, etone) and Bulu. Agriculture is the main activity of the population while hunting and fishing is also practiced. The major land uses in the traditional farming system of the study sites are the *essep*, banana farm, *afup owondo*, and cocoa or coffee plantation (Kanmegne, 2004).

Figure 3. 1: Map of Cameroon showing the study sites.

3.2.2 Questionnaire description and data collection

This study was conducted with the aid of agricultural extension workers, a socio-economist and village heads. Sampling was designed to maximize on spatial coverage in order to capture variability in socio-economic and agro-ecological circumstances that span the study site. A two stage stratified sampling procedure was applied. In the first stage, each study site formed a sampling stratum. In each site, two focus groups were constructed. The groups included both women and men of various ages. Focus group discussions with 6-10 farmers per group were carried out during periods when the farmers are less busy in their farms (December and January). In the second stage semi-structured questionnaires were administered (January – March 2013) after the focus group discussion (FGD) to a total of 165 farmers. The questionnaires were administered by two agricultural extension workers, two technicians and a village head with good knowledge of the local language and English. Respondents were purposefully selected on basis of having cultivated cowpea or a grain legume in the past or presently cultivating cowpea.

Each respondent represented household heads, or in their absence, household members responsible for the farm management. A total of 44 respondents were interviewed in Asso'oseng, 35 in Nkoemvone, 17 in Nkolfoulou II, 38 in Nkometou II, and 31 in Nkometou III making a total of 165 respondents. The main questions focused on were: source of cowpea seeds; number of cowpea varieties cultivated; utilization of cowpea grown; preferred varieties; criteria for selecting variety; cropping system practiced; cowpea production constraints; soil fertility diagnosis method; indicators of declining soil fertility; soil fertility amendments strategies; role of legumes in cropping systems; perceptions on legume nodulation; seasons and soils with enhanced plant nodulation. Demographic questions included personal details such as gender, age, level of education, position in the house hold, and household size. Respondents were also asked about their major and subsidiary income sources.

3.2.3 Statistical analysis

Data collected was analyzed using Statistical Package for Social Scientist (SPSS) version 17.0 (SPSS, Inc., Chicago IL). Participatory rural appraisal (PRA) techniques such as mean, rank and frequency, were used to analyze the data.

3.3 Results

3.3.1 Social and economic characteristics of the respondents

Following the FGD a total of 165 questionnaires were assigned to farmers in the five villages. Forty four (44) respondents came from Asso'oseng, 35 from Nkoemvone, 17 from Nkolfoulou II, 38 form Nkometou II and 31 from Nkometou III (Table 3.1).

Table 3. 1: Distribution of respondents in the five study sites

Location	Frequency	Percent
Asso'oseng	44	26.7
Nkoemvone	35	21.2
Nkolfoulou II	17	10.3
Nkometou II	38	23
Nkometou III	31	18.8
Total	165	100

The age of the respondents ranged from 18 to 70 years with the majority falling between 36-45 years, representing 58% of the respondents (Table 3.2). Seventy six percent of the respondents were females while 24% were males. The major activities carried out by the respondents included: hunting, driving, trading, civil service and farming. Farming was the main occupation involving 87% of the respondents (Figure 3.2).

Table 3. 2 : Age distribution of respondents

Age range	Frequency	Percent
[18-25]	11	6.7
[26-35]	29	17.6
[36-45]	58	35.2
[46-55]	36	21.8
[56-65]	22	13.3
[65+]	9	5.5

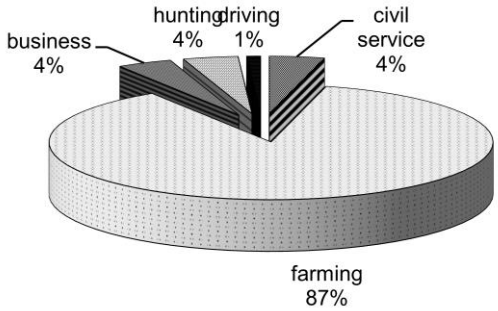


Figure 3. 2: Principal occupation of the respondents

3.3.2 Relative importance of crops grown

Farmers indicated that their important food crops grown in decreasing order were maize (*Zea mays* L.), cassava (*Manihot esculenta* Cranz), groundnuts (*Arachis hypogaea* L.), plantain (*Musa* spp), cowpea (*Vigna unguiculata L. Walp*), soybean (*Glycine max*) and cocoyams (Colocasia esculenta) as indicated on Figure 3.3. There were also minor crops observed on their fields like okra *(Abelmoschus esculentus), sw*eet potatoes [*Ipomoea batatas* L. (Lam.)], pepper (Capsicum annuum), and some vegetables.

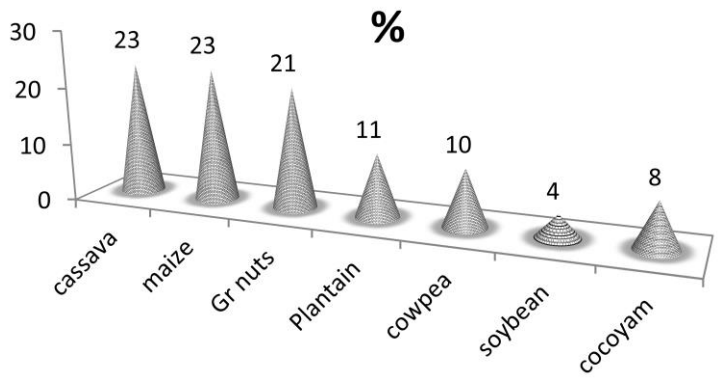


Figure 3. 3: Relatve importance of major crops grown in the study sites

Among the respondents, 88% were presently cultivating cowpea while 12% had cultivated cowpea in the past but later stopped. The reasons they gave for abandoning cowpea cultivation included infestation by pests and diseases, difficulty in harvesting, poor nature of their soils, low yields produced by the varieties they cultivated, lack of good seeds and transformation knowledge. Ranking these factors, farmers identified low yield as the major problem in Asso'oseng, Nkoemvone and Nkolfoulou II, while lack of transformation knowledge and

difficulty in harvesting, were the main reasons in Nkometou II and Nkometou III, respectively (Table 3.2).

3.3.3 Farmer preferred cowpea traits

Generally the farmers grew four varieties of cowpea: brown, black, speckled and white. White was the dominant (75%) and preferred variety. They also grew and preferred mostly the erect and early maturing cowpea type (94%). In two of the locations in the survey area, the local name for cowpea varies. Cowpea is generally called "koki" in Asso'oseng, Nkoemvone, and Nkometou II, while known as Ekoki and wak in Nkometou II and Nkolfoulou II, respectively (Table 3.4). The seeds cultivated also came from various sources including the market, other farmers and research institutions like the Institute of Agricultural Research for Development (IRAD) and the International Institute of Tropical Agriculture (IITA) as indicated in Table 3.4.

Table 3. 3: Ranks of reasons for abandoning cowpea cultivation in the study area.

Factors	**Location**				
	Asso'oseng	Nkoemvone	Nkolfoulou II	Nkometou II	Nkometou III
Pest and diseases	3	2	2	*	2
Difficulty in harvesting	2	4	3	2	1
Poor soil	*	3	*	*	*
Low yield	1	1	1	*	3
Lack of seeds	4	*	*	*	*
Lack of transformation knowledge	*	*	*	1	*

*not a factor; 1 = most important; 4 = Least important

3.3.4 Cowpea cropping system and production constraints

Cowpea was mostly intercropped (69%) with cereals and other crops while a minor proportion of the farmers practised sole cropping (29%) and rotation or shifting cultivation (2%). In cultivating

cowpea, these respondents faced production constraints including, poor soils, low yields, pest and diseases, weeds that compete with their crops, harvest difficulty, lack of seeds and lack of fertilizer (Figure 3.4). Among these constraints, poor soils (30%) was the most important, followed by low yields (29%) and pests and diseases (23%).

Table 3. 4: Local names, types of cowpea cultivated and source of seeds.

Location	Local name for cowpea		Cowpea cultivated		Source of cowpea seeds	
	Name	Percentage of respondents	Variety	Characteristics	Source	Percentage of respondents
Asso'oseng	Koki	56.8 (25)[a]	Lori-niebe	White seeds	Market	63.6 (28)
Nkoemvone	Koki	85.7(30)	Lori-Nebe	White seeds	IRAD	62.9 (22)
Nkolfoulou II	Wak	58.8 (10)	Dsch MMBr Dsch MMBl	Brown, Black	Market	70.6 (12)
Nkometou II	Koki	92.1(35)	MTA 22	Variegated	Other farmers	76.3 (29)
Nkometou III	Ekoki	74.2(23)	MTA 22, Lori-Nebe	Variegated, White seeds	IITA	96.8 (30)

[a] Numbers in brackets represent the number of respondents .

3.3.5 Fertilizer application and awareness on role of legumes in soil fertility improvement

The farmers identified poor soils as the main cowpea production constraints. Since they could not distinguish between soil physical properties like texture, it was considered that poor soils referred to infertile soils. To redress the problem of soil infertility, only 4.5% of the respondents in Nkoemvone did apply some kind of fertilizers .The maximum percentage (19.2%) of fertilizer users were registered in Nkometou II. Some of the farmer respondents were aware of the role of legumes in soil fertility improvement. On average, less that 30% of the respondents were aware except in Nkometou III where 50% of the farmers surveyed did have some knowledge on the role legumes play in soil fertility restoration. Interestingly, all the farmers were ready to cultivate grain legumes for soil fertility restoration purposes if this can be demonstrated on-farm. Most of

the farmers (69%) respondents had observed root nodules on their crops, especially ground nuts and soybean but very few (6%) knew the role these nodules play in soil fertility improvement. To most of the farmers (90%), the nodules represented organs that harbor disease agents, which they referred to as soil "cysts" and help to render the soil poor. They could not also distinguish between root knots caused by nematodes on tomatoes and root nodules of legumes.

3.3.6 Farmers' perception on soil fertility

According to the respondents, soil infertility is caused by soil "cysts", short fallow length, lack of fertilizers, and continuous cultivation which was ranked first by the respondents in all the villages (Table 3.5). Because of demographic pressure and other land use, the fallow length varied from village to village. Some farmers (37%) left their lands to fallow for only two years (Table 3.6) while others (15.2%) had fallow lengths of more than 5 years.

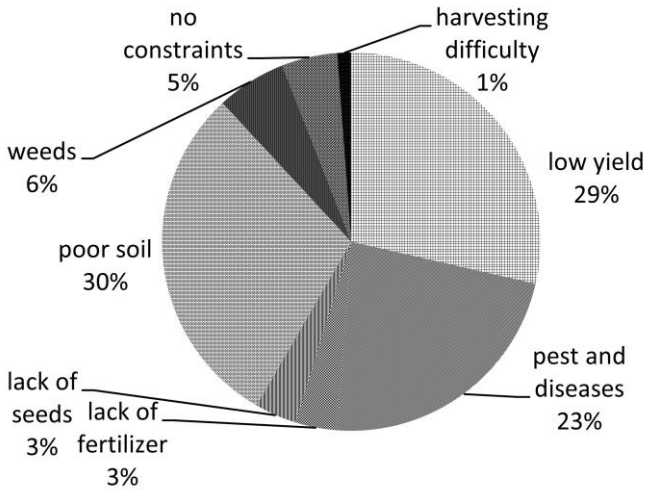


Figure 3.4: Cowpea production constraints in the study sites

Table 3. 5: Farmers' ranking of causes of soil infertility in the study area.

Factors	Location					Overall rank
	Asso'oseng	Nkoemvone	Nkolfoulou II	Nkometou II	Nkometou III	
Continuous production	1	1	1	1	1	1
Short fallow period	2	3	2	2	2	2
Lack of fertilization	4	4	3	4	4	4
Soil "cysts"	3	2	4	3	3	3

1 = most important; 4 = Least important

Table 3. 6: Lengths of fallow periods in the study areas.

Fallow length	Frequency	Percent
[1 year]	18	10.9
[2 years]	62	37.6
[3 years]	29	17.6
[4 years]	13	7.9
[5 years]	18	10.9
[5 years +]	25	15.2
Total	165	100.0

Most famers (83 %) could distinguish between fertile and infertile soils and had local names for the two types of soils (Table 3.7). Fertile and infertile soils were known as "Atek-si" and "Ayet-si", respectively, in two of the sites (Asso'oseng and Nkoemvone) and "Abeng-si" and "Eyat-si", respectively, in two other locations (Nkometou II and III). In Nkolfoulou II, the farmers termed fertile soil, "Kimye" and infertile soil, "komniket". Farmers used plant characteristics and soil properties as indicators of infertile soils (Figure 3.5). Forty three (43) percent of the respondents based their assessment on the hardness of the soil to identify infertile soil.

Table 3. 7: Local names in the beti language for the different soil types .

Location	Local name for soil type …….		
	Fertile soil	**Infertile soil**	**Percentage of respondents**
Asso'oseng	Atek-si	Ayet-si	88.6 (39)
Nkoemvone	Atek-si	Ayet-si	80 (28)
Nkolfoulou II	kimye	komniket	88.2(15)
Nkometou II	Abeng si	Eyat si	78.9 (30)
Nkometou III	Abeng si	Eyat si	87.1 (26)

The color of the soil and nature of the plants are also important indicators. According to 18% of the respondents, red soils were infertile and soils presenting thin plants were also considered infertile. To render infertile soil fertile again, most farmers (75 %) just left the land as natural fallow while very few (3%) planted legumes before allowing the land to fallow (Figure 3.6).

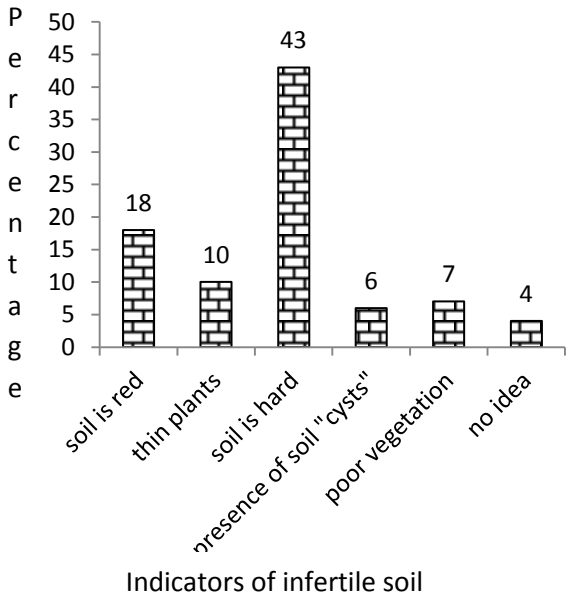


Figure 3. 5 Percentage response for indicators of infertile soil

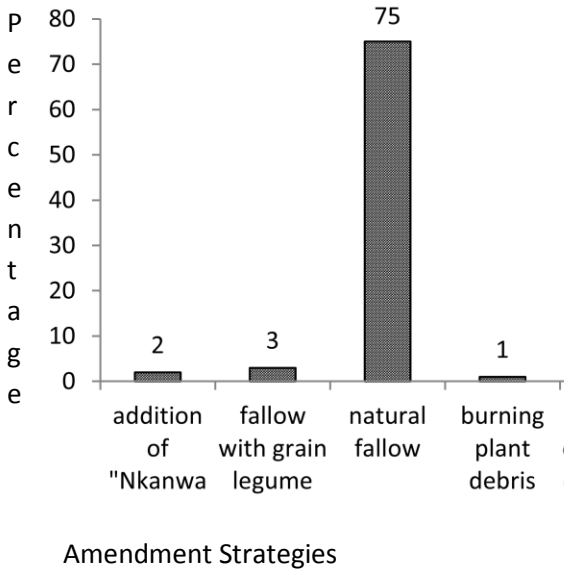


Figure 3.6 Percentage response for soil amendment strategies employed by respondent farmers.

3.4. Discussion

Some of the farmers in Nkoemvone and Nkometou III have been exposed to some training and knowledge on soil fertility management from researchers and extension workers from IRAD and IITA working in these benchmark villages. This exposure made it easier to work with and understand these farmers compared with those from Asso'oseng, Nkolfoulou II and Nkometou II who were very reserved at the beginning of the focus group discussion but later got interested. However, the peculiarity of Asso'oseng and Nkometou II was that the women have formed common initiative groups that worked towards a common goal. This makes it easier for them to adopt a new technology like the use of grain legume in soil fertility management improvement that was being introduced to them. Seventy six percent of the respondents were females. This is very normal as cowpea is not a cash crop and therefore is mostly grown by women for food. The main occupation involving 87% of the respondents was farming usually carried out as mixed cropping of different crop types including cereals, legumes, root and tubers. The farmers ranked cowpea in the fifth position and twelve percent (12%) had even abandoned the cultivation of cowpea. The reasons they gave centered around poor grain yields and other related factors. However, cowpea is a multi-purpose crop that can be used for food, as fodder, and to improve soil fertility due to its nitrogen fixation capacity (Timko *et al*., 2007). If these farmers were aware of the other uses of cowpea apart from grains, they could have gone in for other varieties or grow the poor yielding types and benefit from other parts of the plant than the grains. Difficulty in harvesting was also a major concern and the reason was because the farmers grew indeterminate and climbing cowpea varieties whose flower production and podding spread over time. According to the farmers, harvesting was difficult because they had to harvest the pods

many times from the same plant as they matured. They considered this process cumbersome and time consuming as opposed to groundnuts or soybean that is usually uprooted.

The results of the survey revealed that the farmers preferred erect and early maturing cowpea varieties that they could harvest just once comparable to how they harvest ground nuts and soybean.

The seeds also came from diverse sources including the market, other farmers and research institutions like the Institute of Agricultural Research for Development (IRAD) and the International Institute of Tropical Agriculture (IITA). This makes it difficult for the farmers to have good quality seeds since IITA had stopped working in these villages. The problem of lack of quality seeds was serious especially in the benckmark villages.

Cowpea was mostly intercropped (69%) with cereals and other crops. The farmers explained that they were bound to include a variety of crops on their field for security reasons such that if some failed, the remaining crops would be used to feed their families. In cultivating cowpea, the respondents faced production constraints including poor soils, low yields, pest and diseases, weeds that compete with their crops, harvesting difficulties, lack of seeds and fertilizer. Among these constraints, poor soil (30%) was the most important, followed by low yields (29%) and pests and diseases (23%). Low yields can be the consequences of poor soil as well as pest and disease manifestation. Therefore emphasis was laid on poor soils. With regards to the yields, low yields referred to cases where the farmer cannot harvest three buckets of cowpea from a field of about $200m^2$. The measuring buckets are usually 15 litres.

Participatory rural appraisal (PRA) techniques have previously been employed to assess farmers' perception on soil quality, soil fertility problems and indigenous soil fertility management practices (Elias, 2002; Desbiez *et al.*, 2004). The farmers identified poor soils as the main

cowpea production constraint but very few did apply fertilizers. In some cases only 4.5% of the respondents did applied some kind of fertilizers. Some of the farmer respondents were aware of the role of legumes in soil fertility improvement. On the average, less that 30% of the respondents were aware except in Nkometou III where 50% of the farmers surveyed did have some knowledge on the role legumes play in soil fertility restoration. Nkometou III being one of the benchmark villages of IITA, the farmers were well educated on soil fertility management.

Most respondents 69% had observed root nodules on their crops, especially ground nuts and soybean but very few (6%) knew the role these nodules play in soil fertility improvement. To most of the farmers (90%), the nodules represent organs that harbor disease agents, which they called soil "cysts" and help to render the soil poor. The farmers could not also distinguish between root knots (caused by nematodes) and root nodules and this could explain why some thought nodules did harbor disease causing agents leading to soil infertility. However, after observing fresh nodules on roots of the grain legumes they cultivateed and comparing with root knots on tomato roots, the farmers were convinced nodules have different functions and are ready to adopt high N_2-fixing cowpeas if the residual effect is demonstrated on their farms. According to the respondents, continuous cultivation was the main cause of soil infertility. This result contrasts the findings of Elias (2002) where farmers associated the decline in soil fertility with the dependence of the soil to fertilizer. However, since some farmers (37%) could leave their lands to fallow for two years and others (15.2%) for more than 5 years, other causes of soil infertility had to be looked into. It was interesting to notice that most respondent farmers (83%) could distinguish between fertile and infertile soils. The hardness of the soil was mostly used to assess soil fertility. Very hard soil, referred to infertile soil. Using soil colour, farmers reported that the fertility of red soil is lower than that of black soil. Other farmers suggested that when the

fertility of the soil is exhausted it changes its color from black to red. The results are in agreement with those of Desbiez et *al*. (2004) who reported that farmers in mid-hills of Nepal identified 62 indicators of soil fertility, which were divided into five major related classes i.e., soil characteristics, crop performance, agricultural management, environmental factors and biology. Most farmers reported that soil fertility and the amount of fertilizer used were declining. Along with those who did not totally apply fertilizer, the farmers whose fertilizer use was declining might have been discouraged by the various constraints of fertilizer use, of which high cost, and timely availability of fertilizer are the most important ones. Traditionally, to replenish soil fertility, most farmers (75 %) just left the land under natural fallow, while very few (3%) plant legumes before allowing the land to fallow. Cowpea has been reported to fix 61 - 201 kg N/ha in Ghana (Dakora *et al*., 1987; Belane and Dakora, 2009) and 122.0 kg N.ha–1 in Nigeria (Eaglesham *et al.*, 1981). Though the varieties used in the HFZ of Cameroon fix lower amounts of nitrogen ranging from 36 – 49 Kg N/ha (Jemo *et al.*, 2006), and farmers are gaining awareness on the role of legumes in soil fertility restoration and are willing to adopt cowpea, current breeding programs will incorporate the farmer preferred traits and high nitrogen fixation potentials in future varieties.

3.5 Conclusion

Farmers in the humid forest zone of Cameroon are aware of the soil fertility decline on their farms. They have indicators of poor and fertile soils. These farmers do not apply fertilizers but have other soil amendments strategies. Some respondents lacked knowledge on importance of legumes in cropping system and grew and preferred white coated cowpea with erect growth habit. The farmers were ready to cultivate grain legumes for soil fertility restoration purpose if this could be demonstrated on-farm.

3.6 Implications for research

Farmers need to increase the use of other alternative soil fertility management practices, such as crop rotation with legumes involved in biological nitrogen fixation. They should be encouraged and supported to use such alternative soil fertility management practices by training, research and development programs. Farmers should be encouraged to grow grain legumes expecially cowpea through on-farm research in all the villages to demonstrate the beneficial residual effects of these legumes to other crops like maize.

CHAPTER FOUR

ASSESSMENT OF INDIGENOUS *Bradyrhizobia* spp. POPULATION LEVELS IN THE HUMID FOREST ZONE OF CAMEROON.

4.1 Introduction

Microorganisms constitute a large portion of the biodiversity of soils (Fortin *et al.,* 2008) and their activities influence numerous important ecosystem processes, including nitrogen and carbon cycling (Fortin *et al.,* 2008; van der Heijden *et al.,* 2008), soil formation (Rillig and Mummey, 2006), plant nutrient acquisition and productivity (Dommergues *et al.,*1999, Sene *et al.,* 2010). Interactions between plants and microbes are particularly important since plants represent the main pathway through which elements that severely limit microbial growth, enter the soil (van der Heijden *et al.,* 2006, van der Heijden *et al*., 2008).

Among plant - microbe interactions, the legume – rhizobia symbiosis that converts nitrogen gas (N_2) into ammonia is probably the best studied (Mwend *et al.,* 2011). Legume-nodulating rhizobia play a great role in maintaining soil fertility (Kahindi *et al.,* 1997). However, effective nitrogen fixation in legumes depends on many factors (Voisin *et al.,* 2003, 2007) including the presence of effective and abundant rhizobia in the soil (Giller, 2001). These rhizobia can either be indigenous or applied as inoculum. Legume hosts differ in the range of partners with which they form symbioses. Some legumes nodulate with a restricted number of rhizobia strains or species, while others nodulate with a wide range of fast- and slow-growing rhizobia (Maingi *et al.,* 2006). In addition, factors such as high soil temperature (Giller, 2001), nutrient deficiencies (Beck and Munns, 1984; Watkin *et al.,* 1997; O'Hara, 2001), low levels of soil moisture (Boonkerd and Weaver, 1982), low pH (<5.5), low clay and organic matter (Dudeja and Khurana, 1989; De Mallaro and Izaguirre, 1994) adversely affect rhizobial survival.

Consequently, soils varying in their fertility status will respond differently to rhizobial inoculation.

On the other hand, legume hosts also differ in their response to inoculation. For instance, studies have shown that cowpea (*Vigna unguiculata* L. Walp) is a very promiscuous legume host as it has rarely been found to respond to inoculation unless when grown in a soil where the conditions are not conducive for the survival of rhizobia (Ahmad *et al.,* 1981; Ranga Rao *et al.,* 1985; Giller, 2001). Moreover, from a practical point of view, the use of inoculants is also cumbersome and difficult to exploit by farmers (Hornetz *et al.*, 2000; Kaleem, 2002) who face problems in acquiring and storing inoculants because cooling facilities are not readily available. As a result, cowpea crops as are grown by farmers in Africa receive no inoculants and little or no commercial nitrogen fertilizer. To reduce the need for inoculation, legume varieties that can be nodulated by indigenous rhizobia have to be selected for farmer use. As a prerequisite of this selection process, an assessment of the indigenous rhizobial population levels in low phosphorus soils of the HFZ of Cameroon using cowpea as trap crop was undertaken. The objective was to determine the population of cowpea-nodulating rhizobia in the humid forest zone of Cameroon;

4.2 Materials and Methods

4.2.1 Study site description

Soil samples used in this experiment were collected from two sites. The first site is located at the Institute of Agricultural Research for Development (IRAD) experimental station in Nkoemvone, in the South region of Cameroon. The second site belonging to a farmer is located at Nkometou, one of the benchmark villages of the International Institute of Tropical Agriculture (IITA) in the central region of Cameroon. Both regions are part of the humid forest zone of Cameroon. The agro-ecological characteristics of the two locations are presented in Table 4.1. The IRAD

experimental field at Nkoemvone has a past history of trials involving maize (*Zea mays* L.), cowpea and mucuna (*Mucuna pruriens* L.) rotation. The rotational trials ended in 2004 and since then the field has been cultivated to maize or left to fallow. The dominant species while on fallow was the elephant grass (*Pennisetum purpureum* Schumach). The farmer's field at Nkometou was left to fallow for 6 years with siam weed (*Chromolena odorata* (L.) R. King & H. Robinson) as dominant species; then cassava (*Manihot esculenta* Crantz) and groundnuts (*Arachis hypogaea* Linn.) cultivation followed for two years (March 2010 - December 2011); then maize for two seasons (April – July and September – December) in 2012. Soil samples were collected in December 2012 after the maize was harvested.

4.2.2 Soil sampling

Each study site was divided into 10 sub-sections based on the vegetation, topography and cropping and tillage practices (compact and non compact soils). Ten soil sub-samples were collected from each sub-section in the two study sites, Nkoemvone in the south and Nkometou in the center region. Prior to sampling, surface debris were removed. After clearance of debris from the surface, the soil core was taken up to 20 cm depth with a soil auger (Abaidoo *et al.*, 2002). The 10 samples from each sub-section were then bulked, homogenized, sieved (<2 mm), and divided into two parts. Two composite samples were then produced from bulked samples from all the 10 subsections. One composite sample was air-dried for chemical analyses in the biochemical laboratory of the International Institute of Tropical Agriculture (IITA) and the second was stored at 4 °C in clean paper bags for microbiological analyses.

Table 4. 1: Summary of Agro-ecological characteristics of the two locations.

Parameters	Nkometou	Nkoemvone
***AEZ**	Zone V	Zone V
Longitude	11°15' E	11º20' E
Latitude	3°62'N	2º90'N
Altitude	700(masl)	560(masl)
Climate	Equatorial	Equatorial
Annual Rainfal1	1600 mm	1692,2 mm
Rainfall pattern	Bimodal	Bimodal
Mean Temperatute Minimum	27°C	25°C
Mean Temperature Maximum	29.6°C	26°C
Long Dry months	Mid Dec-Mid Mar	Mid Dec-Mid Mar
Short Dry months	Mid Jun-Mid Aug	Mid Jun-Mid Aug
First Rainy season	Late March –Early June	Late March –Early June
Second Rainy season	Early Sept – Early Dec	Early Sept – Early Dec
Vegetation	Secondary Tropical Rainforest	Tropical RainForest
Soil type:		
FAO	Ferric Acrisols	Ferric Acrisols
USDA	Rhodic Kandiudult	Rhodic Kandiudult

*AEZ = Agro-ecological zones of Cameroon; Zone V = Humid forest with bimodal rainfall; masl = meters above sea level. Adapted from: ICRAF, 1993 and IRAD Nkoemvone, 2014.

4.2.3 Soil analysis

Soil samples were air-dried and ground to pass through a 2 mm mesh. Cations (Ca, Mg, K) were extracted by the Mehlich-3 procedure (Mehlich, 1984); cations were determined by absorption spectrophotometry. Available P was extracted by Bray-1 procedure and analyzed using the molybdate blue procedure described by Murphy and Riley (1962). Soil pH was determined in water at a 2:5 soil: liquid ratio. Organic carbon was determined by chromic acid digestion and spectrophotometry (Heanes, 1984). Total N was determined using the Kjeldahl method for digestion and ammonium electrode determination (Bremner and Tabatabai, 1972; Nelson and Sommers, 1972). Cation Exchange Capacity (CEC) was determined by saturation with 1 N ammonium acetate and extraction of ammonium with 2M potassium chloride (TSBF, 1993). Soil particle size analysis was done by the hydrometer method (Bouyoucos, 1951).

4.2.4 Sterilization and pre-germination of seeds

Seeds of cowpea varieties, Dsch MMBr, Vya niebe and 58-77 were used. Dsch MMBr and Vya niebe are local varieties grown in the western and Northern regions of Cameroon, respectively, while the variety 58-77 was provided by the Senegalese Agricultural Research Institute *(*ISRA*).* These varieties were chosen because they nodulated well in soils from both sites in a previous experiment (Atemkeng, unpublished data). Cowpea seeds were collected, pre-selected and purified in pots. Seeds of good viability (with a germination percentage higher than 80%), undamaged and of uniform colour and size were selected (Maingi *et al.,* 1999). One hundred seeds of each variety were surface sterilized by immersing them into a 3% solution of sodium hypochlorite for 5-10 minutes. The solution of sodium hypochlorite was prepared by adding 10 parts of commercial bleach (5.25% sodium hypochlorite) to 7.5 parts of water. The seeds were rinsed 8 times with sterile distilled water after surface sterilization. They were then soaked in

clean sterile distilled water and allowed to imbibe it for one hour. They were transferred aseptically to 2% water agar plates with a spoon-shaped spatula. Twenty five seeds were placed in each plate. The plates with the seeds were incubated upside down at 28°C to enable the radicles to grow away from the water agar. The incubation period was four days. Seedlings whose radicles attained a length of 1-2 cm after the incubation period were considered ready for transferring to glass tubes.

4.2.5 Plant growth medium and inoculation

The growth medium, used in this study was a mixture of sterile black soil and sand (3:1) sterilized in an autoclave at 121°C for 15 minutes. Five nitrogen-free stock solutions were prepared as described by Beck *et al.* (1993). For each litre of full-strength plant growth solution, 0.5 ml was added from each of the five stock solutions. The pH of the solution was adjusted to 6.8 using NaOH (1.0 M) or HCL (1.0 M). All solutions were sterilized by autoclaving at 121°C for 15 minutes. To prepare the inocula, 10 g of each soil sample was suspended in 90 ml of sterile water and then shaken for 15 to 20 minutes with a wrist shaker. Serial dilutions were made to give from 10^{-1} to 10^{-10}. An aliquot of 1 ml of diluent was used to inoculate pre-germinated, surface-disinfected cowpea seedlings grown in glass tubes. Four tubes were inoculated with each dilution. Uninoculated control plants were included for each dilution to determine if cross contamination of tubes occurred. The growth tubes in rack holders were transferred to a growth chamber in the Regional Biocontrol Laboratory at IRAD Yaounde, Cameroon where the temperature was maintained at 30°C with a 14-h photoperiod provided by fluorescent lighting. Sterile water was added as required through sterile straws. The racks were positioned 60 cm apart in the growth chamber in a completely randomized design. Application and regular

checking of levels of nitrogen–free nutrient solution was done on daily basis to ensure that the seedlings were adequately moistened.

4.2.6 Plant infection counts

The rhizobial populations in each soil were estimated using the Most Probable Number (MPN) technique as described by Somasegaran and Hoben (1994). Cowpea was used as the trap host to check for the abundance of indigenous cross nodulating *Bradyrhizobium* spp. Data were collected 8 weeks after planting. At harvest, the stems of the plants were cut at the level of the growth medium. The plants from the glass tubes were scored for the presence or absence of nodules. The roots were carefully washed using a gentle stream of water to remove sand, taking care not to destroy the roots and nodules. Plants were scored as positive or negative for nodulation and the number of nodulated (+) plant units was recorded beside each dilution. The presence of a single nodule in a tube was considered a positive score. The total number of nodulated units was obtained by summing up the nodulated units at each dilution level. Uninoculated controls were used to check for sterile conditions.

The MPN was calculated from the most likely number (m) obtained from the MPN tables according to the formula: $MPN = (m \times d)/v$

Where: m is the most likely number from MPN tables, d is the lowest dilution in the series and v is the aliquot used for inoculation (Somasegaran and Hoben, 1994).

4.3 Results

4.3.1 Soil characteristics

Results of soil analysis are reported in Table 4.2. Both soils showed low levels of P (7.51 ± 0.91 ppm); N (0.12 % ± 0.03) and had a pH water of 4.52 and 5.49 at Nkoemvone and Nkometou,

respectively. The soils were also characterized as clay at Nkoemvone, and sandy clay loam at Nkometou. The bulk density was 1.29 Mg/m^3 and field capacity 0.36 cm^3 water/cm^3 soil at Nkoemvone while at Nkometou, the bulk density and field capacity was 1.41.29 Mg/m^3 and 0.29 cm^3 water/cm^3, respectively (Table 4.3).

Table 4. 2: Soil chemical properties of Nkometou and Nkoemvone at 0-20cm.

	Site	
Characteristic	**Nkoemvone**	**Nkometou**
pH (water)	4.52	5.49
Ca cmol(+) /kg	0.58	1.92
Mg cmol(+) /kg	0.32	1.30
K cmol(+) /kg	0.18	0.13
Na cmol(+) /kg	0.28	0.29
Al cmol(+) /kg	2.33	0.40
CEC cmol(+) /kg	8.07	7.27
Available P ppm or ug/g	7.51	3.07
Mn ppm or ug/g	1.26	123.20
Fe ppm or ug/g	106.00	65.70
Org C %	1.73	1.69
Total N %	0.12	0.12
C/N	13.92	14.32

Table 4. 3: Soil physical properties of Nkometou and Nkoemvone 0-20cm.

Site	Sand (%)	Clay (%)	Silt (%)	Textural class	Bulk density (Mg/m^3)	Field capacity (cm^3 water/cm^3 soil)
Nkoemvone	42.3	46.4	11.4	clay	1.29	0.36
Nkometou	53.3	34.3	12.4	sandy clay loam	1.4	0.29

4.3.2 Soil rhizobia population

The estimated total *Bradyrhizobium* spp. population in Nkoemvone soil ranged between 1.0 x 10^5 and 5.8 × 10^5 cells per gram of soil sample (Table 4.4), while the population size at Nkometou was between 5.8 x 10^3 and 1.0 x 10^4 cells per gram of soil sample (Table 4.5). Using the cowpea variety Dsch MMBr as trap crop, the *Bradyrhizobium* spp population estimated was 5.8 x 10^3 and 1.0 x 10^5 cells per gram of soil sample, respectively, in Nkometou and Nkoemvone soils. The population estimate was the same in Nkometou but increased in Nkoemvone when the trap crop was changed to Vya niebe. A population of 5.8 x 10^3 and 3.1 x 10^5 cells per gram of soil sample was recorded in Nkometou and Nkoemvone, respectively. The cowpea variety, 58-77 formed more nodules more than the two other varieties in both soils.

Table 4. 4: Most probable number of rhizobia in Nkoemvone soil.

Serial Dilution	Number of nodulated units by each variety		
	.....		
	Dsch MMBr	**Vyu niebe**	**58-77**
10^{-1}	4	4	4
10^{-2}	4	4	4
10^{-3}	3	4	4
10^{-4}	3	3	3
10^{-5}	2	2	3
10^{-6}	2	2	2
10^{-7}	1	1	1
10^{-8}	0	1	1
10^{-9}	0	0	0
10^{-10}	0	0	0
control	0	0	0
Total	19	21	22
MPN (cells per gram of soil sample)	**1.0×10^5**	**3.1×10^5**	**5.8×10^5**

Number of replications, n = 4; dilution steps, s = 10; number of nodulated units, (+) = 19; 21; and 22. Lowest dilution in the series, d = 10^{-1}; v = 1ml.

Table 4. 5: Most probable number of rhizobia in Nkometou soil

Serial Dilution	Number of nodulated units by each variety		
	Dsch MMBr	**Vyu niebe**	**58-77**
10^{-1}	4	4	4
10^{-2}	3	4	4
10^{-3}	2	2	3
10^{-4}	2	2	2
10^{-5}	2	1	1
10^{-6}	1	1	1
10^{-7}	0	0	0
10^{-8}	0	0	0
10^{-9}	0	0	0
10^{-10}	0	0	0
control	0	0	0
Total	14	14	15
MPN (cells per gram of soil sample)	**5.8×10^3**	**5.8×10^3**	**1.0×10^4**

Number of replications, n = 4; dilution steps, s = 10; number of nodulated units, (+) = 14;14;15. lowest dilution in the series, d = 10^{-1} ; v = 1ml;

4.4 Discussion

The most probable number technique based on plant infection count is commonly used to estimate numbers of rhizobia in soil or to determine the quality of inoculants produced in sterile conditions (Beck *et al.*, 1993; Somasegaran and Hoben, 1994). Empirical models have been used to describe the response to inoculation of legumes (Thies *et al.*, 1991). These models indicate

that population density as estimated by the MPN- plant infection assay is one of the primary factors determining the magnitude of legume response to indigenous soil rhizobia. This is one of the main reasons why the *Bradyrhizobia* populations had to be determined in the two field sites before evaluating cowpea for nitrogen fixation related traits. In this study, the cowpea varieties used formed nodules following inoculation with serial dilutions of soils from the two study sites. However, there were variations in the number of nodulated units per variety and per study site. Indeed, using the cowpea variety, 58-77 as trap crop, a *Bradyrhizobium* spp population size of 1.0×10^4 cells per gram of soil sample was estimated in Nkometou as compared to 5.8×10^5 in Nkoemvone. Similar high rhizobia populations of 4.9×10^2, 3.5×10^3 and 4.3×10^4 cells g-1 of soil were recorded from three soils in West Africa (Ahmad *et al.*, 1981). However, the cropping history of the soils was not provided.

Generally, population sizes of rhizobia using the three varieties were higher in Nkoemvone soil than in Nkometou soil. These results showed that the levels of *Bradyrhizobia* populations recorded in the two study sites were adequate to give satisfactory outputs on nodulation and nitrogen fixation without inoculation. This is in agreement with earlier findings by Nambiar *et al*. (1983) where it was shown that most cultivated tropical soils have a rhizobial population of more than 100 rhizobia cells per gram of soil capable of nodulating the legumes grown on such soils. More so, in soils where naturalized rhizobial populations are high ($>10^3$ Rhizobium bacteria per g soil), introduction of new strains can be difficult and often unsuccessful (Thies *et al.*, 1991; Brockwell *et al.*, 1995).

The Nkoemvone site had higher *Bradyrhizobia* populations in all the categories than the Nkometou site. This difference could be linked to the cropping history of both sites as well as the soil physico-chemical properties especially the pH and soil P level. Both soils had been

cultivated to legumes in the past. However, while cowpea had been cultivated at Nkoemvone, the site at Nkometou had only been cultivated to groundnut, that is known to be nodulated only by a subgroup of *Bradyrhizobium* spp. (Yousef *et al.*, 1987).

Legumes serve to maintain rhizobia in the soil through rhizosphere effects and senescence of nodules. Studies by Woomer *et al.* (1988) and Yousef *et al.* (1987) highlighted the effects of legumes on rhizobial populations. They reported that groundnut rhizobia which prefer a pH ranging from 7.6 to 8.1, are not favored by increasing soil organic carbon over 1 %. In this study, soil samples collected in Nkometou displayed low pH (5.49) and organic C content above 1% (1.69 %). This implies that the survival of *Bradyrhizobium* spp that nodulate groundnut in Nkometou was hampered as is evident by the lower soil rhizobia population estimated**.** Available P in the soils of Nkometou was also very low (3.07 ppm) compared to Nkoemvone (7.51 ppm). Since cowpea is more tolerant to phosphorus deficiency than are most grain legumes (Alkama *et al.*, 2009), the growth, nodulation and rhizobia survival in previous experiments with cowpea in Nkoemvone may have been less affected as was groundnuts in Nkometou soil with very low P . The fact that cowpea could be nodulated by soil dilutions from both sites corroborated the idea that cowpea is a promiscuous legume, and rhizobia are facultative symbionts which in the saprophytic state, are independent of their host legumes (Woomer *et al.*, 1988).

4.5 Conclusion

Bradyrhizobium spp. also referred to as cowpea miscellany were detected in the soils from the two study sites. However the numbers varied based on site and the type of trap crop used. Good infectivity was observed using the cowpea variety 58-77 in this study. This implies that this variety may have high nitrogen fixation potential and can be cultivated in the HFZ of Cameroon to improve soil fertility. From this study, the rhizobia population from both sites was above 10^3

cells per gram of soil. This strongly suggests that cowpea production in the HFZ of Cameroon does not require inoculation. The low soil pH and P status can reduce the benefits of inoculating cowpea in this zone. This knowledge of indigenous bradyrhizobia populations in the HFZ of Cameroon will be very valuable for developing strategies to improve Biological Nitrogen Fixation (BNF) for increasing cowpea yields at low costs since inoculation is proven not to be necessary.

CHAPTER FIVE

MORPHOLOGICAL CHARACTERIZATION OF COWPEA CULTIVARS GROWN UNDER LOW PHOSPHORUS CONDITIONS FOR NITROGEN FIXATION RELATED TRAITS.

5.1 Introduction

Biological nitrogen fixation (BNF) potentials of legumes including cowpea is limited in heavily weathered acid soils that prevail in the humid forest zone (HFZ) of Cameroon, since phosphorus (P) is important in nodulation, is generally deficient (Waluyo *et al.,* 2004). There are different types of stresses that affect plants and one prominent in the HFZ of Cameroon is soil nutrient deficiency including phosphorus (P). Plants can also be grouped according to their nutrient efficiency. Gerloff (1977) proposed a system which separates cultivars into four groups based on their response to P. The groups are 1) efficient, responder; 2) inefficient, responder; 3) efficient, non-responder, and; 4) inefficient, non-responder. In this context, an efficient cultivar has higher yield than the other cultivars under low nutrient supply, while a responder cultivar has higher yield under high nutrient supply. This classification groups cultivars based on performance under low (efficient vs.inefficient) and high (responder vs. nonresponder) nutrient supply, and allows the identification of those cultivars with adaptation to a range of soil nutrient conditions. Hammond (2009) also reported groups of plants based on P use efficiencies.

When selecting plants for increased N_2 fixation in grain legumes, according to Bliss (1991), selection must be practiced under soil nitrogen (N) conditions that allow discrimination between high and low N_2-fixing lines. According to Bliss (1991), field trials should be carried out without application of large amounts of N fertilizer to facilitate selection and simulate farming conditions where low inputs are common. In growth media with minimal or no-N, plant biomass, total plant

N and grain yield are useful indicators of N_2 fixation. Other studies have shown that estimates of N_2 fixation potential can be based on nodulation traits such as nodule mass, nodule number, nodulation score, etc. (Rosas and Bliss, 1986). Given that sampling for biomass of both nodules and roots are difficult in the field and destructive (as some plants have to be dug up before maturity in order to sample for nodulation capacity and N-fixed), some studies have used above-ground biomass to replace nodule/root biomass, based on the relationship between these two variables (Denison *et al.,* 1985; Bell *et al.*, 1994; Yu *et al.,* 2002). Moreover, the heritability for these traits is usually low to moderate as environmental and genotype x environment effects are large (Bliss, 1993). Studies indicate that the amount of nitrogen fixed correlates with the %Ndfa (percentage of nitrogen derived from the atmosphere) and nodulation traits (number of primary root nodules, number of secondary root nodules, total number of root nodules, nodule score and nodule dry weight) in mungbean and soybean (Rosario *et al.,* 1997; Greder *et al.,* 1986) indicating that any of the traits or %Ndfa can be used as effective selection criteria for biological nitrogen fixation potential.

Several methods can be used to quantify N_2 fixation. The N difference, N yield, ^{15}N, acetylene (C_2H_2) reduction and xylem solute (ureide) methods have all been used to quantify N_2 fixation in selection and breeding programs. Although there is no single best method for measuring legume N_2 fixation, the limitation of each method determines its appropriateness for large-scale plant selection and breeding (Unkovich *et al.,* 2008). Consistently, legume dry matter and N yield have served as measures of N_2 fixation. The current methods used are the ^{15}N isotope methods. The

^{15} N method is a direct measure of N_2 fixation. However, its utilization is limited due to the high cost of sophisticated measuring equipment (mass spectrometer), the technical skills required to accurately determine the isotopic composition of plant samples and the expense of analyses. The

accuracy of the ^{15}N natural abundance method depends ultimately on the levels and uniformity of the ^{15}N in the soil (Unkovich *et al.,* 1994).

Challenges of recurrent climate change and soil fertility decline demands the search for crop varieties that will have the intrinsic capacity to withstand these stresses and still be highly productive. It is reported that cowpea [*Vigna unguiculata* (L.) Walp] has high protein value, high variability and high adaptability (Siambi *et al.,* 1992; Udensi *et al.,* 2011a, b). However, its landraces have not been fully exploited in research, which has caused serious losses of superior genes that could have been used during crop genetic manipulation. The availability of knowledge on genetic diversity of indigenous and cultivated germplasm can greatly enhance the genetic improvements of cowpea. Cowpea as a single crop species has varietal requirements in terms of plant type, maturity date, seed type (colour preference), and its uses are extremely diverse from region to region, making breeding programmes for cowpea more complex than for other crops (Barrett, 1987; Paul *et al.,* 1988; Timsina, 1989; Akundabweni *et al*., 1990; Silva, 1990; Tian and Xu, 1993). Since no single variety can suit all conditions, there is a need to develop varieties with different attributes and resistance to major biotic and abiotic constraints, to suit the specific needs of different regions and cropping systems. Therefore, characterization of diversity in germplasm collections is important for plant breeders, germplasm curators and farmers.

Recent advances in biotechnology and other related fields have enhanced the improvement of crops for adverse conditions. This notwithstanding, conventional techniques involving the use of biometrical approaches with morphological markers has also proved indispensable as a complementary tool to the modern techniques (Udensi *et al*., 2010, 2011a). Distance measures between entries can be based on biochemical marker traits, pedigree information, and quantitative or qualitative morphological traits. Although variation in morphological traits is

influenced by environmental factors, their efficiency for selection is still extremely useful. Morphological attributes have traditionally been employed in establishing phylogenetic relationships among genotypes between and within species and for various other purposes including identification of duplicates, studies of genetic variation patterns, and correlation of characteristics of agronomic importance. Marechal *et al*. (1978) used morphological diversity to study taxonomic relationships between genotypes belonging to the genera *Phaseolus* and *Vigna*. Lush (1979) carried out a study on the flower morphology of wild and cultivated cowpea. Pasquet (1993) carried out an intraspecific classification on *Vigna unguiculata* using their morphological traits. Obute (2001) used morphological traits (plant height, number of leaves, leaf length, the number of pods per plant, pod length and number of seeds per pod) to characterize an aneuploid *Vigna unguiculata* from the other cytotypes. So far, several types of Cameroonian cowpea have been promoted by IITA, IRAD and other Non governmental organizations (NGOs). However, information on the genetic diversity of these cowpea germplasm is limited. Recently, Gonné *et al.* (2013) evaluated the diversity of 18 traditional cowpea varieties grown by farmers in the Soudano-Sahelian Zone of Cameroon. Their results indicated that the genotypes were predominantly of the spreading type with rough and white seeds and formed five distinctive groups on the basis of morphological discriminant descriptors. The findings are interesting but further studies are necessary as only landraces from northern Cameroon were evaluated. Traditionally, farmers conserve and develop local phytogenetic resources by preserving landraces and associated local knowledge. However, ex situ conservation of potentially useful populations requires a clear understanding of the genetic variation and distinctiveness of these populations. Careful characterization of accessions is a necessary first step to guide efforts to conserve biodiversity and facilitate breeding.

The objectives of this study were to:

a) determine the varietal differences in cowpea based on their P use efficiency (Hammond grouping) and nitrogen fixing potentials

b) identify relalated traits and indices that correlate highly with nitrogen fixed, and

c) identify cowpea genotypes that are contrasting for biological nitrogen fixation related traits.

5.2 Materials and methods

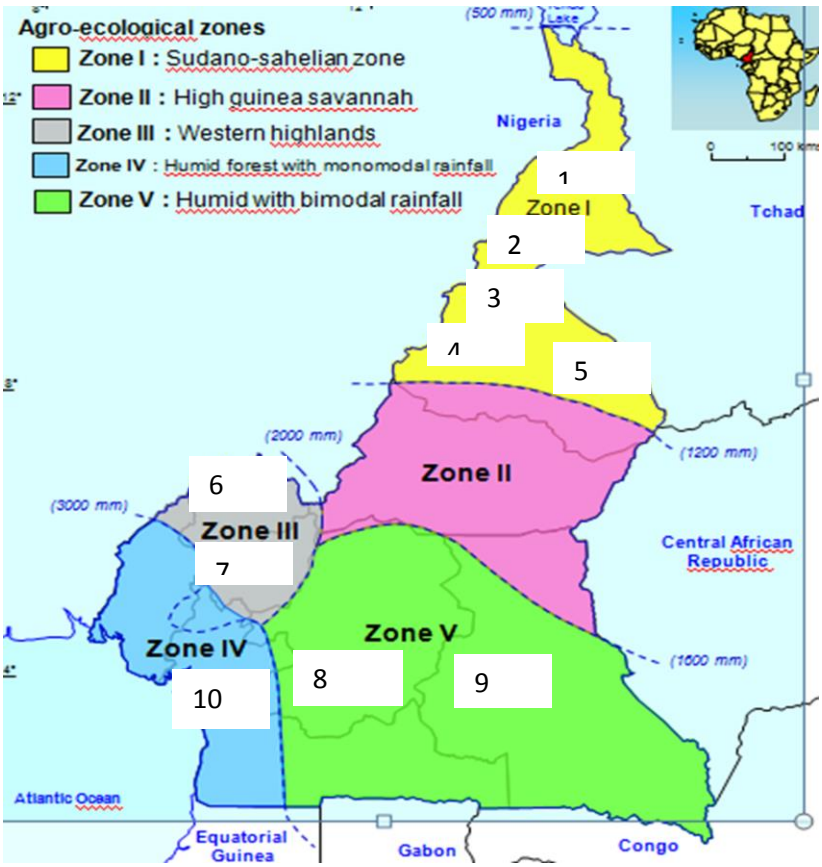
5.2.1 Plant material

A total of 50 genotypes of cowpea were collected during 2012 from various sources (Table 5.1). Among the collection were seeds of ten Cameroon landraces obtained from farmers in the different agro-ecological zones, research stations, and village markets in Cameroon (Fig 5.1). The exotic genotypes came from the International Institute of Tropical Agriculture (IITA), Senegalese Agricultural Research Institute (ISRA), University of California, Riverside (UCR); and one genotype evaluated previously at the study site (Danilla). The seeds were conserved ex situ, dried and stored at -20°C and then multiplied at IRAD Nkoemvone, during which each landrace was self-pollinated for purification.

5.2.2 Experimental design and layout

The experiment was carried out in pots in the screen house at Nkolbisson (11° 36’ E; 344’ N) IITA, Cameroon. The experimental design was a two-factor factorial experiment in a randomized complete block design with two replications. Each entry was represented by two pots in each replication. The factors were fertilization level and the genotypes. Plant nutrient application was as follows: N_0P_0 (no Nitrogen and no Phosphorus applied; N_0P_{30} (0 mg N and 30 mg P per kg of soil); and $N_{90}P_{30}$ (90 mg N and 30 mg P per kg of soil). All pots received potassium fertilizer at

equal dose (80 mg K per kg of soil). Nitrogen, phosphorus and potassium were supplied as ammonium sulphate $(NH4)_2SO_4$, KH_2PO_4 and muriate of potash, respectively. For each treatment, ten pots were planted with early maize variety (TZEEI 42) from IRAD Maroua, to serve as reference crop for the quantification of nitrogen fixation.



1. Ahel
2. Kodek
3. Lori-niebe
4. Mozongo
5. Vyu-niebe
6. Nkol Cr White
7. Dsch MMBl
8. Yde 2
9. Yde 6
10. Tiko

Figure 5. 1 Map of Cameroon showing locations of landrace collection.

Table 5. 1: Source of 50 *Vigna unguiculata* germplasm characterized.

No.	Accession	Source	No.	Accession	Source
1	58-77	Senegal	28	IT08K-161-2	IITA
2	Ahel	North Cameroon	29	IT08K-162-3	IITA
3	Bambey 21	UCR	30	IT08K-189-8	IITA
4	CB46	UCR	31	IT08K-193-14	IITA
5	DANILLA	UCR	32	IT08K-193-6	IITA
6	Dsch MMBL	West Cameroon	33	IT08K-215-2	IITA
7	FRIJOL BAYO	UCR	34	IT84S-2246-4	IITA
8	Grif 14286	UCR	35	IT89KD-288	IITA
9	IT00K-1263	IITA	36	IT90K-277-2	IITA
10	IT00K-835-45	IITA	37	IT95K-105-2	IITA
11	IT04K-227-4	IITA	38	IT95K-1090-12	IITA
12	IT06K-111	IITA	39	KODEK	North Cameroon
13	IT06K-275	IITA	40	LORI-Niebe	North Cameroon
14	IT07K-202-2-10	IITA	41	Mouride	UCR
15	IT07K-210-1-1	IITA	42	Mozongo	North Cameroon
16	IT07K-241-1-2	IITA	43	NKOL CR white	Centre Cameroon
17	IT07K-243-1-10	IITA	44	Tiko	Southwest Cameroon
18	IT07K-298-9	IITA	45	UCR 287	UCR
19	IT07K-302-10	IITA	46	UCR 3373	UCR
20	IT07K-309-44	IITA	47	Vya-NIEBE	North Cameroon
21	IT08K-125-18	IITA	48	YACINE	Senegal
22	IT08K-126-2	IITA	49	yde 2	Centre Cameroon
23	IT08K-134-11	IITA	50	Yde 6	Centre Cameroon
24	IT08K-134-12	IITA			
25	IT08K-149-1	IITA			
26	IT08K-149-3	IITA			
27	IT08K-150-24	IITA			

IITA = International institute of tropical agriculture, UCR = University of California Riverside.

5.2.3 Soil sampling

A composite soil sample was collected from the field on low N plots to a depth of 20 cm after removing surface litter. The low N plot was created by planting maize for two seasons at very high densities (100,000 plants /ha).

5.2.4 Plant and soil analyses

Soil samples were analyzed for pH, organic C, N, exchangeable Ca, Mg, K, and extractable P as presented in section 4.2.3. For plant samples, all fresh biomass samples were oven-dried at 65^{o} C, then ground to pass through a mesh of 0.5 mm, and digested according to Novozamsky *et al.* (1983). Total N was determined with an ammonium sensitive electrode (Powers *et al.*, 1981). Total P was determined by the malachite green colorimetric procedure (Motomizu *et al.*, 1983). Soil particle size was also determined as presented in section 4.2.3. For P, 0 – 20 ppm is classified as low, 20 – 40 as moderate and more than 40 as high (Nelson *et al.*, 1995). Soil N less than 0.2% is classified as low, 0.2 – 0.4 % as moderate and more than 0.4 % as high.

5.2.5 Cultural practices

Prior to sowing, seeds were surface sterilized with 95% ethanol for 1 min, and 3% H_2O_2 for 5 min, then rinsed with sterile water (Vincent, 1970). Three seeds of each genotype were sown in each pot and thinned to one plant per pot one week after emergence. Considering the bulk density of the soil, 4.3kg of soil was introduced to each pot. Before sowing, N, P and K nutrients were applied as mentioned in section 5.2.2. One mililiter of a combination of micronutrients (Vincent, 1970) per kg soil was also applied. The micronutrients included: Boric Acid (H_3BO_3); Manganese sulphate ($MnSO_4.7H_2O$); Zinc sulphate ($ZnSO_4.7H_2O$); Sodium molybdate (Na_2MoO_4); Ferric chloride ($FeCl_3.6H_2O$); Cobalt sulphate ($CoSO_4.6H_2O$); and Lactic acid (88%). Pots were watered to field capacity at planting. Field capacity was maintained by weighing three random pots each time and replacing the amount of water lost.

5.2.6 Determining soil bulk density and gravimetric moisture content to approximate field capacity moisture

The gravimetric moisture was determined by the method proposed by Somasegaran and Hoben, (1985). A 2000 ml transparent plastic cylinder was used and a hole drilled at the bottom. The hole allows air to escape when water is added to the cylinder. The cylinder was filled with a random subsample of air-dried soil. It was tapped to a similar consistency as used in the pots for the screen house experiment. The surface of the soil was covered with a filter paper disc and a small quantity of water (100 ml) poured slowly onto the surface. The vessel was covered to avoid evaporation and kept for 24 hours. Precaution was taken for the water not to reach the bottom of the cylinder. After 24 h equilibration period a sharp line where the water stopped moving in the soil column could be observed. A sample of soil for moisture determination was then collected from about 5 cm above the wetting front. This sample (wet soil) was placed in a weighed dish, and reweighed. The soil was dried at 100°C until it reached a constant weight. The oven-dry soil and dish were weighed again.

The gravimetric moisture fraction (GMF) on an oven-dried basis was calculated as follows:

GMF = (weight of wet soil on dish- weight of oven-dry soil on dish) / (weight of oven-dry soil on dish -dish weight).

The core method was used to determine the soil bulk density. A good site without roots was selected. Soil was sampled for bulk density at 0-20 cm). Samples were taken by driving a thin-walled metal cylinder of known volume (V) and weight (W1) into the vertical face of an excavation made in each plot during soil monolith collection, with a wooden mallet. Excess soil from the cylinder ends was trimmed with a knife and corked thoroughly and stored in a case.

Samples were dried at 105^{o}C for 48 hours. The dry weight (W2) was then measured. The bulk density (D) was calculated (Anderson and Ingram, 1993) as: $D = (W2 – W1) \times V^{-1}$

5.2.7 Data collection

Data on the following traits were collected:

1) Days to 50% flowering (Dflw) – Data was collected on number of days from sowing to when 50% of the plants of same genotype in a treatment showed the first fully open flower.

2) Leaf Colour Score (LCS) - three leaves per genotype in a replication were sampled at 6 weeks after planting and chlorophyll content measured. The LCS was measured with a chlorophyll meter (Markwell *et al.,* 1995).

3) Nodule score (NSC) – one plant per genotype in a replication was harvested at early podding stage (5-8weeks after planting). To examine the roots, the plants were carefully removed from the pots manually. The soil was separated from the roots by carefully shaking to loosen the soil followed by washing with tap water. The nodule scoring followed a 0-5 scale (Corbin *et al.*, 1997; Chapagain and Riseman, 2012)

4) Nodule Number (NN): After scoring, the total number of nodules per plant was counted.

5) Number of non-functional nodules (NFN): the functional nodules were identified by their color after cutting the nodules. All nodules with green or white colour were considered non-functional.

6) Number of functional nodules (FN): After selecting the non-functional nodules the rest of the nodules were counted as functional.

7) Nodule dry weight (NDW) - Dry weight was obtained by drying nodules in an oven at 70°C for 72 h. The value was expressed in milligram per plant.

8) Shoot fresh weight (SFW): At harvest, the fresh weight of the shoot was taken on the field and expressed in grams per plant.

9) Shoot dry weight (SDW) – At the early podding stage, after collecting data on nodulation, above ground parts were harvested and dried in the oven at 70°C for 72 h. These dried plants were weighed and value recorded in grams per plant.

10) Root dry weight (RDW): roots were dried in the oven at 70°C for 72 h. These dried plants were weighed and value recorded in gram per plant.

11) Nodule size (Nsize): The nodule size (Betts and Herridge, 1987; Kuang *et al.*, 2005) was estimated as nodule dry weight divided by nodule number (NDW/NN).

12) Nodulation index (NI): The nodulation index was calculated as number of nodules per gram of the total fresh biomass (Manier *et al.*, 2009).

13) Number of pods per plant (Npod) – At harvest, when pods turned completely brown on a plant, the pods per pot were counted. The dry pods were threshed by hand and seeds dried for 24 h at 40°C.

14) Number of seeds per pod (Sdpod): the number of seeds from 10 randomly selected pods per plant were counted and the average recorded. The moisture content of seeds was 12% after oven drying.

15**) Grain yield per plant (YLDplt)** – Grain yield was measured per pot and expressed in gram plant^{-1}.

16) Shoot phosphorus content (Shoot P) – Dry shoots were ground and analysed for P. Total P was analyzed using Murphy Riley reagent and read on a UV-VIS spectrophotometer.

17) **Delta N of the legume (δ^{15}N leg):** is the ^{15}N natural abundance of legume. It was calculated according to Unkovich *et al.* (1994): $\delta^{15}N = [(atom\%^{15}Nsample - atom\%^{15}Nair)/atom\%^{15}Nair] *1000$.

18) Percentage N derived from atmosphere (%Ndfa) – Maize was used as the reference crop. After drying the plants, 5 g of finely ground and sieved (2mm) plant material (Leaves of cowpea and maize reference plant) were weighed into labeled plastic bags and mailed to the stable light isotope laboratory, University of Cape Town for quantification of δ^{15}N and **Shoot N** (mg N per g plant) and eventual estimation of nitrogen fixed by the δ^{15}N natural abundance method (Peoples and Craswell, 1992; Adjei-Nsiah *et al.*, 2008; Shearer and Kohl, 1986; Unkovich *et al.*, 1993). Samples were collected from the legume and reference plants at the same period, as described by Unkovich *et al.* (1994). The ^{15}N natural abundance (δ^{15}N) of the plant sample was calculated as (Unkovich *et al.*, 1994): $\delta^{15}N = [(atom\%^{15}Nsample - atom\%^{15}Nair)/atom\%^{15}Nair] *1000$.

The proportion of N derived from fixation (%Ndfa) was estimated as (Shearer and Kohl, 1986, Unkovich *et al.*, 1994): $\%Ndfa = [(\delta^{15}N\ ref - \delta^{15}N\ leg)/ (\delta^{15}Nref - B)] *100$.

Where δ^{15}Nref is the ^{15}N natural abundance of reference plants, δ^{15}Nleg is the ^{15}N natural abundance of legume, and B represents the δ^{15}N of legume plants relying entirely on symbiotic N_2 fixation for their N nutrition. The B value incorporates the isotopic fractionation associated with nitrogenase activity during N_2 fixation and replaces the value of atmospheric N_2 (Shearer and Kohl, 1986; Unkovich *et al.*, 1994).

The B value was calculated by taking the average value from five accessions collected from different geographic areas. This measurement was done on sterile-grown plants of each accession raised in pots (Vincent, 1970) and inoculated with rhizobia isolated from the nodules of that accession. Shoot ^{15}N was analyzed, and the $\delta^{15}N$ value used as the B value in the calculation of %Ndfa of the accessions.

19) Shoot N (mg N per g plant): Dry shoots were ground and analysed for N along side the ^{15}N isotope.

20) Nitrogen fixed (N-fixed): The amount of N-fixed was calculated as the product of %Ndfa and legume shoot N.

5.2.8 Data analysis

Analysis of variance (ANOVA) was performed on all the traits using the GLM of SAS version 9.2. Information from the ANOVA was used to determine phenotypic and genotypic variances and coefficients of Variation. The format of ANOVA used is presented in Table 5.2.

Table 5. 2: Format of ANOVA used.

Source	DF	MS	Expected mean square (EMS)
Genotype (G)	49	MS_1	$\sigma^2_e + RP\,\sigma^2g + R\,\sigma^2gp$
P-Fertilization (P)	2	MS_2	$\sigma^2_e + RG\,\sigma^2P + R\,\sigma^2gp$
G*P	98	MS3	$\sigma^2_e + R\,\sigma^2gp$
Error	99	MS_4	σ^2_e

σ^2e = error term; σ^2g = genotypic variance; σ^2gp = variance due to genotypes by phosphorus (environment) interaction; MS_1 = mean square for genotype; MS_2 = mean square for phosphorus level; MS_3 = mean square for genotypes by phosphorus interaction; MS_4 = mean square error.

The Phenotypic Coefficient of Variation (PCV) and Genotypic Coefficient of Variation (GCV) values of less than 10%, 10%-20% and greater than 20% are considered to be low, intermediate and high, respectively (Khorgade *et al.*, 1985). The phenotypic and genotypic variation and coefficient of variation were calculated following the formula suggested by Singh and Chaundhary (1997) and Allard (1991) as follows:

V_G (genotypic variance) = $(MS_1 – MS_3)/RP$;

V_{GP} (Genotype by environment interaction variance) = $(MS_3-MS_4)/R$;

V_P (phenotypic variance) = $\sigma^2g + \sigma^2gp/P + \sigma^2e/rp$;

PCV = $\sqrt{(VP \times 100)/GM}$;

GCV = $\sqrt{(VG \times 100)/GM}$;

ECV (Environmental Coefficient of Variations) = $\sqrt{(\sigma^2e \times 100)/ GM}$;

GECV (Genotype by Environment Interaction Coefficient of Variation) = $\sqrt{(\sigma^2gp \times 100)/ GM}$;

where: GM is the population mean respectively for the traits considered.

Heritability: Broad sense heritability (h^2_b) was estimated for each trait by using the following equation: $h^2_b = V_G/(V_P)$, where V_G is the variance between genotypes and V_P is the phenotypic variance (Zhu, 2002).

Estimation of Correlation Coefficient: The Pearson's correlation coefficients between all possible pairs of quantitative traits were tested for their significance using XLSTAT 2015.

Principal component analysis (PCA) was also used to evaluate the traits contributing to the phenotypic variation among the genotypes. Based on the PCA-biplot analysis the genotypes were classified.

Genotypes were grouped according to the different Phosphorus Use Efficiency (PUE) as described by Hammond *et al.*, (2009):

Agronomic P use efficiency (APE, g DM g^{-1} P_f) = (Yhigh–Ylow)/ ΔPapp;

P uptake efficiency (PUpE, g P g^{-1} P_f) = [(Phigh x Yhigh)–(Plow x Ylow)]/ ΔPapp;

P utilization efficiency (PUtE, g DM g^{-1} P) = (Yhigh–Ylow)/[(Phigh x Yhigh)–(Plow x Ylow)];

Physiological P use efficiency (PPUE, g2 DM g^{-1} P) = Yhigh/Phigh or Ylow/Plow;

P efficiency ratio (PER, gDM g^{-1} P) = Yhigh/(Phigh x Yhigh) or Ylow/(Plow x Ylow);

Where;

Yhigh=yield on a high P; Ylow=yield on a low P;

Phigh=tissue P concentration on a high P;

Plow=tissue P concentration on a low P;

ΔPapp=difference in amount of P applied as fertilizer between high and low P treatments;

DM=dry matter;

Pf=fertilizer P.

Cluster analysis: Scatter plots of each variable in a pair-wise manner was performed to check if the data needed transformation prior to cluster analysis. The data was linearly transformed as the scatter plots showed poorly separated and in some cases elongated elliptical patterns. Some of the variables also had different units warranting transformation. The Proc Aceclus program of SAS was used for transformation. The dendrogram was constructed based on the hierarchical method using Euclidean test in SAS version 9.2.

5.3 Results

5.3.1 Soil characteristics

The soil was low in available P (7.51 ± 0.91 ppm) and N (0.12 % ± 0.03) according to Nelson *et al.* (1995) and had a pH water of 4.52 (Table 4.2). It was also characterized as clay with bulk density of 1.29Mg/m^3 and field capacity of 0.360 cm^3 water/cm^3 soil (Table 4.3).

5.3.2 Variation in nitrogen fixation and yield related traits among 50 genotypes of cowpea.

The combined analysis of variance across environments (P-fertilization levels) showed significant effects for most of the traits (Table 5.3, 5.4 and 5.5). In this study, the interaction between genotype and P-fertilization was significant ($p< 0.05$) for Nsize (Table 5.5). This interaction was also very significant ($P< 0.01$) for LCS, SFW, SDW, RDW, shootp (Table 5.3), NN, NFN, FN, NDW, NI, delta N of the legume (δ^{15}N leg), %Ndfa, Shoot N, N-fixed (Table 5.5) and YLDplt (Table 5.4). The genotypes were significantly different for all the traits studied at $P< 0.01$.

5.3.3 Variation in phosphorus environments

The contrast analysis indicated (Table 5.6) that the low P environment was not significantly different from high P only for NSc. Low P and high P environments were significantly different ($P<0.05$) for shoot P, and very significantly different on the rest of the 18 traits. When N was added to the P fertilization to create the third environment (high P and N), there was no significant difference between low P and this third environment for NSc, NDW, and shoot P, while a higly significant difference ($P< 0.01$) was observed for the other 17 traits.

Table 5. 3: Analysis of variance for cowpea agronomic traits from 50 genotypes.

Trait	Source of variation			Coeff Var	R-Square
	Genotypes (G)	P-fert	Genotype * P-fert		
Dflw	83.279**	978.509**	19.193ns	7.1	0.76
LCS	92.015**	4657.409**	32.834**	9.9	0.85
SFW	88.219**	7957.470**	75.224**	27.3	0.90
SDW	6.244**	223.149**	4.934**	46.0	0.79
RDW	0.084**	0.953**	0.056**	36.6	0.80
Shootp	0.007**	0.009*	0.004**	20.3	0.69

** Significant at the probability 0.01 level; * Significant at the 0.05 probability level, ns non significant. Dflw = Days to 50% flowering, LCS = Leaf Colour Score, SFW = Shoot fresh weight, SDW = Shoot dry weight, RDW = Root dry weight and Shoot P= Shoot phosphorus content.

Table 5. 4: Analysis of variance for cowpea Yield and yield components using 50 genotypes.

Trait	Source of Variaton			Coeff Var	R-Square
	Genotypes (G)	P-fert	genotype * p-fert		
Npod	7.469**	153.840**	0.679ns	34.4	0.87
Sdpod	17.354**	258.757**	3.547ns	31.7	0.74
YLDplt	123.167**	800.337**	12.382**	13.5	0.91

** Significant at the probability 0.01 level; * Significant at the 0.05 probability level, ns = non significant. Npod = Number of pods per plant, Sdpod = Number of seeds per pod, and YLDplt = Grain yield per plant.

Table 5.5: Analysis of variance for cowpea nitrogen fixation related traits.

Trait	Source of Variation			Coeff Var	R-Square
	.....				
	Genotypes (G)	P-fert	genotype * p-fert		
NSC	38.449ns	77.810ns	35.715ns	259.6	0.51
NN	453.821**	10830.721**	106.820**	32.6	0.91
NFN	30.437**	670.925**	20.957**	86.3	0.73
FN	316.255**	16333.238**	137.196**	41.1	0.93
NDW	5951.260**	135655.518**	1725.713**	48.5	0.90
Nsize	12.672**	46.622**	7.222*	94.2	0.64
NI	1.780**	109.761**	1.752**	30.4	0.94
δ^{15}N leg	0.046**	11.263**	0.033**	4.7	0.99
%Ndfa	38.063**	24586.748**	26.727**	0.8	0.99
Shoot N	24939.189**	2697688.528**	23900.977**	0.8	0.99
N-fixed	15763.463**	142502.777**	10953.339**	0.7	0.99

** Significant at the probability 0.01 level; * Significant at the 0.05 probability level, ns non significant. NSC= Nodule score, NN = Nodule Number, NFN= Number of non-functional nodules, FN = Number of functional nodules, NDW= Nodule dry weight, Nsize = Nodule size , NI = Nodulation index, δ^{15}N leg = Delta N of the legume, %Ndfa = Percentage N derived from atmosphere, ShtN = Shoot N, and N-fixed = Nitrogen fixed.

5.3.4 Grouping of cowpea genotypes and traits based on principal components.

The PCA revealed three main principal components (PCs) explaining 55.34% of total variance among the 50 genotypes of cowpea (Table 5.7). These principal components explained 27.27, 17.49 and 10.57, percent of the total variance, respectively. Strong correlations were observed between PCs and phenotypic traits (Table 5.8).

Table 5. 6: Contrast analysis for three environments (fertilization levels)

Trait	low P vs high P and N	low P vs high P	high P vs high P and N	low P vs all
Dflw	1756.200**	1115.146**	73.205*	1883.814**
LCS	8138.455**	237.271**	5596.502**	3718.317**
NSC	100.111ns	2.101ns	131.22ns	24.401ns
NN	1131.452**	11506.445**	19854.266**	1807.176**
NFN	530.483**	172.98**	1309.312**	32.538ns
FN	3239.521**	14424.511**	31335.683**	1330.793**
NDW	797.010ns	190368.563**	215800.98**	55510.056**
SFW	14105.497**	508.262**	9258.650**	6656.290**
SDW	384.205**	276.383**	8.858*	437.439**
RDW	1.849**	0.787**	0.223**	1.683**
Nsize	49.053**	85.728**	5.085ns	88.159**
NI	62.834**	47.294**	219.156**	0.367ns
Npod	294.031**	28.88**	138.611**	169.070**
Sdpod	509.134**	188.702**	75.940**	439.447**
YLDplt	1526.566**	145.934**	728.512**	872.163**
Shootp	0.0001^{ns}	0.012*	0.015*	0.003*
δ^{15}N leg	0.0467**	0.010**	11.263**	0.033**
%Ndfa	38.063**	634.554**	24586.748**	26.727**
ShtN	2642199.468**	389660.537**	5061205.579**	334171.477**
N-fixed	246603.507**	6175.727**	174729.097**	110276.457**

** Significant at the probability 0.01 level; * Significant at the 0.05 probability level, ns non significant. Dflw = Days to 50% flowering, LCS = Leaf Colour Score, NSC= Nodule score, NN = Nodule Number, NFN= Number of non-functional nodules , FN = Number of functional nodules, NDW= Nodule dry weight, SFW = Shoot fresh weight , SDW = Shoot dry weight, RDW = Root dry weight , Nsize = Nodule size, NI = Nodulation index, Npod = Number of pods per plant, Sdpod = Number of seeds per pod, YLDplt = Grain yield per plant, Shoot P= Shoot phosphorus content, δ^{15}N leg = Delta N of the legume, %Ndfa = Percentage N derived from atmosphere , ShtN = Shoot N , and N-fixed = Nitrogen fixed .

PC1 is well-correlated with nodulation traits, such as total nodule number, number of functional nodules and nodule dry weight, as well as biomass related traits like shoot fresh weight, shoot dry weight and root dry weight and finally yield component, that is, the number of pods. The second, PC2 correlated with leaf color score, nodulation index, delta N of the legume, percentage of nitrogen derived from atmosphere and N-fixed, while PC3 correlated only with shoot N. Among the 20 phenotypic traits used in the current study, the PCA identified 13 phenotypic traits with strong correlations with the three principal components. The means of traits highly correlated with the three main PCs are found in Table 5.9.

Table 5. 7: Eigenvalue of first three principal components

	PC1	PC2	PC3
Eigenvalue	5.455	3.498	2.115
Variability (%)	27.277	17.491	10.573
Cumulative %	27.277	44.768	55.341

The biplot of the PC1 and PC2 for the genotypes (Figure 5.2) distributes the 50 genotypes into four quadrants. Quadrants I and III contain genotypes with high grain yields (16-20 g plant^{-1}) and N_2-fixing capacity (400-536 mg N-fixed per plant) while genotypes in the fourth quadrant are very low in grain yield (5-7 g plant^{-1}) as well as N-fixed (220-290 mg N-fixed per plant). Most of the genotypes were grouped in quadrant II with moderate grain yield and N_2-fixing capacity.

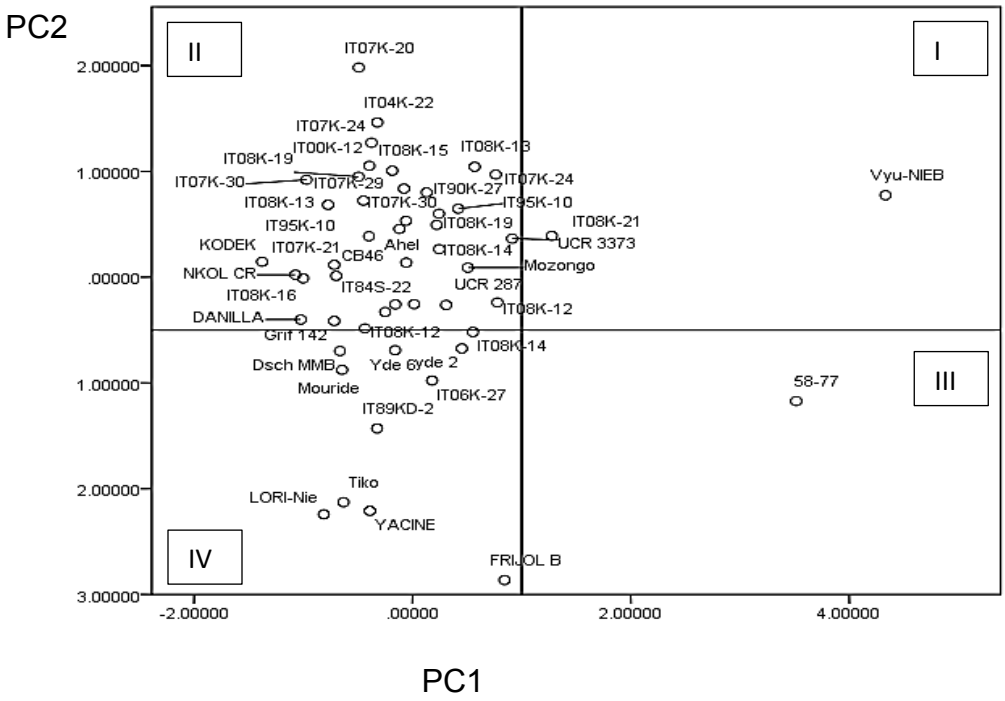


Figure 5. 2 PCA-biplot of 50 cowpea genotypes.

5.3.5 Association among the 13 important cowpea traits in low P

The correlation studies with the 13 retained traits from the PCA (Table 5.10) indicate that N-fixed correlated positively with %Ndfa (r=0.545) and shoot N (r = 0.971) and negatively with δ^{15}N leg (r = -0.591). Shoot N also correlated positively with %Ndfa (r = 0.364) and negatively with δ^{15}N leg (r = -0.391). Similarly, %Ndfa and δ^{15}N leg were negatively correlated (r = -0.849). It is worth mentioning that shoot N also correlated positively (data not reported) with N-fixed under high P (r = 0.997) and high P and N environments (r = 0.491). The nodulation trait, nodule number correlated positively with other nitrogen fixation related traits like functional nodules (r = 0.879), nodule dry weight (r = 0.641), shoot fresh weight (r = 0.51), shoot dry weight (r = 0.472), root dry weight (r = 0.374) and nodulation index (r = 0.772). Nodule dry weight also had positive correlation with shoot fresh weight(r = 0.45), shoot dry weight (r = 0.47), root dry weight (r = 0.411), nodule size (r = 0.716) and nodulation index (r = 0.461).

Table 5.8: Relative contributions of phenotypic traits in three main principal components generated using 20 traits.

Trait	PC1	PC2	PC3
Dflw	0.214	0.242	0.189
LCS	0.204	**-0.456**	-0.240
NSC	0.109	0.223	-0.292
NN	**0.829**	0.420	0.073
NFN	-0.186	0.282	0.300
FN	**0.877**	0.289	-0.049
NDW	**0.840**	0.245	0.249
SFW	**0.772**	-0.184	-0.457
SDW	**0.801**	-0.182	-0.442
RDW	**0.720**	-0.058	-0.472
Nsize	**0.529**	0.081	0.182
NI	0.412	**0.657**	0.442
Npod	0.574	0.283	0.455
Sdpod	0.351	0.319	0.020
YLDplt	0.349	-0.438	-0.029
Shootp	-0.006	-0.032	0.230
δ^{15}N leg	-0.366	**0.750**	-0.134
%Ndfa	0.322	**-0.728**	0.115
Shoot N	0.243	-0.570	**0.581**
N-fixed	0.285	**-0.694**	0.539

Values in bold are different from 0 with a significance level alpha = 0.05. Dflw = Days to 50% flowering, LCS = Leaf Colour Score, NSC= Nodule score, NN = Nodule Number, NFN= Number of non-functional nodules , FN = Number of functional nodules, NDW= Nodule dry weight, SFW = Shoot fresh weight , SDW = Shoot dry weight, RDW = Root dry weight , Nsize = Nodule size, NI = Nodulation index, Npod = Number of pods per plant, Sdpod = Number of seeds per pod, YLDplt = Grain yield per plant, Shoot P= Shoot phosphorus content, δ^{15}N leg = Delta N of the legume, %Ndfa = Percentage N derived from atmosphere , ShtN = Shoot N , and N-fixed = Nitrogen fixed .

The biomass related trait, the shoot dry weight, correlated positively with root dry weight (r = 0.835), nodule size(r = 0.232) and %Ndfa (r = 0.197) and negatively with δ^{15}N leg (r = -0.242).

Table 5.9: Means and standard deviations of highly correlated variables in low P

Trait	LCS	NN	FN	NDW	SFW	SDW	RDW	Nsize	NI	δ^{15}N leg	%Ndfa	Shoot N	N-fixed
Mean	38.8	13.8	10.3	26.6	9.3	1.4	0.2	1.6	1.5	0.48	62.9	703.7	455.4
StD	5.3	8.3	8.6	29.9	3.6	0.7	0.1	1.3	0.8	0.18	3.3	118.6	85.9

LCS = Leaf Colour Score, NN = Nodule Number, FN = Number of functional nodules, NDW= Nodule dry weight, SFW = Shoot fresh weight , SDW = Shoot dry weight, RDW = Root dry weight , Nsize = Nodule size, NI = Nodulation index, δ^{15}N leg = Delta N of the legume, %Ndfa = Percentage N derived from atmosphere , ShtN = Shoot N , and N-fixed = Nitrogen fixed .

5.3.6 Phenotypic and Genotypic variability of 13 cowpea traits

Comparatively, maximum phenotypic coefficient of variation (PCV) values (Table 5.11) were observed for nodule dry weight (68.6%), nodule size (60.3%), functional nodules (54.3%), and nodule number (50.1%). Intermediate PCV values were observed for δ^{15}N leg, nodulation index, shoot dry weight, root dry weight, and shoot fresh weight. However, nitrogen fixed, leaf colour score, shoot N and percentage of N derived from the atmosphere exhibited low PCV values, 11.9, 8.9, 8.7 and 4.3%, respectively (Table 5.11).

Shoot N had the lowest (1.7%) estimate of genotypic coefficient of variation (GCV) while nodule dry weight recorded the highest value (57.8%). The GCV was also low for traits such as leaf colour score, shoot fresh weight, nodulation index, percentage of N derived from atmosphere and nitrogen fixed. Intermediate GCV values were observed for nodule number, functional nodules and nodule size.

The highest genotype *x* environment interaction coefficient of variation (G x ECV) was obtained for nodulation index (59.8%), nodule dry weight (57.7%), delta N of the legume (55.6%) and functional nodule (55.1%). Those traits had high to moderate PCV values. The G x ECV values for most of the traits considered in this study were found to be higher than the GCV values. Apart from nodule size that had a high value (82.7%) for the environment coefficient of variation (ECV), the ECV values for the rest of the traits were low to moderate.

5.3.7 Broad Sense Heritability (h^2_b) estimates

Estimates of broad sense heritability (h^2_b) ranged from 1.5% for nodulation index to 76% for nodule number (Table 5.11). Hence, the highest heritability estimates were observed for nodule number (76.4%), nodule dry weight (71.0%), leaf color score (64.3%) and functional nodules (56.6%). Traits with moderate broad sense heritability included shoot dry weight (20.9%), root dry weight (33.6%), nodule size (43.0%), delta N of the legume (28.5%), percentage of N derived from atmosphere (29.7%), and N-fixed (30.5%). Nodulation index and shoot N had the lowest broad sense heritability of 1.5 and 4.0%, respectively.

5.3.8 Phosphorus Efficiencies of cowpea genotypes (Hammond grouping).

Based on Duncans mean separation, the top and bottom 20% of genotypes were selected based on the amount of N-fixed. Following the mean separation, the genotypes 58-77 and Danilla were among the top 20% while Yacine and Lori-niebe fell among the bottom 20%. The estimates of the five phosphorus efficiencies (PEs) are presented in Table 5.12. The genotypes 58-77 and Danilla have relatively lower phosphorus effiency ration (PER) as presented on Figure 5.3 compared to Yacine and Lori-niebe with low N_2-fixing capacities.

Table 5.10: Associations among traits

Variables	LCS	NN	FN	NDW	SFW	SDW	RDW	Nsize	NI	δ^{15}N leg	%Ndfa	ShtN	N-fixed
LCS	**1**												
NN	0.007	**1**											
FN	-0.001	**0.879**	**1**										
NDW	-0.027	**0.641**	**0.71**	**1**									
SFW	**0.214**	**0.51**	**0.56**	**0.45**	**1**								
SDW	**0.206**	**0.472**	**0.57**	**0.47**	**0.904**	**1**							
RDW	0.186	**0.374**	**0.493**	**0.411**	**0.714**	**0.835**	**1**						
Nsize	-0.049	0.196	**0.28**	**0.716**	**0.208**	**0.232**	**0.252**	**1**					
NI	-0.133	**0.772**	**0.568**	**0.461**	-0.069	-0.049	-0.023	0.15	**1**				
δ^{15}N leg	**-0.198**	-0.048	-0.118	-0.111	**-0.3**	**-0.242**	-0.129	-0.072	**0.203**	**1**			
%Ndfa	0.142	0.041	0.111	0.087	**0.242**	**0.197**	0.088	0.051	-0.16	**-0.849**	**1**		
ShtN	0.147	-0.021	0.012	0.128	0.06	0.12	0.064	0.119	-0.053	**-0.391**	**0.364**	**1**	
N-fixed	0.167	-0.027	0.025	0.128	0.116	0.154	0.078	0.124	-0.115	**-0.591**	**0.545**	**0.971**	**1**

Values in bold are different from 0 with a significance level alpha = 0.05. LCS = Leaf Colour Score, NN = Nodule Number, FN = Number of functional nodules, NDW= Nodule dry weight, SFW = Shoot fresh weight , SDW = Shoot dry weight, RDW = Root dry weight , Nsize = Nodule size, NI = Nodulation index, δ^{15}N leg = Delta N of the legume, %Ndfa = Percentage N derived from atmosphere, ShtN = Shoot N , N-fixed = Nitrogen fixed.

Table 5.11: Heritabilty, Phenotypic and Genotypic variability of 13 important cowpea traits.

Traits	VG	VGP	VP	PCV	GCV	ECV	GECV	h^2_b
LCS	9.864	6.548	15.336	8.933	7.164	10.134	5.837	64.317
NN	57.834	36.803	75.637	50.124	43.829	33.216	34.964	76.462
FN	29.843	54.257	52.709	54.328	40.879	40.076	55.120	56.618
NDW	704.258	701.757	991.877	68.683	57.875	39.146	57.772	71.003
SFW	2.166	27.359	14.703	23.988	9.207	28.330	32.722	14.730
SDW	0.218	1.359	1.041	32.300	14.796	47.143	36.905	20.982
RDW	0.005	0.018	0.014	31.648	18.369	37.842	35.729	33.689
Nsize	0.908	1.624	2.112	60.333	39.565	82.758	52.909	43.006
NI	0.005	0.770	0.297	37.128	4.616	31.453	59.808	1.546
δ^{15}N leg	0.002	0.017	0.008	38.108	20.348	5.510	55.672	28.511
%Ndfa	1.889	13.200	6.344	4.350	2.374	0.987	6.274	29.781
Sht N	173.035	11925.764	4329.567	8.762	1.751	0.936	14.542	4.001
N-fixed	801.687	5472.385	2627.244	11.967	6.611	0.683	17.272	30.514

VG = genotypic variance; VGP = Genotype by environment interaction variance; VP = phenotypic variance; PCV = Phenotypic Coefficient of Variation; GCV = Genotypic Coefficient of Variation; ECV = Environmental Coefficient of Variations; GECV = Genotype by Environment Interaction Coefficient of Variation; h^2_b = Broad sense heritability..Values of less than 10%, 10%-20% and greater than 20% are considered to be low, intermediate and high, respectively (Khorgade *et al.*, 1985).

Table 5.12: Phosphorus efficiencies (PEs) of top and bottom 20% of cowpea genotypes selected based on amount of N-fixed.

Genotype	APE	PUpE	PUtE	PPUE	PER
58-77	342.167	170.527	2.007	41.978	2.781
Danilla	41.333	22.256	1.857	54.918	3.699
Dsch MMBL	-28.833	-21.580	1.336	26.134	3.149
Frijol Bayo	114.667	24.401	4.699	26.675	3.999
IT00K-1263	51.167	8.275	6.184	78.481	6.007
IT00K-835-45	111.333	30.694	3.627	18.036	3.611
IT07K-202-2-10	77.500	8.040	9.639	76.374	5.088
IT08K-134-11	136.333	27.022	5.045	74.453	4.987
IT08K-189-8	45.472	-28.069	-1.620	65.672	3.124
IT08K-193-6	98.167	53.552	1.833	58.583	3.920
IT08K-215-2	-62.667	-20.147	3.110	54.052	3.269
IT84S-2246-4	-7.333	-11.578	0.633	78.389	4.638
Kodek	153.833	33.670	4.569	63.837	5.070
Lori-Niebe	505.833	85.355	5.926	40.681	8.892
Mozongo	-137.000	-21.010	6.521	90.003	4.715
Nkol CR white	33.167	12.012	2.761	56.434	5.972
Tiko	26.500	-15.030	-1.763	35.195	4.235
Yacine	408.000	77.990	5.231	56.272	8.337
Yde 2	58.000	10.154	5.712	41.716	5.221
IT90K-277-2	4.000	14.854	0.269	71.861	4.401

APE = Agronomic P use efficiency; PUpE = P uptake efficiency; PUtE = P utilization efficiency; PPUE = Physiological P use efficiency, PER = P efficiency ratio.

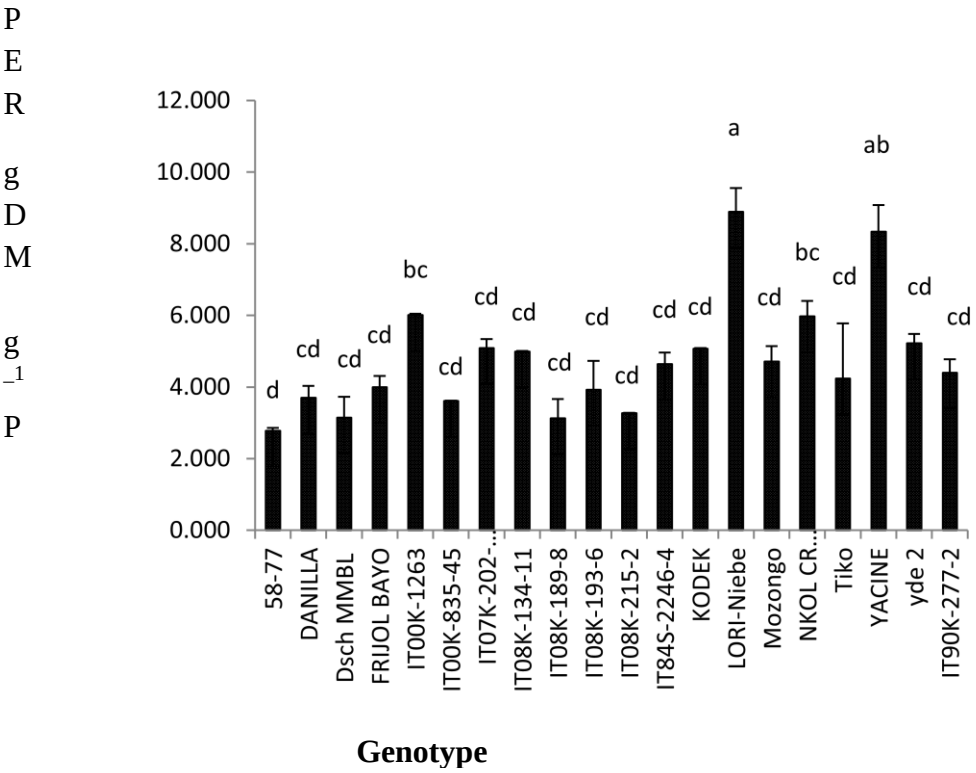


Figure 5.3 Phosphorus efficiency ratio of top and bottom 20% of cowpea genotypes selected based on the amount of N-fixed.

5.3.8.1 Association between phosphorus efficiencies and nitrogen fixed under low P

Since N-fixed correlated positively and strongly with shoot N in all the environments, and the P efficiencies (calculated based on yield and shoot P in low and high P) are also used to select crop plants on low P soil, these PEs were correlated with shoot N and N-fixed under low P (Table 5.13). The results indicated that among the five PEs, only Phosphorus efficiency ratio (PER) correlated negatively with shoot N (r = -0.490) and N-fixed (r = - 0.491). PER also correlated positively with the agronomic phosphorus use efficiency (r = 0.594) and phosphorus utilization efficiency (r = 0.500). While APE also had a positive correlation with phosphorus uptake efficiency (r = 0.823).

Table 5.13: Association between P efficiencies Shoot N and N_2-fix in low P.

Variables	APE	PUpE	PUtE	PPUE	PER	Sht N	N-fixed
APE	**1**						
PUpE	**0.823**	**1**					
PUtE	0.232	0.161	**1**				
PPUE	-0.287	-0.239	0.222	**1**			
PER	**0.594**	0.237	**0.500**	0.167	**1**		
Sht N	-0.101	0.157	-0.204	0.096	**-0.490**	**1**	
N-fixed	-0.140	0.111	-0.187	0.141	**-0.491**	**0.996**	**1**

Values in bold are different from 0 with a significance level alpha=0.05. APE = Agronomic P use efficiency, PUpE = P uptake efficiency, PUtE = P utilization efficiency, PPUE = Physiological P use efficiency, PER = P efficiency ratio, Sht N = shoot nitrogen content, and N-fixed = Nitrogen fixed.

5.3.9 Phylogenetic relationships between cowpea in different phosphorus environments

The phenogram shows the dissimilarity among the accessions grown under low P condition (Figure 5.4). The phenograms for high P and high P and N are not presented but the information is summarized on Table 5.14.

5.3.9.1 Phylogenetic relationships between cowpea under low phosphorus condition

Under low P (Figure 5.4), at a dissimilarity coefficient of 1.8, the phenogram divides the 50 accessions into two major clusters (I, II) as sumarrised in Table 5.14. Cluster II has only one accession (Kodek). The first major cluster (I) had four sub clusters (A, B, C, and D) at a dissimilarity coefficient of 0.6. Sub cluster A of cluster I comprised of 10 accessions and were the high nitrogen fixers, sub cluster B, 31 accessions, sub cluster C only one accession (Vya

niebe with high grain yield), and sub cluster D, 7 accessions (with low nitrogen-fixing potentials). The genotypes 58-77 and Danilla that were among the top 20% based on N-fixed fell in sub cluster I -A while yacine and Lori-niebe that belonged to the bottom 20% group were in sub cluster I -D.

Table 5.14: Relatedness of 50 cowpea accessions based on 20 traits in three environments.

Environment					
Low P		**High P**		**High P and N**	
Cluster	**No. of accessions**	**Cluster**	**No. of accessions**	**Cluster**	**No. of accessions**
sub cluster **I** -A	10	sub cluster **I** -A	3	**Cluster I**	18
sub cluster **I** -B	31	sub cluster **I** -B	25	**Cluster 2**	24
sub cluster **I** -C	1	sub cluster **I** -C	2	**Cluster 3**	3
sub cluster **I** -D	7	sub cluster **I** -D	15	**Cluster 4**	1
		sub cluster **I** -E	4		
Cluster II	1			**Cluster 5**	5
		Cluster II	1		

5.3.9.2 Phylogenetic relationships between cowpea under high phosphorus conditions

When P fertilizer was applied, the grouping changed. The number of clusters and structure of phenogram II did not change but the accessions were regrouped compared to grouping under low

P. Here at a dissimilarity level of 1.8, the phenogram also divides the 50 accessions into two major clusters (I, II) as indicated in Table 5.14. Cluster II had only one accession (Frijol Bayo). Considering sub-clustering at a dissimilarity level of 0.6, the first major cluster (I) had five sub-clusters (I-A, 1-B, 1-C, 1-D and 1-E). In sub-cluster A of cluster I, there are 3 accessions, in sub cluster B, 25 accessions, sub clusters C only two accessions, sub cluster D, 15 accessions, and sub cluster E, 4 accessions.

5.3.9.3 Phylogenetic relationships between cowpea under high P and high N

Here the maximum dissimilarity level between the accessions was 1.6 so grouping was done only at a dissimilarity level of 0.6. The addition of N fertilizers to the high P pots completely changed the phenogram. The accessions were grouped into 5 clusters (Table 5.14). Cluster I had 18 accessions, cluster II, 24 accessions, cluster III, 3 accessions, cluster 4, 1 accession, and cluster 5, 5 accessions.

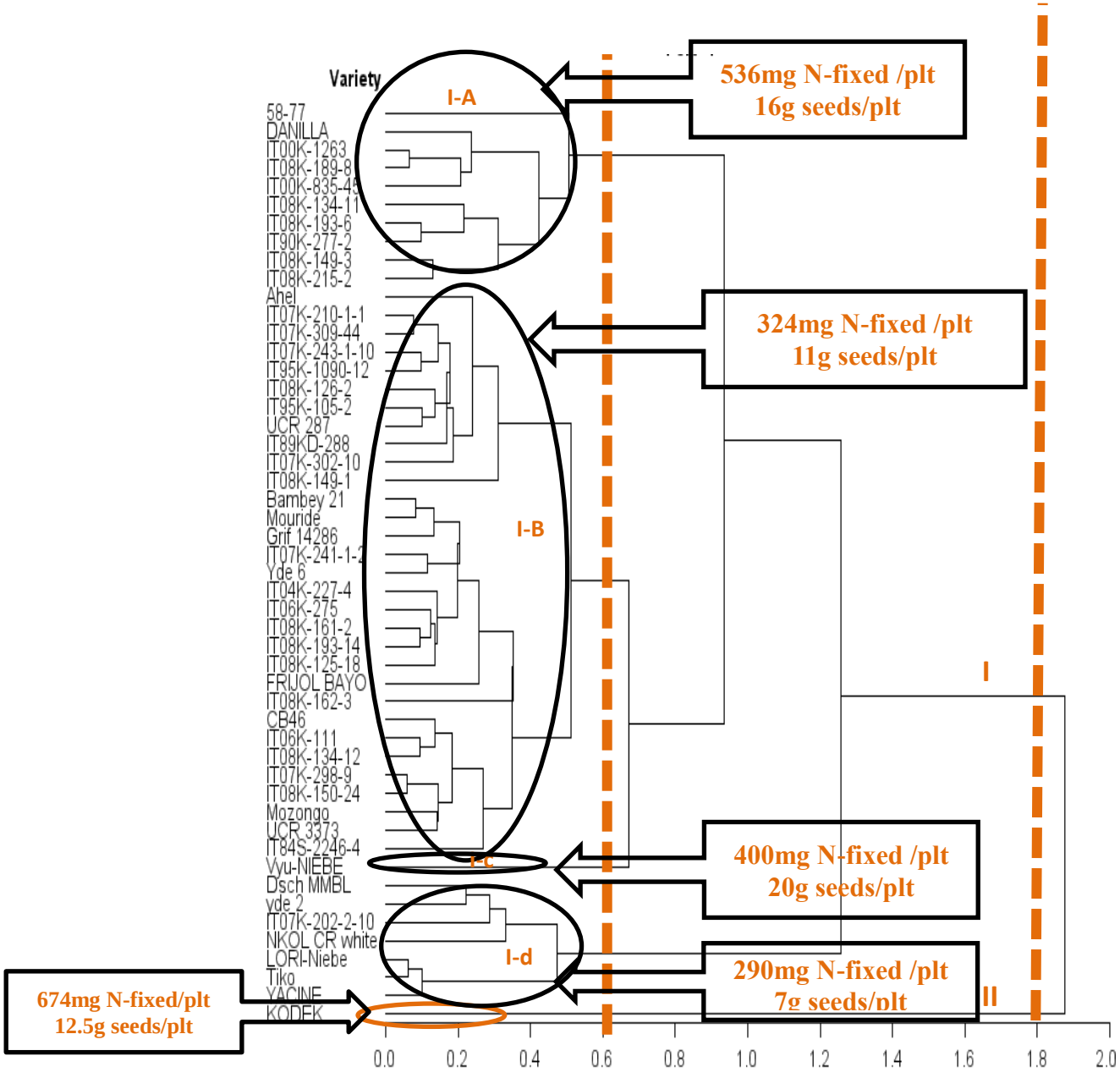


Figure 5. 4 Phenogram of 50 cowpea accessions grown in low P

5.4. Discussion

According to Nelson *et al.* (1995) P soil content of 0 – 20, 20 – 40 and more than 40 ppm is classified as low, moderate and high, respectively. Whilst soil N less than 0.2% is classified as low, 0.2 – 0.4 % as moderate and more than 0.4 % as high. The physico-chemical properties of the soil used indicated that it was a low P and N soil making it appropriate for screening for the efficient use of those traits. This is following recommendation by Bliss (1991), that when selecting plants for increased N_2 fixation in grain legumes, selection must be practiced under low soil Nitrogen conditions that allow discrimination between high- and low-fixing lines. Moreover, according to (Ezekiel and Adigun, 2005), one of the prerequisites of varietal screening for mineral stress is that the growth medium should be deficient in the nutrient under study. The low available P concentration in the soil can be attributed to low pH (5.0) in H_2O. At this acidic pH, orthophosphate anions $H_2PO_4^-$ and HPO_4^{2-} will be in soil solution just as Al and Fe. The Fe and the Al may have, therefore, precipitated the orthophosphate anions from solution. The two cycle of soil depletion with high density maize population also greatly reduced the soil N content. Genotypic, environmental factors and genotype by environmental (GxE) interaction differences were observed in this study. These differences affected crop yields and yield components (Yan *et al.,* 2001). The existence of significant effects of G x E interaction on quantitative and qualitative traits response in cowpea has previously been reported (Ansah, 2012; Sanginga *et al.,* 2000; Krasilnikoff *et al.,* 2003 and Jemo *et al.,* 2006). Crop genotypes also have different efficiencies in their use of available growth resources (George *et al.,* 2002). This variation in genetic constitution of crop genotypes influences the expression and level of heritable traits between and within environment (Acquaah, 2007). The present findings are in agreement with these reports wherein genotype and P-fertilization effects had significant effect

on yield and yield components and nitrogen fixation related traits. Because of the variation of trial environment as seen in the contrast analysis, the accuracy of the results will depend on repetitions across representative environments.

Principal component analysis (PCA) has been applied as a statistical approach to identify major variance components, their contributions and correlated traits (Heberger *et al.*, 2003). This study identified three major principal components explaining 55.341% of the total variance. This is in line with a study on agro-morphological characterization of different accessions of cowpea by Gonne *et al.* (2013) where three main components accounting for 83.66% of the total genetic variability were identified. The difference in percentage of total variation explained may be due to the traits considered.

This method assists in reducing the number of variables in the data collection in a breeding and selection process. The PCA enabled the reduction of the number of variables from 20 to 13. Consequently, it saves time and resources and improves the selection responses in crop improvement programmes (Johnson and Wichern, 2007). Through this method, fewer variables explaining variations among individuals can be screened among various characters (Shimelis *et al.*, 2013). Therefore, the PCA provides valuable information when there are several correlated traits, by reducing the costs of screening.

Principal component analysis assists in determining the relationships between traits, and the independent principal components that are effective on plant traits (Beheshtizadeh *et al.*, 2013). Results from this study showed that PC1 was well-correlated with nodule number, number of functional nodules and nodule dry weight, shoot fresh weight, shoot dry weight, root dry weight and finally the number of pods per plant. The second, PC2 was correlated with leaf color score, nodulation index, delta N of the legume, percentage of nitrogen derived from atmosphere and N-

fixed, while PC3 correlated only with shoot N. This is in conformity with experiments carried out by Jolliffe, (1972, 1973) where variables that loaded highest on each component were retained. The biplot results also indicated the direction of the correlation between the two PCs.

The results of the correlation analysis indicated that N-fixed correlated positively with %Ndfa (r =0.545) and shoot N (r =0.971) and negatively with δ^{15}N leg (r= -0.591). In addition, most trait associations were positive and this positive correlation could result from the presence of common genetic elements that control the characters in the same direction. Positive and reported significant correlation due to effect of genes can be the result of the presence of strong coupling linkage between their genes or the characters may be the result of pleiotropic genes that control these characters in the same direction (Kearsey and Pooni, 1996). The positive and significant correlation coefficient observed between shoot N and N-fixed may imply that there is the potentials to improve N_2 fixation through conventional improvement of shoot N. However, the low heritability of shoot N will require that large populations be used or molecular breeding should instead be employed.

Nodule number correlated positively with nodule dry weight (r = 0.641), shoot fresh weight(r = 0.51), shoot dry weight(r = 0.472), root dry weight (r = 0.374) and nodulation index (r = 0.772). These results are similar to other studies in which nodule number had a strong positive correlation with total nodule weight, with correlation coefficients ranging from 0.57 to 0.74. Tanya *et al.* (2005) also reported that the correlation between nodule number and total weight (g plant^{-1}) was 0.58 in soybean. Santos *et al.* (2013) found that the correlation between nodule number and total weight (mg plant^{-1}) was 0.64 in soybean. In pea, Bourion *et al.* (2010) reported that the correlation between nodule number and total weight (g plant^{-1}) ranged from 0.74 to 0.77.

Sinclair *et al.* (1991) also found that both nodule weight per plant and nodule number per plant significantly correlated with shoot dry weight in soybean.

Phenotypic and genotypic coefficients of variation were very low for leaf colour score, percentage of nitrogen derived from the atmosphere, shoot N and amount of N-fixed. These traits also had lower environmental and genotype x environment interaction coefficients of variation. The most probable reason could be the phenotypic plasticity occurring in those traits is the main source of variation than the genetic variance (Guillen, *et al.*, 1999). This result also shows that selection is not effective for these traits because of the narrower genetic variability. Most of the nodulation traits (NN, FN, NDW and Nsize) considered in this study had moderate to high GCV values. This implies that there is a potential natural genetic variability among cowpea genotypes and hence varietal improvement through conventional breeding based on these traits is possible. However, this result is contrary to that of Ansah (2012) who reported low PCV and GCV for nodule number in a pot experiment. This could be due to the different population used or the inefficiency in rhizobia quantity and quality in the soil used.

In order to improve the phosphorus use efficiency in crop plants, it is important to explore genetic variation for all its associated traits or indices (Shenoy and Kalagudi, 2005). The results of this study show that only the P efficiency ratio correlated negatively with shoot N and N-fixed and positively with P utilization efficiency. Gentoypes with high N_2-fixing capacity had lower PER and phosphorus utilization efficiency (PUtE). The results are in contrast with that of Ansah (2012) who found that PUtE correlated negatively with all the traits with the exception of phosphorus uptake efficiency (PUpE).

Under low P, the phenogram grouped accessions according to their geographic origin or specific collection sites. For example, the sub-cluster I-D had seven accessions, with five (5/7) of them

constituting Cameroon landraces. Sub-cluster B of cluster I, grouped 31 accessions including all the accessions from the University of California Riverside (UCR) and the majority (22/30) of the accessions from IITA. The two accessions (Vya niebe and Kodek) that were singled out in sub-cluster I-C and cluster II are from the northern part of Cameroon. Vya niebe is a high grain yielding genotype while Kodek is a good N_2 fixer under low P conditions. As was the case of grouping under low P, the phenogram under high P grouped accessions according to their geographic origin or specific collection sites. This can be observed in the sub-cluster I-B with 25 accessions, including the majority (7/10) of Cameroon landraces. The peculiarity of phenogram III summarized in Table 5.11 is that all the landraces fell in two of the clusters. Cluster I with 4/10, and cluster 2 with the remaining 6/10 landraces. Previous studies on cowpeas using morphological traits such as plant pigmentation, plant habit, root traits, leaf traits, pod traits, seed traits, grain quality, and yield have been done. These traits were all found to be of great importance to distinguish genetic variability, and have led to a better classification of cowpea genotypes (Gonne *et al.*, 2013; Fery and Dukes, 1994; Karkannavar *et al*., 1991; Roquid and Patnaik, 1990). As in earlier studies, this study also found that agro-morphological traits especially those related to nitrogen fixation are still valuable tools for cowpea genetic diversity studies.

5.5 Conclusion

Highly significant differences among genotypes for all the traits were recorded indicating the presence of genetic variability which is necessary for an effective breeding programme. The polygenic variation are phenotypic, genotypic or due to genotype by environment interactions. Thus, its relative values give an idea about the magnitude of variability. This study revealed that some morphological traits discriminated more efficiently between the accessions than others. It

would therefore be very important to identify beforehand the right agro-morphological characters (those with a high discrimination capacity) before undertaking any genetic diversity studies based on morphological traits to save time and money.

There were also correlation among traits in both the positive and negative directions and this can be exploited in the selection process. Shoot N and PER can be used as selection criteria in breeding for enhanced N_2-fixing capacity but must be done with a high population because of the low GCV.

Cluster analysis substantiated the existence of diversity among the 50 accessions for the morphological traits studied. The clustering pattern showed that the Cameroon landraces were not genetically more distant from each other like the exotic accessions. Furthermore, dissimilarities were observed among accessions from the three different geographical regions, presenting a great possibility for the development of suitable varieties for the various agro-ecological zones of Cameroon. That is to say, different cultivars can be developed for specific agro-ecological regions by making use of the available potential of the germplasm. It can therefore be concluded that in this study, the clustering process did reflect the source of the accessions when grown in the natural environment (low P conditions). Selection for P-efficiency can easily be done on this soil or field from which soil was collected. Some of the landraces did have unique characteristics that need to be exploited. Generally, the accession 58-77 from Senegal occupied a specific position in all the three phenograms. It also has high grain yield and nitrogen-fixing capacity. This accession seems to possess traits that are not affected by environment. Therefore, P efficient genotypes like 58-77 and Vya niebe can be exploited for grains, while Kodek and 58-77 that can be used for soil N restoration. In conclusion, the use of

materials from different geographical origins in any cross breeding programme aiming to develop suitable varieties with specific characters is therefore strongly recommended.

CHAPTER SIX

INHERITANCE OF TRAITS RELATED TO BIOLOGICAL NITROGEN FIXATION IN COWPEA UNDER LOW PHOSPHORUS CONDITION.

6.1 Introduction

Cowpea has the unique ability to fix atmospheric nitrogen through its root nodules in poor soils with more than 85% sand, less than 0.2% organic matter and low levels of phosphorus. It also covers the ground so well that it controls soil erosion. As a leguminous crop, it fixes about 70 – 240 kg nitrogen per ha (Singh *et al*, 2003).

Genotypic variability for nitrogen fixation and related traits in cowpea germplasm have been well documented (Sanginga *et al.,* 2000, Jemo *et al.*, 2006a). These quantitative differences in efficiency offer great opportunity for development of superior N_2-fixing cultivars. Some research on inheritance of various qualitative and quantitative characters has been done (Lachyan and Dalvi, 2015). However, the genetics of these characters is not well understood and there is controversy with regards to the nature of inheritance i.e. number of genes controlling the character, type of gene action and segregation pattern (whether independent assortment, interactions or linkages). There is a lot of scope for improvement in nitrogen fixation by gene manipulation. This is possible only when the genetic structure of the plant is known. As such the knowledge of the inheritance of various characters in cowpea is a prerequisite as it is in other crop plants.

The genetic basis for nitrogen fixation related traits in cowpea have been studied to an extent. In a study on the parameters of variability, correlations, and gene action in cowpea, characters like total nodule weight and nitrogen content per plant recorded high heritability estimates (Sreekumar, 1995). Moderate heritability was recorded for the characters viz number of nodules

in the primary root, number of nodules in the secondary roots, total number of nodules, weight of effective nodules in the primary root, and weight of nodules in the secondary root. Plant dry weight had low heritability indicating the greater influence of environment over this character (Sreekumar, 1995). Genetic advance was found to be high for number of nodules in the secondary roots, weight of effective nodules in the primary root, weight of nodules in the secondary roots, and total nodule weight and moderate for number of nodules in "the primary root and total number of nodules. Low genetic advance was recorded by plant dry weight and nitrogen content per plant (Sreekumar, 1995). Hence traits such as number and weight of nodules in the primary root, number and weight of nodules in the secondary roots, total number and weight of nodules may be controlled by additive genes whereas nitrogen content in plant may be controlled by non-additive genes. Weight of nodules in the primary root and total nodule weight were positively correlated with the nitrogen content in plant. Both additive and non-additive gene actions were important for all the characters under study (Sreekumar, 1995). Many authors have studied genetic variation and heritability for nitrogen fixation and related traits in other legumes like peanut (Isleib *et al*., 1980; Arrendell *et al*., 1986; Phudenpa, 2002). Working with peanuts, Arrendell *et al.* (1985) found that broad sense heritability estimates for nodule number, nodule weight, nitrogenase activity, shoot weight and fruit weight were moderate to high. Nigam *et al.* (1985) also reported predominant nature of non-additive genetic variances for nitrogenase activity and other traits. Provorov and Tikhonovich (2003) in their review reported high heritability of nitrogenase activity in many leguminous crops, suggesting that selection for this activity may be highly effective. Bado *et al.,* (2006) also observed significant correlation ($p<0.05$, $R^2 = 0.94$) between total N yield of peanut and N derived from the atmosphere. Bhardwaj *et al*. (2005) using two crosses of cowpea reported that additive and dominance gene

effects were highly significant. In both crosses, additive gene effects for the number of pods per plant and seed yield per plant were greater than the dominance gene effects. They also recorded epistatic gene effects for all studied traits, except for seed yield per plant in both crosses. This information is important for breeders to formulate effective and efficient breeding strategies. However, genetic variation and heritability estimates are unique to a particular population. In addition, these genetic properties are dependent on variation due to environment and genetic background of breeding populations.

The efficiency of a breeding programme increases by careful choice of parents and populations capable of producing progeny with desirable trait combinations (Abreu *et al.,* 2002; Cristina *et al.,* 2002). Selection of the appropriate breeding method for better exploitation of the genetic potential of different traits depends on the type of genes controlling the trait and its inheritance (Akhtar and Chowdhry, 2006). In most crop plants the types of genes and their effects have been studied (Lamkey and Lee, 2005). Specialized biometrical techniques are required to study the type of genetic variability associated with a trait. These biometrical techniques are dependent on different mating designs such as diallel, line x tester; North Carolina design and generation mean analysis for the estimation of type of genetic variability. Among these tools, generation mean analysis has been the most powerful biometrical analysis since it gives additional information about the epistatic interactions. Generation mean analysis is an approach which provides information about the nature and magnitude of gene actions involved and used to estimate the component of variance which provides information about the predominant type of gene action for the important characters of crop species (Ganesh and Sakila, 1999). Besides gene effects, breeders would also like to know how much of the variation in a crop is genetic and to what extent this variation is heritable, because efficiency of selection mainly depends on additive

genetic variance, influence of the environment and interaction between genotype and environment. Generation mean analysis has been used in common bean to study the inheritance of complex traits such as leafhopper insect resistance (Kornegay and Temple, 1986), rate of ethylene production (Sauter *et al.*, 1990), bean pod morphology (Chung *et al.*, 1991), and most recently heat tolerance (Rainey and Griffiths, 2005) and climbing ability (Checa *et al.*, 2006). Plant breeders are interested in the analysis of phenotypic data to measure genetic effects and heritability of quantitative traits and predict gain from selection. Measurement of phenotypic values of 6 related generations (parents, F_1, F_2, and backcrosses) allows for the simultaneous analysis of both Mendelian and quantitative traits. Variance of the F_2 provides an estimate of phenotypic variance, whereas the mean variance of the nonsegregating generations (P_1, P_2, and F_1) provides an estimate of environmental variance (Wright, 1968). In addition, main and epistatic gene effects contributing to the phenotypic expression of a quantitative trait can be partitioned according to the model proposed by Hayman (1958) and reviewed by Gamble (1962).

In breeding to improve the inherent nitrogen-fixing potential of a crop plant, the selection criterion may be nitrogen fixed or some of the morphological components of nitrogen fixation. An understanding of the mode of inheritance of the nitrogen fixation components, the correlations among them, and the association between each component with N-fixed is necessary for the intelligent choice of breeding procedures for developing high N_2-fixing varieties.

Objectives

The aim of this study was to identify gene effects controlling nitrogen fixation related traits through the generation mean analysis of traits. Other objectives of this study were to estimate

variance components of generations, broad-sense (h^2_b) and narrow-sense (h^2_n) heritabilities and number of effective genes involved.

6.2 Materials and methods

6.2.1 Site description

Field evaluation was done at Nkoemvone in the HFZ of Cameroon while crosses were generated in pots in the screen house at IRAD, Nkolbisson. The Nkoemvone field station, IRAD Cameroon, is located on latitude 2°90'N, longitude 11°20 'E, at an altitude of 615 metres above sea level (masl). The average annual rainfall over 20 years is 1820 mm with a bimodal distribution. Nkoemvone received an average of 1687.20 mm of rainfall in 2014 with minimum and maximum mean temperature of 23.5^o C in the month of August and 27 ^{o}C in March, respectively. Soil analysis for the experimental site showed it was low in P (7.51 ± 0.96 ppm); N (0.12 % ± 0.02) and had a pH water of 4.52. Phosphorus was determined according to Mehlick as explained by Nelson *et al.,* (1995), N was determined by regular macro – Kjeldahl method (Black, 1995). The soils were characterized sandy clay loam with bulk density of 1.35Mg/m^3 and field capacity of 0.290 cm^3 water/cm^3 soil.

6.2.2 Plant material

The parents were selected based on the results from a preliminary screening pot experiment at IRAD, Nkolbisson in 2012 and the characterization studies in 2013 (section 5). These parents exhibited a wide range of expression of nitrogen-fixing related traits and also represent diversity in origin and botanical type. The parents included one high N_2-fixing genotype, 58-77 and one low N_2-fixing type Lori-niebe. The genotype 58-77 is a black small-seeded local cultivar from Senegal, resistant to pest and diseases with high N_2-fixing capacity. Lori-niebe is a large white-

seeded advanced breeding line from IRAD Maroua, Cameroon with high yields in the sahelian zone but low yield in the HFZ. In the HFZ, Lori-niebe has very poor nodulation and low N_2-fixing potentials.

6.2.2.1 Development of F_1 seed

The parental material was sown in pots in January 2014 under optimum conditions. Normal production package and crop husbandry techniques were followed to raise the crop. Crosses were made between 58-77 and Lori-niebe, using 58-77 as female to produce F_1seeds.

6.2.2.2 Development of F_1, F_2, BC_1P_1, BC_1P_2 generation

In May 2014, F_1 seeds and all the two parents were planted in a field. The F_1 seed was used to grow F_1 generation. At flowering stage, F_1 plants were allowed to self to produce F_2 seeds. The F_1 plants were also crossed with the first parent (female) to produce BC_1P_1; they were also crossed with the second parent (male) to produce seed of BC_1P_2. In the same season, parents were again crossed to produce fresh F_1 seed.

6.2.3 Manual crossing

To make the crosses, every evening between 3pm - 5pm light green buds that were about two days old were selected and incisions made along the side. The buds were opened to make sure the pollen sacs had not released pollen yet. The pollen sacs were removed, stigma kept intact and labels attached. The next morning, between 7:30 and 8am, open flowers from the male donor plant were removed and anthers covered with pollen were used to dust the stigma of mother plant prepared the previous evening. To avoid stray pollen contamination, stigma was covered with a soda straw tube. To be sure the F_1 seeds used for further crosses were from a successful cross and not from self pollination, part of the F_1 seeds were planted the next season while generating

the other generations. The F_1 plants whose seeds showed no segregation at maturity were eliminated and all crosses from that lot destroyed. Crosses and self-pollinations were made in the greenhouse. The crosses are presented in Table 6.1.

Table 6.1: Crosses made to produce 6 cowpea generations

Parents	No. of plants
P_1 = 58-77	21
P_2 = Lori-niebe	21
F_1 = 58-77 x Lori-niebe	20
F_2 = F_1 selfed	84
BC_1P_1 = 58-77 x F_1	32
BC_1P_2 = Lori-niebe x F_1	32

6.2.4 Experimental design and treatments

The six treatments (P_1, P_2, F_1, F_2, BC_1P_1, and BC_1P_2) were planted in a split plot design with phosphorus level as the main plot and generations assigned to sub-plots in two replicates. The six generations were grown under two contrasting phosphorus levels, Low P (0 kg P per ha) and high P (30 kg P per ha). P source was triple super phosphate. Plots were fertilized uniformly with K (KCl) at 80 kg ha^{-1}. Lime [$Ca(OH)_2$] at the rate of 924kg of CaO per ha was incorporated into the soil during soil preparation. This dose followed the recommendations of Kamprath (1970). Fertilizers were applied at planting.

6.2.5 Field evaluation

Planting was done in the second growing season (September-December, 2014). Since the non-segregating generation represents the homozygous population while segregating generation represents heterozygous population, the number of plants used for the different generations was

varied. Each replication consisted of five plants of the P_1, P_2, and F_1 generations, and 21 plants of the F_2. Eight plants were evaluated per replicate for both BC_1P_1 and BC_1P_2. Plot size was a single 2m row plot for the P_1, P_2, and F_1 generations, double row plots for the $BC1P_1$ and BC_1P_2 generations and four row plots for the F_2 generations. The rows were kept at 0.5 m apart. Within row spacing (distance between plants) was 50cm m to enable good sampling of nodules.

Each hill had a single plant. Seeds were treated with fungicide (Captan) at rate of 5 g kg-1 (seed). No rhizobium inoculant was applied because cowpea-type rhizobia, are ubiquitous in this soil (Atemkeng and Begoude, 2014). Insecticide Thiodan® (endosulfuran organochlorine) was applied when necessary to obtain optimum growth and yield. Hand weeding was done at 15 and 35 days after planting. A total and mean precipitation of 812.9 and 203.2 mm respectively was recorded during the crop growth period (September to December, 2014). Mean minimum and mean maximum temperatures ranged from 25°C in September to 26°C in December (IRAD Nkoemvone, 2014).

6.2.6 Data collection

Eight weeks after planting, plants were sampled. Data were recorded on each plot for nitrogen fixation parameters, viz. nodule number, and nodule dry weight, shoot dry weight, and shoot nitrogen content. The method used per trait is as follows:

a) **Nodule number-** nodules on single plant basis were counted to determine the average number of nodules per plant. This was done using three inner plants per replicate for the P_1, P_2, and F_1 generations, five plants/rep in each backcross and 15 plants/rep in each F_2 generations. To examine the roots, the plants were carefully dug out of the soil with a spade. The soil was separated from the roots by careful shaking to loosen the soil followed by washing with tap water.

b) Nodule dry weight (NDW) - Dry matter was obtained by drying nodules from the above sampled plants. The nodules were dried in an oven at 70°C for 72 h and value expressed in milligram per plant.

c) **Shoot dry weight (SDW)** – At nodule collection, above ground parts from same plants was harvested and dried in the oven at 70°C for 72 h. The dried shoots were weighed and the value converted to gram per plant..

d) Nitrogen content in shoots –Dry shoots of the cowpea plants were ground and analysed using the Kjeldahl method for digestion and ammonium electrode determination (Bremner and Tabatabai, 1972; Nelson and Sommers, 1972). Values were measured in percentage of total N. In a previous (characterization, chapter five) experiment using same soil in pots, shoot N was found to correlate strongly with N-fixed. So in this experiment, shoot N was considered to represent N-fixed.

6.2.7 Data analysis

a) Analysis of variance: Data were analyzed using split plot analysis of variance. There were two factors i.e., generations and phosphorus levels. Generations comprised of 6 levels while there were 2 phosphorus levels (low P and high P). Variations among the generations were further broken down into parents, F_1's, F_2's, BC_1P_1's and BC_1P_2's. Analysis of variance depicted significant variation among generations and generation ×Phosphorus level. Therefore, data were subjected to the generation mean analysis to determine the type of genetic variation associated with the traits under study under low phosphorus level. Analyses of variance of the phenotypic data of the six treatments or generations for each cross were performed using PROC GLM (SAS v. 9.02, SAS Institute Inc. 2010). Generations were considered as fixed effects.

b) **Mean comparison:** Mean comparision was performed by Duncan's multiple range test (Gomez and Gomez, 1984).

c) **Univariate analysis** was done and the distribution of the nitrogen fixation related traits plotted.

d) **Phenotypic correlation:** Correlations between nitrogen fixation related traits were investigated based on family means. Phenotypic Pearson's correlations were conducted by using PROC CORR (SAS Institute Inc. 2010).

e) **Generation mean analysis. :** Data were subjected to the generation mean analysis to determine the type of genetic variation associated with each trait under the low phosphorus level. To obtain information on the nature of gene action governing the traits under study, all the six components of generation means were computed.

The Haymans' model was applied. $Y = m + \alpha [a] + \beta [d] + \alpha^2 [aa] + 2 \alpha \beta [ad] + \beta^2 [dd]$,

where

Y = The mean of one generation

m = The mean of all generation

a = The sum of additive effects

d = The sum of dominance effects

aa = The sum of additive x additive interaction (complementary)

dd = The sum of dominance x dominance interaction (duplicate)

ad = Sum of additive x dominance

α, β, $2\alpha\beta$ and $\beta 2$ represent the coefficients of genetic parameters.

The estimated (m, [a], [d], [aa], [dd], [ad]) values were tested by t-test at the 0.05 and 0.01 levels of probability.

Narrow (h^2_n) and broad (H_b^2) sense heritability were calculated according to Warner (1952).

$$h_b^2 = [V_{F2} - (V_{P1} + V_{P2} + V_{F1}) / 3] / V_{F2}$$

$$h^2_n = [2V_{F2} - (V_{BC1P1} + V_{BC1P2})] / V_{F2.}$$

With V representing corresponding variances.

In the presence of epistasis, generation mean analyses were carried out according to Hayman (1958). The significance of the different parameters was tested with the help of 't' values, which were calculated for each component by dividing the gene effect of respective components by their standard errors (SE). GMA was done according to Marther and Jinks (1982) models in the SASQuant program (Gusmini, *et al.*, 2007) using the SAS package (version 9.2). The number of effective factors was determined following five methods (Wright 1968; Lande 1981; Mather and Jinks 1982).

a) Computational Methods used by the SASQuant program

SASQuant consists of two SAS macros. The first macro, DIST, plots the data from the F_2 generation to verify the normal distribution of the F_2 phenotypic data. In a second step, the DIST macro tests the homogeneity of variances of the overall F_2 data (Ostle and Malone, 1988; Steel *et al.*, 1997) using the Bartlett's test. The second macro, ESTIM, calculates means and variances by generation. Generation means and variances are then combined to estimate genetic variances, heritabilities (narrow and broad sense), number of effective factors, and gene effects. SASQuant estimates phenotypic (P), environmental (E), genotypic (G), and additive (A) effects from generation variances according to Warner (1952); and Wright (1968). SASQuant estimates the

number of effective factors as described by Wright (1968); Lande (1981); and Mather and Jinks (1982). SASQuant partitions additive, dominance, and epistatic effects based on the Hayman's mean separation analysis procedure (Hayman, 1958; Gamble, 1962), and computes the standard errors for the estimates as square root of their variances. SASQuant tests the hypothesis that the estimates are significantly different from zero, performing a Fisher's t-test. It estimates the degree of freedom as an average of the segregating generations used to estimate the gene effect. Where ever the formulas used by SASQuant produced negative estimates, they were considered equal to zero (Robinson *et al.*, 1955). Both negative and positive estimates were reported to permit unbiased estimates and proper future interpretation of genetic parameters (Dudley and Moll, 1969).

6.3 Results

6.3.1 Variations between cowpea generations

Analysis of variance indicated significant variation among generations and generation × P- level (data not shown). Therefore, data were subjected to the generation mean analysis to determine the type of genetic variation associated with the traits under low P conditions. Highly significant differences were detected among generations for all traits measured (Table 6.2). Duncan's multiple comparison of generation means showed that both parents were contrasting for all traits (Table 6.3). Substantial generation-wide variation was observed for all traits (Figure 6.1A-D). The means for all the traits of parent 58-77 (P_1) as shown in Table 6.3 and Figure 6.1A-D were higher than those of Lori-niebe (P_2). The F_1s showed increased performance (Figure 6.1A-D) in NN, NDW, SDW and Shoot N compared to the P_2 (Lori-niebe). The F_1's shoot N out yielded the better parent (parent 1), while for the other traits, no significant differences were observed between the F_1 and parent 2 (Table 6.3). The F_2, and BC_1P_1 generation means for NN, NDW,

SDW and shoot N were lower than the means of the parents and F_1, generation. The BC_1P_1 generation mean for all the traits was higher than the means of the two parental populations (Figure 6.1A-D). In addition, the BC_1P_1 population mean was higher than any other population for SDW (Table 6.3). The F_1, mean for shoot N exceeded the high parent, while the F_2 population mean was significantly less than the mean of the lower parent. The BC_1P_2 population mean for shoot N was also low, while the BC_1P_1 mean exceeded the midparent value (Table 6.3).

Table 6.2: Analyses of variance for generations under low P.

Source of variation	DF	NN	NDW	SDW	ShootN
Generation	5	10540.421***	398561.89***	3545.242***	67.096***
Error	204	302.958	4344.565	52.524	0.582

*** Significant at the probability 0.001 level

6.3.2 Phenotypic correlation of nitrogen fixation related traits

Correlation coefficient (r) using data from all genotypes in all generations indicated positive and strong correlation among all the traits. Shoot N, that correlated strongly and positively with N-fixed in previous studies with cowpea (Table 5.10) correlated positively with NN (r= 0.94), NDW(r= 0.98), and SDW(r=0.90).

6.3.3 Estimates of Variance components and gene factor number.

The estimates of additive, dominance, and environmental components of variance for the nitrogen fixation related traits are presented in Table 6.4. Apart from estimates for NN, for all other traits, the variance due to genotypes was lower than the environmental variance. Signs associated with the variances indicate the influence of each parent.

Table 6.3: Duncan's multiple generation means comparison for nitrogen fixation related traits.

Gen		NN(number plant^{-1})		NDW (mg plant^{-1})		SDW(g plant^{-1})		Shoot N (%)	
	N	Mean	CV	Mean	CV	Mean	CV	Mean	CV
BC_1P_2	32	18.77±1.2 **C**	36.13	33.56±1.7 **C**	29.6	6.75±0.6 **C**	51.33	2.19±0.1 **C**	32.11
BC_1P_1	32	48.36±2.7 **B**	32.23	198.4±17.9 **B**	51.03	29.12±1.5 **A**	30.42	4.06±0.1 **B**	13.51
F_1	20	55.3±2.6 **BA**	21.38	279.56±27.3 **A**	43.71	24±2.02 **B**	37.56	5.17±0.1 **A**	14.37
F_2	84	21.84±2.5 **C**	108.57	35.72±3 **C**	77.09	6.83±0.6 **C**	85.29	1.68±0.1 **D**	54.25
P_2	21	26.2±1.28 **C**	22.35	38.13±3.0 **C**	36.89	8.19±0.9 **C**	55.25	2.46±0.1 **C**	23.32
P_1	21	61.7±2.6 **A**	19.32	250.38±22.6 **A**	41.41	23.08±2.7 **B**	54.32	4.37±0.1 **B**	14.45

Numbers followed by the same letter within a column are not significantly different at $p > 0.05$. Gen = generation, N = number of plants.

Table 6.4: Genetic variances and heritability for nitrogen fixation related traits derived from 58-77 x Lori cross.

Trait	V_P	V_E	V_G	V_A	V_D	H^2_b	H^2_n
NN	562.73	113.93	448.8	834	-385.2	0.8	1.48
NDW	758.3	10201	-9443	-8831	-611.7	-12.45	-11.65
SDW	33.91	85.04	-51.13	-22.63	-28.5	-1.51	-0.67
Shoot N	0.83	0.46	0.37	0.86	-0.49	0.45	1.04

V_P = Phenotypic Variance ; V_E = Environmental Variance ; V_G = Genetic Variance; V_A = Additive Variance; V_D = Dominant Variance; H^2_n = Narrow-Sense Heritability; and H^2_b = Broad-Sense Heritability. Negative estimates were assumed to be zero (Robinson *et al.*, 1955).

A negative estimate of dominance genetic variance was found for all traits (Table 6.4). The additive variance estimates were higher than the dominance variance for NN, NDW and shoot N .Broad sense heritability ranged from 0 to 0.80 and the narrow sense from 0 to 1.48 for all traits.

Nodule number and shoot N had high narrow sense heritabilities of 1.48 and 1.04, respectively, while shoot N had moderate broad sense heritabilities of 0.45.

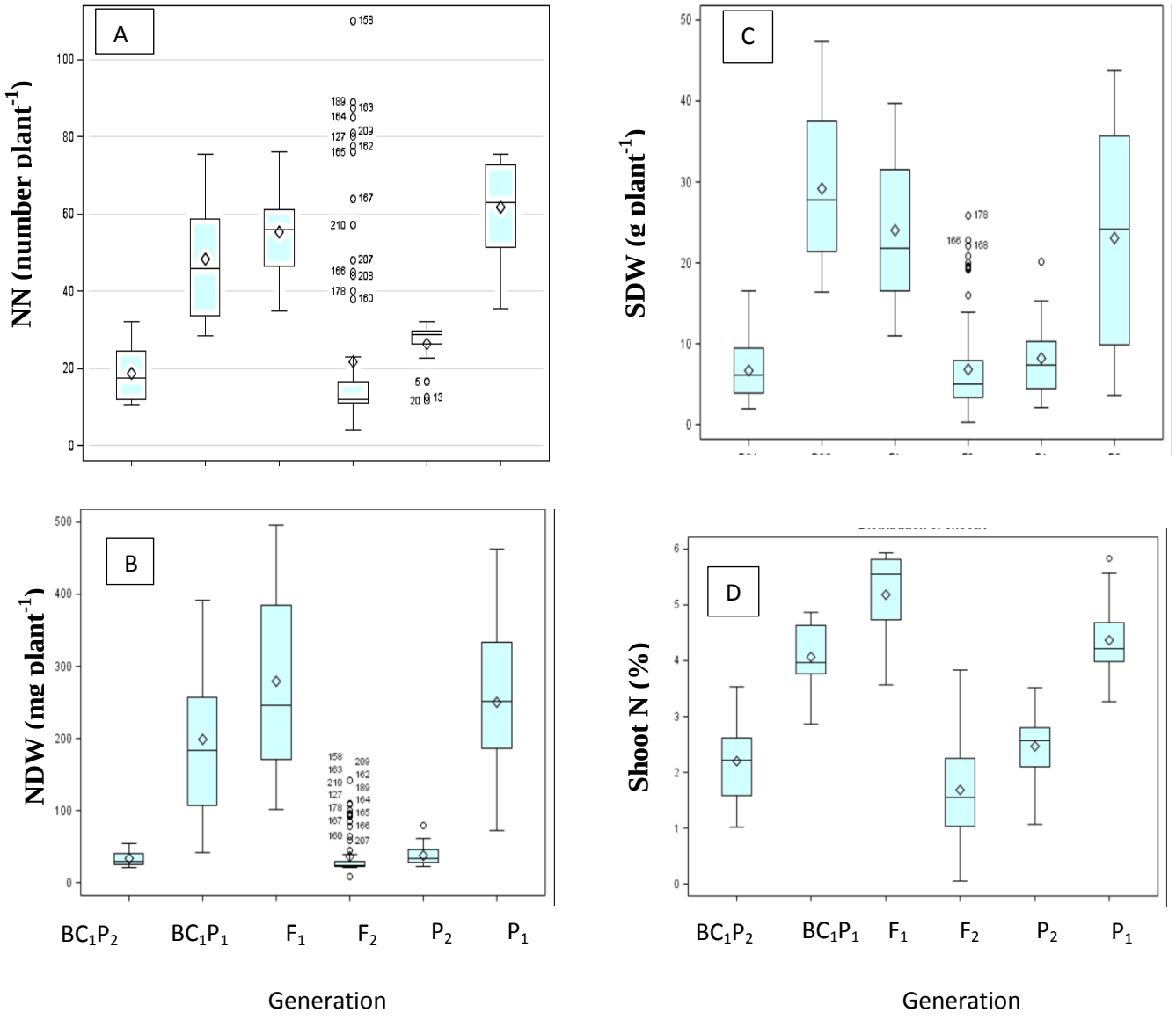


Figure 6.1 A-D: Distribution of nitrogen fixation related traits in 6 cowpea generations from 58-77 x Lori-niebe cross.

A= Nodule number (NN) , B = nodule dry weight (NDW) , C = Shoot dry weight (SDW) , D = Shoot N .Boundaries of the box closest to and farthest from zero indicate the 25th and 75th percentiles, respectively. The continuous line and geometric shape (lozenge) within the box indicate the median and mean, respectively. Error bars indicate the 10th and 90th percentiles. Circles indicate outliers.

In this study, multiple methods were used to estimate the minimum number of effective factors. The results indicate that, two methods (those of Wright and Mather) provided higher estimates of the minimum number of effective factors determining the traits, while Lande's methods provided lower estimates and were not considered in calculating the average number of effective factors (Table 6.5). The minimum number of effective factors was 0.0 for SDW and NDW. For NN, and shoot N different number of effective factors were estimated. The average was 0.8 for NN, and 0.7 for shoot N.

6.3.4 Estimates of main and epistatic gene effects

Using a six-parameter model and the six means, the main and epistatic gene effects were estimated. Results of the six-parameter model indicated significant and positive additive x additive *[aa]* epistatic effects for all traits (Table 6.6). The additive x dominance *[ad]* component was significant but negative for shoot dry weight and shoot N. In addition, the dominant *[d]* and additive *[a]* effects were significant and positive for all the traits (Table 6.6).

Table 6.5: Minimum number of effective factors (genes) for nitrogen fixation related traits derived from 58-77 x Lori cross.

Trait	EF_1	EF_2	EF_3	EF_4	EF_5	EFm
NN	0.4	0.8	0.3	0.2	2.5	0.8
NDW	-1.1	-2.6	-0.6	-0.6	-1.1	-1.2
SDW	-0.9	-4.9	-0.5	-1.2	-0.3	-1.6
Shoot N	3.3	2.1	1.2	0.5	-3.8	0.7

EF_1 = Wright's method, EF_2=Mather's method, EF_3 = Lande's method I, EF_4 Lande's method I I , EF_5= Lande's method I I I, and EF_m= average estimate considering EF_1 and EF_2 only. Negative estimates were assumed to be zero (Robinson *et al*., 1955).

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Table 6.6: Estimates of main and epistatic gene effects for nitrogen fixation related traits derived from 58-77 x Lori cross.

Gene effect	NN	NDW	SDW	Shoot N
m	21.92± 2.59***	35.72±3***	6.83±0.64***	1.68±0.10***
[a]	29.41±3.69***	164.8±19.65***	22.37±2.18***	1.87±0.22***
[d]	58.10±22.85*	456.34±91.49***	52.78±10.77***	7.55±1.14***
[aa]	46.90±18.28*	321.04±51.32***	44.42±6.90***	5.8±0.84***
[ad]	11.69±5.89ns	58.72±32.50ns	14.93±4.04**	0.91±0.35*
[dd]	17.33±35.35ns	62.67±170.96ns	-36.89±19.01ns	-1.13±1.88ns

*** Significant at the probability 0.001 level; ** Significant at the probability 0.001 level;

* Significant at the 0.05 probability level, ns = non significant.

6.4 Discussion

The means for all the traits of the high N_2-fixing parent (P_1) were higher than those of the low N_2-fixing parent (P_2). These results are in line with reports from Toomsan *et al*. (1991) that high nitrogen-fixing lines generally give high values for weight and number of nodules and shoot dry weight. The backcross generations were similar to those of their respective recurrent parents. The F_1 showed increased performance in NN, NDW, SDW and Shoot N compared to the P_2 which is an indication that effective selection is possible using these parants. The F_1 shoot N out-yielded the better parent (parent 1) indicating the presence of over dominance in the expression of this trait. The F_2, and BC_1P_2 generation means for NN, NDW, SDW and shoot N were lower than the means of the parents and F_1 generation due to segregation. The BC_1P_1 generation mean for all the traits was higher than the means of the two parental populations which indicated the contribution of 58-77 in increasing the performance in all traits studied.

In addition, the BC_1P_1 population mean was higher than any other population for SDW. Kosgei, (2014) found similar results working with chickpea. The F_1 mean for shoot N exceeded the high parent, while the F_2 population mean was significantly less than the mean of the lower parent. The overall results suggest that significant epistatic interactions are involved in the inheritance of these traits. The results agree with the findings of Philips *et al.* (1989) from peanut research.

In the previous characterization experiment, shoot N proved to be a good predictor of nitrogen fixation in low P soils. This result confirms the findings of Toomsan *et al*. (1991) where fixed nitrogen correlated with total nitrogen (0.97) and nodule weight (0.60) in peanuts and those of Sreekumar (1995) where weight of nodules in the primary root and total nodule weight were positively correlated with the nitrogen content in plant. The strong and positive association between NN, NDW and SDW with Shoot N, suggest that it could be possible to improve nitrogen-fixing ability through selection for these nitrogen fixation associated traits. These results confirm that shoot N, a predictor of nitrogen fixed, among this parental cowpea is controlled in large part by the plant's ability to form large, efficient nodules as well as good vegetative matter. A significant correlation was also observed between nodule weight and number (0.97). Miller *et al.* (1986) also reported similar results working with cowpea.

The results indicate that the dominance variance component had negative values for all traits. According to Khodambashi *et al.* (2012) negative dominance indicate reductive alleles involving the dominant phenotype. Negative variances are not uncommon and are often found for dominance variance components (Hallauer and Miranda 1988). Negative dominance variances suggest the presence of epistatic interactions in the traits measured, which could be due to a dominance bidirectional effect resulting in the cancellation between positive and negative effects of the alleles that control the trait (Fatokun *et al.*, 1992; Lark *et al.*, 1995; Maughan *et al.*,

1996). Therefore, epistasis effects would seriously bias any attempt to partition the genetic variances of the segregating generations into additive or dominance components.

The heritabilities of the traits were low to moderate and are in line with the reports of Bliss (1993) that the heritability for nitrogen fixation traits is usually low to moderate as environmental and genotype x environment effects are large, especially when measured on a single plant basis. The results indicated heritability values ranging from 0.0 to 1.48 with narrow-sense heritability values greater than one. Heritability is determined based on additive and environmental variance. High magnitudes of dominance and additive variances in addition to presence of epistasis probably caused the narrow-sense heritability to be more than one. This result confirms findings by Coates and White (1998) that exaggerated heritability could be due to epistasis and environmental influence. Samineni *et al.* (2011a) found higher heritability that ranged from 108 to 164% in chickpea under saline and control environments. Heritability values ranging from 0.22 to 0.76 have also been reported and in many of the studies few progeny performed better than the superior N_2-fixing parent (Graham *et al.*, 2004). The high broad and narrow sense heritability estimates for Shoot N are consistent with the additive variance component estimates observed. Sreekumar, (1995) also found high values for heritability estimates for plant N content in cowpea. However, estimation of variance components and narrow sense heritability estimates in multiple environments may be needed for a correct quantification of these values. For NN, NDW and shoot N different number of effective factors were estimated. These results indicate that different number of genes are responsible for the expression of NN, and Shoot N traits, while NDW and SDW probably had the same number of genes. The average was 0.8 for NN, and 0.7 for shoot N. In cowpea this is the first information

on number of genes controlling nitrogen fixation related traits based on field evaluation and calculations.

Mather and Jinks (1982) three-parameter model assumes that the genes involved are independent of each other (or epistasis is negligible), i.e., exhibit simple autosomal inheritance. The results however, indicate the presence of epistasis. For all four traits the additive x additive effect was significant and positive, while the additive x dominace effect was also significant and positive for shoot dry weight and shoot N content. Mather and Jinks (1982) proposed the association or dispersion of genes in the parents based on signs associated with epistasis gene effects such as additive x additive and additive x dominance. A positive sign for any of these parameters is an indication of interaction between increasing alleles, hence presence of association in the parental genotype. This result indicates that positive alleles were dominant and the epistatic interaction was of the duplicate gene action type rather than complementary epistasis. In peanuts, results indicate that differences in nodulation and N_2 fixation were heritable and influenced mainly by additive-gene effects (Wynne, 1978). The results of this study showed contrasting findings as epistasis is involved. Epistatic effects generally were found to be also as important as the additive effects for all traits. These findings agree with those of Sreekumar, (1995) who reported that nitrogen fixation traits in cowpea including plant N content is controlled by non-additive genes. Among the three types of epistasis, the *[aa]* component was the most important and was positive. Additive gene effects are fixable indicating that selection for such traits is effective.

6.5 Conclusion

As expected, mean values of the backcrosses tended to be located close to those of their respective recurrent parents. Based on heritability estimates, there is the possibility to improve nitrogen fixation and by selecting families from the cross using Shoot N as selection criteria.

Phenotypic correlations indicated that nodule number, nodule dry weight, shoot dry weight and shoot N may be useful as selection criteria for high nitrogen fixation and may help in reducing research cost, since direct measurement of nitrogen fixed may not be necessary. The inheritance of the traits indicates that both additive and non-additive gene actions were important for all the characters under study. All the traits had significant additive and/or addiditve x aditive gene effects leading to the conclusion that selection should not be done at early generation to take advantage of homozygozity when additive and additive variances are prevailing. In a nut shell, with improving N_2 fixation as a goal, plant breeders need to understand the inheritance of the traits affecting fixation so an appropriate breeding strategy can be formulated.

CHAPTER SEVEN

QUANTITATIVE TRAIT LOCI ANALYSIS OF EPISTASIS AND PHOSPHORUS ENVIRONMENT EFFECTS FOR BIOLOGICAL NITROGEN FIXATION AND YIELD RELATED TRAITS IN COWPEA.

7.1 Introduction

Breeding for increased N_2 fixation can improve legume crops that are normally dependent on N fertilizer for significant yields, and promote the development of low-input cropping systems. One of the main constraints for BNF improvement is that quantitatively inherited traits such as nodulation are greatly influenced by the environment (Bliss, 1985). Two main options for improving N_2 fixation include the management of the legume to maximize growth and minimize stresses such as mineral nitrogen shortage (Tsai *et al.*, 1998b) and other soil factors (Peoples *et al.*, 1995), and breeding legumes with enhanced capacity for N_2 fixation.

Cowpea [*Vigna unguiculata* (L.) Walp] is a warm weather, drought-tolerant crop well-adapted to the drier regions of the tropics, where other food legumes do not perform well. This makes cowpea an important component of traditional intercropping systems, especially in the complex, subsistence farming systems of the dry savannas in sub-Saharan Africa (Sanginga, 2003). With its greater tolerance to heat, drought, and low soil fertility (Hall, 2004) and yet close evolutionary relatedness to other economically important grain legumes such as common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), cowpea can serve as a model species for crop adaptation to these stresses. Cowpea (*Vigna unguiculata*) is also closely related to mung bean (*Vigna radiata*) and shares more distant common ancestry with common bean (*Phaseolus vulgaris*), soybean (*Glycine max*), and pigeon pea (*Cajanus cajans*) (Choi *et al*,. 2004). Cowpea germplasm is

notably diverse, especially when considering tolerance to several biotic and abiotic stresses; however, the genetics of these traits are not sufficiently understood in the context of modern, marker-assisted, breeding. Many agronomically important traits display a continuous phenotypic distribution. These quantitatively inherited traits are typically influenced by several loci and the environment, and are difficult to breed using conventional methods reliant on phenotypic assessments. Associations between genotype and phenotype can expedite development of improved varieties containing favorable alleles for several traits through streamlined approaches to breeding.

There have been few reports of nodule traits being genetically mapped in soybean, (Nicola´s *et al.,* 2006; Santos *et al.,* 2013; Tanya *et al.*, 2005). For example, Nicola´s *et al.* (2006) identified QTL for nodule weight, nodule number, and individual nodule weight in an F_2 population of 160 plants. Tanya *et al.* (2005) also evaluated nodule related traits in 136 RILs from an F_5-derived population using 85 SSR markers. Santos *et al.* (2013) described QTL for nodule number and individual nodule weight on a population of 157 F_2:$_7$-derived lines. In cowpea, QTL have been detected for many traits such as seed weight and pod shattering (Andargie *et al.,* 2011), thrips resistance (Muchero *et al.,* 2010), heat tolerance (Lucas *et al.,* 2013), and aphid resistance (Huynh *et al.,* 2015) but to date no QTL have been reported for nitrogen fixation related traits. BNF has been characterized as a quantitatively-inherited (McFerson, 1983), complex trait (Mytton, 1984), and no simply-inherited characters have been described with BNF (Rosas and Bliss, 1986). Selection for such traits can greatly be enhanced by the use of genetic markers. Key genomic resources facilitating marker–trait associations are now available for cowpea. These include single nucleotide polymorphism (SNP) genotyping assays (Muchero *et al.*, 2009), a consensus genetic map containing 1107 transcript-derived SNP markers (Lucas *et al.*, 2011), and

knowledge of synteny with crop and model species (Wanamaker and Close, 2011). However, due to technical difficulties in evaluating phenotypic traits related to nitrogen fixation (nodulation and nitrogen fixed), the mechanisms and genetic control of these traits are still poorly understood. An interesting and informative way to dissect the genetic architecture is to map quantitative trait loci (QTL). As mentioned above, this approach has been broadly used in soybean to identify traits of interest, such as yield potential, disease resistance, contents of protein and oil in seeds (Soybase, 2012). Preliminary studies about genomic regions controlling BNF in soybean have been performed (Tanya *et al.*, 2005; Nicolas *et al.*, 2006; Santos *et al.*, 2006). There is also information about other legumes, such as pea (Bourion *et al.*, 2010) and common bean (*Phaseolus vulgaris* L.) (Nodari *et al.,* 1993; Tsai *et al.*,1998b). These studies have demonstrated the potential of mapping QTL for BNF traits, but no information is available for cowpea on BNF related traits.

Until recently only limited progress had been made in basic gene discovery and gene regulation in cowpea, with such information available for *Vigna* species being 2- to 5-fold less than for pea (*Pisum sativum*), common bean (*Phaseolus vulgaris*), and alfalfa (*Medicago sativum*) and 500-fold less than for soybean (*G. max*) (Timko et al, 2008). This work used recently developed SNPs linkage map between RILs developed from two cowpea varieties 58-77 (high N_2-fixing) and Yacine (low N_2-fixing) for genotypic differentiation. Identification of genetic loci affecting quantitative trait loci (QTL) such as number of root nodules formed (NN), nodule dry weight (NDW), shoot dry weight (SDW), number of pods per plant (Npod), weight of hundred dry seeds (HUN_SDW) and their possible interactions with one another and the environment were studied in F_{11}-RILs, grown at two levels of phosphorus nutrition on low phosphorus fields and in the screen house with soils from same fields.

The objectives of this study were to:

1) identify QTL associated with nitrogen fixation related traits of cowpea F_{11}-RIL population under low P condition.

2) detect QTL for number of pods per plant and dry weight of hundred seeds in cowpea F_{11}-RIL population under low P condition, and

3) analyze epistasis, epistasis by P environment and QTL by P environment effects.

7.2 Materials and Methods.

7.2.1 Plant material

A population consisting of 118 Recombinant Inbred Lines (F_{11}; RILs) derived from a cross between the contrasting cowpea genotypes Yacine and 58-77 that were previously evaluated for nodulation capacity in the Humid Forest Zone (HFZ) of Cameroon were used in the study. Out of 118 RILS evaluated, 88 RILs with complete data in all eight environments were finally used in QTL mapping. These RILs were developed in the Senegalese Agricultural Research Institute (ISRA*)* through inbreeding and single Seed descent. The genotype 58-77 is a black small-seeded local cultivar from Senegal resistant to pests and diseases with good nodulation and nitrogen-fixing properties while Yacine is also a variety developed in Senegal that has large brown seeds, and very poor nodulation and low nitrogen-fixing potentials.

7.2.2 The experimental site

The study was conducted at two sites.The first site was at the IRAD (Institut de la Recherche Agricole pour le Développement) Research Station, in Nkoemvone, in the HFZ of Cameroon (situated between longitude 11^{o} 6’ E and 11 ^{o} 10 ’ E, latitude 2^{o} 53’ N, and 2^{o} 57’ N, and altitude of 615 m). The average annual rainfall is 1820 mm with a bimodal distribution and mean daily temperature of 23.5^{o} C. The soils are Ferric Acrisols characterized by a low base saturation and a

low cation exchange capacity (Table 4.2). The soils for the study area are highly acidic, with pH (1:1 H_2O) 4.5. The vegetation consists of secondary humid forest. The second site, Nkometou, is a village in the Yaoundé neighborhoods, in the HFZ of Cameroon. Geographically, the study area is situated between latitude 3º51' and 3º53' N, and longitude 11º25' E and 11º27' E and has an altitude of 813 m. The climate is Equatorial with two rainy seasons corresponding to two cropping seasons: March to June and August to November. The average rainfall is 1692 mm with bimodal distribution; the mean daily temperature ranges from 19.2 to 28.6ºC. The soils are also Ferric Acrisols, characterised by low base saturation and a low cation exchange capacity. The vegetation is evergreen forest, severely degraded by human activities, especially agriculture and timber exploitation.

7.2.3 Soil sampling

A composite soil sample was collected from different locations in each of the two fields. The soil was collected from low N plots to a depth of 20 cm after removing surface litter. The low N plots were created by planting maize for two seasons at very high densities (100,000 plants /ha).

7.2.4 Plant and soil analyses

Soil samples were analyzed for pH, organic C, N, exchangeable Ca, Mg, K, and extractable P as earlier presented in section 4.2.3. For plant samples, all fresh biomass samples were oven-dried at 65^{o} C, then ground to pass a mesh of 0.5 mm, and digested according to Novozamsky *et al.* (1983). Total N was determined with an ammonium sensitive electrode (Powers *et al.*, 1981). Total P was determined by the malachite green colorimetric procedure (Motomizu *et al.*, 1983). Soil particle size was also determined as earlier presented in section 4.2.3.

7.2.5 Phenotyping 1: Field Evaluation

Evaluation of cowpea RILs was done on low nitrogen plots. The experimental design was a split-plot with two replicates. The main plots were two phosphate levels: 0 P and 30 Kg P ha^{-1} (Triple super phosphate, TSP), while the 118 RILs and the two parents constituted the sub-plots randomized in a 12 x 10 α-lattice design. Plots were fertilized uniformly with K (KCl) at 80 Kg ha^{-1}. Lime $Ca(OH)_2$ at the rate of 924kg of CaO per ha was incorporated into soil during land preparation. This dose followed the recommendations by Kamprath (1970). On the field, each of the 118 RILs was planted in a single row of 5m length at a spacing of 50cm between rows and 50 cm within rows to allow sampling of plants to record data for nodulation. All plants were sprayed twice (before flowering and after pod setting) with the insecticide Thiodan® (endosulfuran organochlorine) at a concentration of 0.33 mg/L. The experimental area was bordered on either side by guard rows in order to minimize border effects. The field was hand weeded twice, two and four weeks after planting.

7.2.6 Phenotyping 2: Pot experiment

Two screen house experiments were carried out at IITA Cameroon with soils collected from low N plots at Nkometou and Nkoemvone at 0– 20 cm depth. The soil was air dried, sieved through a 2mm screen and homogenized. The 118 RILs and their parents were grown. Plants were grown in 5L capacity pots containing 4.3 kg of non-sterile soil with one plant growing per pot after thinning. The experimental design was a factorial randomized complete block design with two factors, and two replications (two pots per RIL per replication for nodulation and yield traits, respectively). The factors were phosphorus level (0 and 30 mg P per Kg soil) and genotypes. Seed sterilization and cultural practices were done as earlier described in section 5.2.5. Data collected was same as for field experiment. Bulk density and gravitational soil moisture were

measured as described in section 5.2.6. Phosphorus and potassium were supplied as KH_2PO_4 and muriate of potash, respectively. Prior to sowing, seeds were surface sterilized with 95% ethanol for 1 min, and 3% H_2O_2 for 5 min, then rinsed with sterile water (Vincent, 1970). Three seeds of each genotype were sown in each pot and thinned to one plant per pot one week after emergence. Before sowing, P and K nutrients were applied as mentioned above. One milliliter of a combination of micronutrients per kg soil was also applied (Vincent, 1970). Pots were watered and maintained at field capacity. Soil rhizobial population was estimated using the Most Probable Number (MPN) method (Weaver and Frederick, 1972; Vincent, 1970). The soil rhizobia population was found to be high (>10^3 rhizobium bacteria per g soil) which made artificial inoculation unnecessary for the soils (Thies *et al.,* 1991; Brockwell *et al.,* 1995).

7.2.7 Data collection

Data was collected on nitrogen fixation and yield related traits. It included nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), taken at early podding stage (section 5.2.7), number of pods (Npod) and dry weight of hundred seeds (HUN_SDW) collected at the RH(80% maturity) stage.

7.2.8 Data analyses of phenotypic data

Phenotypic data were subjected to univariate analysis using SAS 9.2 (SAS Institute Inc., Cary, NC, USA, 2008). Normality was tested per trait and per environment. The means of parents were compared using a student t test. A 5 % false-positive value was chosen as a significant criterion.

7.2.9 Genotyping

The parent inbreds, Yacine and 58-77, and 141 RILs developed from their cross had been prevously genotyped for 1,536 SNPs using the GoldenGate assay (Muchero *et al.,* 2009, Lucas *et*

al., 2011). Out of the 1,536 SNPs genotyped, 435 SNPs specific for Yacine – 58-77 population was mapped on the recent cowpea consensus genetic map (Lucas *et al.*, 2011). This genotype data was used for this study. However, confirmation of the genotype data for the population that was phenotyped for this study was done by sending leaf samples from all RILs and parents to KBiosciences, UK for re-genotyping with some genome-wide markers that were run on the Golden Gate assay (Illumina) in 2011. This exercise consisted of extracting DNA from 120 leaf samples and genotyping with 120 polymorphic SNPs from among the 435 SNPs mapped on the recent cowpea consensus genetic map (Lucas *et al.*, 2011). The DNA samples were initially tested at four different dilutions to ensure that the DNA was of the correct quality to genotype. The overall quality was regarded to be sufficient and samples were then screened with 120 assays. Genotyping for SNP analysis was performed by KBioscience using the competitive allele specific PCR (KASP) chemistry coupled with a FRET-based genotyping system http://www.kbioscience.co.uk/reagents/KASP/KASP.html. Genotyping data was viewed usingSNPviewer (KBioscience, Hertfordshire, UK), allowing graphical viewing of the clusters that group the allele calls. The two sets of data (genome wide genotyping (Kbioscience, 2014) and illumine gold, 2011 were checked by pairwise genotype comparisons of the common SNPs and found to be 99% similar. Then 372 polymorphic SNPs were finally used after removing SNPs that occupied the same position on the linkage group.

7.2.10 QTL mapping of nitrogen fixation and yield related traits

Win cartographer version 2.5 was used to map QTL per environment using composite interval mapping. The QTL were named beginning with "q" standing for QTL, followed by trait name and the linkage group number. In cases where there were more than one QTL on a linkage group for the same trait, the serial number was added after the linkage group number separated by a

dot. A threshold of 2.5 was retained for all traits after 1000 permutations. QTLnetwork 2.1 (Yang *et al.*, 2005) that uses a model that includes the effects of multiple QTL, epistasis, QTL-by-environment interactions and epistasis-by-environment interactions was also used. The map distances were estimated based on the Kosambi function. This mapping strategy is based on marker interval selection, detection of marker interval interactions and genome scans, to evaluate putative locations of multiple QTL and their interactions. An F-statistics was used for hypothesis tests. In each of the mapping procedures, permutation testing was exploited to control for genome-wide false positive rate, and model selection was used to reduce ghost peaks in F-statistic profile. The thresholds of the QTL (LOD scores) were obtained at p = 0.05 by 1,000 random permutations of the trait values. Parameters of the full-QTL model were estimated using a Bayesian method via Gibbs sampling. The different stages in QTL mapping using the the QTLnetwork software involved mapping main QTL by one dimensional (1D) genome scan and epistasis by two-dimensional (2D) genome scan.

7.3 Results

7.3.1 Transgressive segregation of traits

The phenotypic behavior of NN, NDW, SDW, Npod and Hun_SDW for the RIL population and its parents under the eight environments are described in Table 7.1 for the pot experiments and Table 7.2 for the field experiments. The parent 58-77 had higher means for NN, NDW, SDW and Npod while Yacine had a higher mean for HUN-SDW than 58-77 in both experiments. Considering a particular trait, the means were different under different environments and transgressive segregants were observed across all eight environments with some RILs higher than the better N_2-fixing parent, 58-77, or lower than the poor N_2-fixing parent, Yacine. The five

traits of the RIL population under study segregated continuously as indicated by the absolute skew and kurt values (Tables 7.1 and 7.2).

Table 7.1: Phenotypic values of nitrogen fixation and yield related traits among parents and RIL population per evnironment in pot experiments.

		Parents		RIL population						
		……...		……						
ENV	**Trait**	**58-77**	**Yacine**	**Mean**	**Max**	**Min**	**Stdev**	**C V(%)**	**Skew**	**Kurt**
PHP1	NN	38±2.12	7±2.12	16.03	92.50	1.50	15.31	95.47	2.18	7.18
	NDW	22.42±1.20	10.35±1.20	39.70	224.85	0.65	50.15	126.33	1.93	3.51
	SDW	2.82±0.74	1.61±0.72	2.61	9.85	0.75	1.51	57.96	2.28	6.95
	Npod	14±4.24	4.5±0.71	7.89	20.00	2.50	2.64	33.41	1.22	4.09
	HUN_SDW	8.16±0.01	18.40±1.41	12.19	18.90	7.95	2.19	17.99	0.44	-0.01
PLP1	NN	15±1.41	5±1.41	14.34	34.00	1.00	8.88	61.98	0.36	-0.89
	NDW	22.42±5.54	8.91±0.58	25.41	150.05	40.05	27.77	109.28	2.05	5.01
	SDW	1.75±0.06	0.495±0.21	1.49	3.71	0.02	0.80	53.67	0.63	0.03
	Npod	7.5±2.12	2±0.00	2.81	6.50	1.00	1.34	47.71	0.90	0.72
	HUN_SDW	5.93±0.19	17.175±0.19	10.26	16.00	6.30	2.10	20.48	0.61	0.04
PHP2	NN	116±9.90	30.5±4.95	65.13	165.50	0.00	39.34	60.40	0.42	-0.38
	NDW	325±49.50	20±14.14	128.81	330.00	0.00	80.12	62.20	0.35	-0.47
	SDW	13.66±1.20	5.89±4.06	8.38	15.74	1.68	3.17	37.81	0.01	-0.36
	Npod	7.5±3.54	3±1.41	9.05	24.00	3.00	3.70	40.89	1.25	2.74
	HUN_SDW	8.76±0.70	20.06±0.70	13.27	21.30	7.60	3.01	22.70	0.57	0.01
PLP2	NN	84.5±6.36	0.00	32.31	91.00	1.00	20.52	63.51	0.93	0.36
	NDW	85±77.78	0.00	34.62	110.00	0.00	25.44	73.47	0.85	0.37
	SDW	7.82±1.58	3.14±0.23	5.43	9.65	1.96	1.84	33.88	0.16	-0.75
	Npod	4.5±2.12	1.5±0.71	3.97	11.00	1.00	1.84	46.42	1.41	2.69
	HUN_SDW	7.04±0.19	18.25±0.19	11.54	19.55	5.85	3.01	26.10	0.57	0.00

ENV = Environment; PHP1 = Nkometou high p, PLP1 = Nkometou low P , PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low p in pot experiments. Stdev = standard deviation and CV is coefficient of variation. The means of the parents are given ± stdev. NN= nodule number, NDW= nodule dry weight, SDW = shoot dry weight, Npod = number of pods per plant, HUN_SDW= hundred seed dry weight.

Table 7.2: Phenotypic values of nitrogen fixation and yield related traits among parents and RIL population per evnironment in field experiments.

		Parents		RIL population						
		.....		.....						
ENV	**Trait**	**58-77**	**yacine**	**mean**	**max**	**min**	**Stdev**	**cv (%)**	**skew**	**Kurt**
FHP1	**NN**	90.5±10.61	40.5±3.54	82.07	374.00	12.50	62.90	76.65	3.57	12.73
	NDW	252.88±20.10	93±47.80	125.24	666.18	88.38	76.08	60.75	5.16	31.33
	SDW	246.27±18.78	91.4±33.87	179.34	485.98	73.53	93.65	52.22	1.51	1.66
	Npod	61.5±4.95	28.5±2.12	37.11	71.00	22.50	10.63	28.66	1.57	2.58
	HUN_SDW	9.09±1.12	20.94±0.83	12.92	22.16	7.18	3.07	23.79	0.71	0.53
FLP1	**NN**	41.5±9.19	11.5±0.71	17.80	87.00	10.50	16.82	94.46	3.22	9.48
	NDW	86±11.31	38.75±1.77	30.15	136.00	20.50	18.72	62.08	3.55	13.99
	SDW	9.37±2.35	1.68±0.25	7.40	104.40	0.90	10.89	147.09	8.31	74.44
	Npod	5.1±0.42	1.2±0.85	3.47	10.50	1.00	1.66	47.96	1.30	3.28
	HUN_SDW	8.42±0.27	19.16±0.44	12.77	21.10	6.97	3.08	24.10	0.58	0.11
FHP2	**NN**	240.24±35.64	92.56±14.02	208.09	483.00	29.40	93.68	45.02	0.28	-0.49
	NDW	722.75±150.26	213.5±229.81	674.96	1151.25	30.15	280.97	41.63	-0.34	-0.76
	SDW	28.32±3.52	9.75±2.97	27.68	83.10	7.43	13.93	50.33	1.27	2.44
	Npod	74.55±7.67	39.7±4.38	62.15	177.50	29.00	32.07	51.61	1.58	2.53
	HUN_SDW	8.85±0.52	20.85±1.25	12.32	19.31	6.80	2.54	20.62	0.90	0.97
FLP2	**NN**	50.2±3.68	29.4±0.28	31.85	63.80	26.40	5.82	18.26	2.97	12.17
	NDW	247.5±17.68	66.75±31.47	73.62	424.25	11.00	64.19	87.20	2.63	9.96
	SDW	19.6±3.32	6.025±2.72	12.13	37.40	2.48	6.49	53.52	1.34	2.69
	Npod	6.15±1.06	1.8±0.85	8.30	29.50	1.00	5.57	67.10	1.28	2.07
	HUN_SDW	8.36±0.25	21.03±1.02	12.33	19.99	8.05	2.18	17.66	0.98	2.13

ENV = Environment; FHP1 = Nkometou high p, FLP1 = Nkometou low P , FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low p in field experiments. Stdev = standard deviation and CV is coefficient of variation. The means of the parents are given ± stdev. NN= nodule number, NDW= nodule dry weight, SDW = shoot dry weight, Npod = number of pods per plant, HUN_SDW= hundred seed dry weight.

7.3.2 Quantitative trait loci for nitrogen fixation and yield related traits identified by Win Cartographer per environment.

Win Cartographer identified a total of 26 QTL for NN (Table7.3), 31 QTL for NDW (Table 7.4), 32 QTL for SDW (Table 7.5), 12 QTL for HUN_SDW (Table 7.7) in seven of the eight environments and 14 QTL for Npod (Table 7.6) in all eight environments. Summary of marker number and positions are presented in Figure 7.1. Constitutive QTL such as qSDW3.4 (Table 7.7, Figure 7.2) was identified in four of the eight environments, while qNN3.3 (Table 7.3), qNDW6.2, qNDW11.2 (Table 7.4) and qNpod1.1 (Table 7.6) were identified in three of the eight environments.

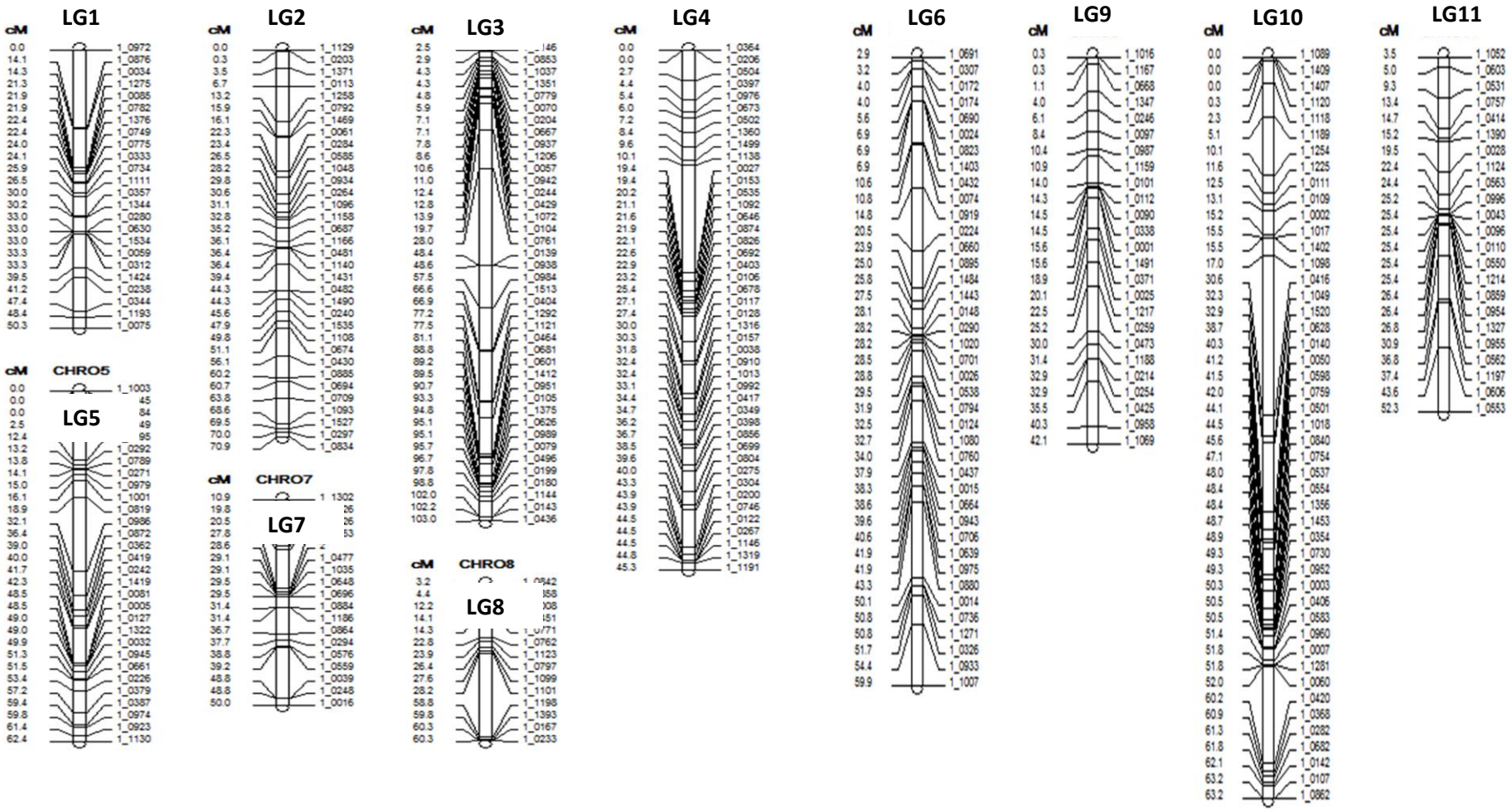

Figure 7. 1 Partial linkage map of cowpea used for QTL mapping.

It was constructed based on genotypic information from 372 EST-SNPs loci of an F_{11} RIL mapping population from 58-77 x Yacine cross. Marker loci are shown on the right side of each linkage group (LG) and genetic distances (cM) are indicated on the left as mapped by Win Cartographer 2.5 using the Kosambi function.

Table 7.3: Nodule number (NN) QTL with main effects identified by Win Cartographer in eight environments.

Trait	EVN	QTL	LG	Marker	Lod score	additive	R^2
NN (26QTL, 7 EVNs)	FHP1	qNN3.3	3	1_0938	21.91	133.62	0.5
	FHP1	qNN6.1	6	1_0224	33.05	-134.23	0.6
	FHP1	qNN7.1	7	1_1186	22.23	133.62	0.5
	FHP1	qNN8.1	8	1_1101	31.42	134.23	0.6
	FHP1	qNN10.1	10	1_1189	2.73	-20.58	0.1
	FHP1	qNN11.2	11	1_1124	23.25	133.62	0.5
	FHP1	qNN11.4	11	1_1197	13.90	133.62	0.5
	FHP1	qNN11.5	11	1_0606	19.15	133.62	0.5
	FLP1	qNN2.1	2	1_0585	6.02	-10.95	0.2
	FLP1	qNN2.3	2	1_1431	3.04	8.06	0.1
	FLP1	qNN3.3	3	1_0938	19.61	29.58	0.4
	FLP1	qNN6.1	6	1_0224	18.16	-29.30	0.5
	FLP1	qNN8.1	8	1_1101	17.99	26.89	0.4
	FLP1	qNN11.1	11	1_1052	19.74	26.89	0.4
	FLP1	qNN11.3	11	1_1327	20.07	26.89	0.4
	FLP1	qNN11.4	11	1_1197	11.77	29.64	0.4
	PHP1	qNN3.2	3	1_0139	3.84	26.15	0.2
	FHP2	qNN7.2	7	1_0559	2.84	-176.78	0.1
	FLP2	qNN3.2	3	1_0139	5.22	8.29	0.2
	FLP2	qNN3.4	3	1_1513	5.10	-2.20	0.1
	FLP2	qNN11.2	11	1_1124	4.80	8.87	0.2
	PHP2	qNN2.2	2	1_1149	3.58	-17.34	0.1
	PHP2	qNN4.1	4	1_0535	4.54	-18.70	0.2
	PHP2	qNN9.2	9	1_1188	6.00	-19.81	0.2
	PLP2	qNN3.3	3	1_0938	5.12	22.60	0.3
	PLP2	qNN9.1	9	1_0473	2.97	-7.47	0.1

Rows highlighted in same colour represent a constitutive QTL detected in more than one Environment. LG= linkage group. ENV = Environment; FHP1 = Nkometou high p, FLP1 = Nkometou low P , FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low p in field experiments . PHP1 = Nkometou high p, PLP1 = Nkometou low P , PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low p in pot experiments. The QTL are named beginning with "q" standing for QTL, followed by trait name and the linkage group number. In cases where there are more than one QTL on a linkage group for the same trait, the serial number is added after the linkage group number separated by a dot.

Table 7.4: Nodule dry weight (NDW) QTL with main effects identified by Win Cartographer in eight environments.

Trait	EVN	QTL	LG	Marker	Lod score	additive	R^2
NDW (31QTL, 7 ENVs)	FHP1	qNDW5.1	5	1_0095	29.84	112.13	0.4
	FHP1	qNDW6.1	6	1_0919	18.90	-96.47	0.2
	FHP1	qNDW6.2	6	1_0224	21.49	-96.47	0.2
	FHP1	qNDW8.1	8	1_1101	21.50	96.47	0.2
	FHP1	qNDW11.1	11	1_1124	24.59	96.47	0.2
	FHP1	qNDW11.2	11	1_0606	20.96	96.47	0.2
	FLP1	qNDW2.1	2	1_0585	4.65	-9.75	0.2
	FLP1	qNDW3.2	3	1_0244	10.89	28.31	0.4
	FLP1	qNDW3.3	3	1_0139	19.81	27.12	0.4
	FLP1	qNDW8.1	8	1_1101	15.83	27.12	0.4
	FLP1	qNDW11.3	11	1_0028	15.33	27.12	0.4
	FLP1	qNDW11.4	11	1_1327	16.38	28.01	0.4
	FLP1	qNDW11.2	11	1_0606	15.35	27.96	0.4
	PHP1	qNDW3.3	3	1_0139	8.65	52.52	0.5
	PHP1	qNDW3.4	3	1_0938	3.92	52.91	0.5
	PHP1	qNDW5.2	5	1_0872	3.16	35.84	0.3
	PHP1	qNDW10.1	10	1_1098	6.89	56.87	0.5
	PHP1	qNDW10.2	10	1_0416	6.59	56.78	0.4
	PHP1	qNDW11.2	11	1_0606	4.01	-20.09	0.1
	PLP1	qNDW1.1	1	1_0972	5.21	33.84	0.4
	PLP1	qNDW3.3	3	1_0139	6.45	28.12	0.4
	PLP1	qNDW6.2	6	1_0224	3.14	-28.45	0.3
	PLP1	qNDW7.1	7	1_1186	3.69	31.43	0.4
	FLP2	qNDW5.3	5	1_0661	4.62	-29.27	0.2
	FLP2	qNDW6.3	6	1_1403	6.52	-35.16	0.2
	FLP2	qNDW6.2	6	1_0224	3.59	-74.55	0.2
	FLP2	qNDW6.4	6	1_0437	3.39	26.68	0.1
	FLP2	qNDW7.1	7	1_1186	2.68	78.73	0.1
	FLP2	qNDW11.1	11	1_1124	4.36	134.04	0.2
	PHP2	qNDW9.1	9	1_1188	3.48	-31.11	0.1
	PLP2	qNDW1.2	1	1_0344	3.13	28.93	0.1

Rows highlighted in same colour represent a constitutive QTL detected in more than one Environment. LG= linkage group. ENV = Environment; FHP1 = Nkometou high p, FLP1 = Nkometou low P , FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low p in field experiments. PHP1 = Nkometou high p, PLP1 = Nkometou low P , PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low p in pot experiments.

Table 7.5: Shoot dry weight (SDW) QTL with main effects identified by Win Cartographer in eight environments.

Trait	EVN	QTL	LG	Marker	Lod score	additive	R^2
SDW (32QTL, 7EVNs)	FHP1	qSDW1.1	1	1_0972	6.58	114.33	0.4
	FHP1	qSDW1.2	1	1_1344	7.29	106.87	0.5
	FHP1	qSDW3.2	3	1_0244	5.71	114.96	0.4
	FHP1	qSDW3.5	3	1_0938	6.96	102.63	0.4
	FHP1	qSDW8.1	8	1_1101	6.30	100.82	0.4
	FHP1	qSDW10.2	10	1_1520	5.19	113.38	0.4
	FHP1	qSDW11.1	11	1_0028	2.74	102.83	0.4
	FHP1	qSDW11.4	11	1_1327	7.09	106.82	0.4
	FLP1	qSDW3.4	3	1_0139	33.44	24.82	0.4
	FLP1	qSDW6.3	6	1_0224	24.79	-24.82	0.4
	FLP1	qSDW8.1	8	1_1101	27.54	24.82	0.4
	FLP1	qSDW11.2	11	1_1124	32.62	24.82	0.4
	FLP1	qSDW11.5	11	1_1197	23.65	24.82	0.4
	PHP1	qSDW1.1	1	1_0972	6.70	2.18	0.3
	PHP1	qSDW3.4	3	1_0139	7.22	2.20	0.3
	PHP1	qSDW5.1	5	1_0095	3.09	1.10	0.2
	PHP1	qSDW7.1	7	1_1186	3.73	2.17	0.3
	PHP1	qSDW10.1	10	1_1098	6.26	1.92	0.3
	PHP1	qSDW10.2	10	1_1520	10.96	2.02	0.5
	PHP1	qSDW10.3	10	1_0754	3.21	0.54	0.1
	PLP1	qSDW3.5	3	1_0938	2.71	-0.35	0.2
	PLP1	qSDW6.1	6	1_0307	2.77	-0.26	0.1
	FHP2	qSDW3.4	3	1_0139	2.64	23.53	0.2
	FHP2	qSDW10.2	10	1_1520	4.55	20.88	0.2
	FLP2	qSDW3.3	3	1_1072	3.22	-2.26	0.1
	FLP2	qSDW3.4	3	1_0139	2.99	8.21	0.2
	FLP2	qSDW6.2	6	1_0919	3.16	-2.99	0.1
	PHP2	qSDW2.1	2	1_1048	3.98	-1.28	0.1
	PHP2	qSDW4.1	4	1_0692	5.71	-1.44	0.2
	PHP2	qSDW9.1	9	1_0987	2.58	-0.94	0.1
	PHP2	qSDW11.3	11	1_0563	4.27	1.32	0.1
	PHP2	qSDW11.4	11	1_1327	7.09	1.37	0.2

Rows highlighted in same colour represent a constitutive QTL detected in more than one Environment. LG= linkage group. ENV = Environment; FHP1 = Nkometou high p, FLP1 = Nkometou low P , FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low p in field experimenst . PHP1 = Nkometou high p, PLP1 = Nkometou low P, PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low p in pot experiments. The QTL are named beginning with "q" standing for QTL, followed by trait name and the linkage group number.

Table 7.6: Number of pods (Npod) per plant QTL with main effects identified by Win Cartographer in eight environments.

Trait	EVN	QTL	LG	Marker	Lod score	additive	R^2
Npod	FHP1	qNpod1.2	1	1_1111	2.87	3.99	0.1
(14QTL, 8	FHP1	qNpod3.1	3	1_0139	5.05	12.64	0.4
EVNs)	FHP1	qNpod10.4	10	1_0142	4.67	-6.81	0.2
	FLP1	qNpod6.1	6	1_1484	2.65	-0.85	0.2
	PHP1	qNpod11.1	11	1_0028	2.53	-0.80	0.1
	PLP1	qNpod1.1	1	1_0972	2.67	1.31	0.4
	FHP2	qNpod4.1	4	1_0504	2.54	15.42	0.2
	FLP2	qNpod6.2	6	1_0326	2.86	4.54	0.3
	FLP2	qNpod10.1	10	1_1098	3.21	4.61	0.4
	FLP2	qNpod10.2	10	1_0416	3.97	4.75	0.4
	PHP2	qNpod1.1	1	1_0972	2.86	6.31	0.3
	PHP2	qNpod1.1	1	1_0972	4.23	2.18	0.4
	PLP2	qNpod5.1	5	1_0032	4.33	2.18	0.4
	PLP2	qNpod6.2	6	1_0326	2.63	2.02	0.4

Rows highlighted in same colour represent a constitutive QTL detected in more than one Environment. LG= linkage group. ENV = Environment; FHP1 = Nkometou high p, FLP1 = Nkometou low P, FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low p in field experiments. PHP1 = Nkometou high p, PLP1 = Nkometou low P, PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low p in pot experiments. The QTL are named beginning with "q" standing for QTL, followed by trait name and the linkage group number. In cases where there are more than one QTL on a linkage group for the same trait, the serial number is added after the linkage group number separated by a dot.

Table 7.7: Hundred seed weight (HUN_SDW) QTL with main effects identified by Win Cartographer in eight environments.

Trait	EVN	QTL	LG	Marker	Lod score	additive	R^2
Hun_SDW (12 QTL, 7 EVNs)	FHP1	qHun_SDW6.1	6	1_0691	6.43	-1.47	0.2
	FHP1	qHun_SDW6.5	6	1_1080	2.65	0.94	0.1
	FLP1	qHun_SDW6.2	6	1_0307	6.32	-1.54	0.2
	PHP1	qHun_SDW5.1	5	1_0819	3.22	-0.83	0.1
	FHP2	qHun_SDW11.1	11	1_1390	3.53	-1.06	0.1
	FLP2	qHun_SDW3.1	3	1_0139	3.88	0.95	0.2
	FLP2	qHun_SDW6.3	6	1_0919	4.10	-3.26	0.4
	FLP2	qHun_SDW6.4	6	1_0895	7.70	-3.44	0.3
	FLP2	qHun_SDW7.1	7	1_0559	2.80	-2.22	0.1
	FLP2	qHun_SDW10.1	10	1_1254	4.69	1.03	0.2
	PHP2	qHun_SDW6.1	6	1_0691	5.23	-1.38	0.2
	PLP2	qHun_SDW6.2	6	1_0307	5.22	-1.37	0.2

Rows highlighted in same colour represent a constitutive QTL detected in more than one Environment. LG= linkage group. ENV = Environment; FHP1 = Nkometou high P, FLP1 = Nkometou low P, FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low P in field experiments. PHP1 = Nkometou high P, PLP1 = Nkometou low P, PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low P in pot experiments. The QTL are named beginning with "q" standing for QTL, followed by trait name and the linkage group number. In cases where there are more than one QTL on a linkage group for the same trait, the serial number is added after the linkage group number separated by a dot.

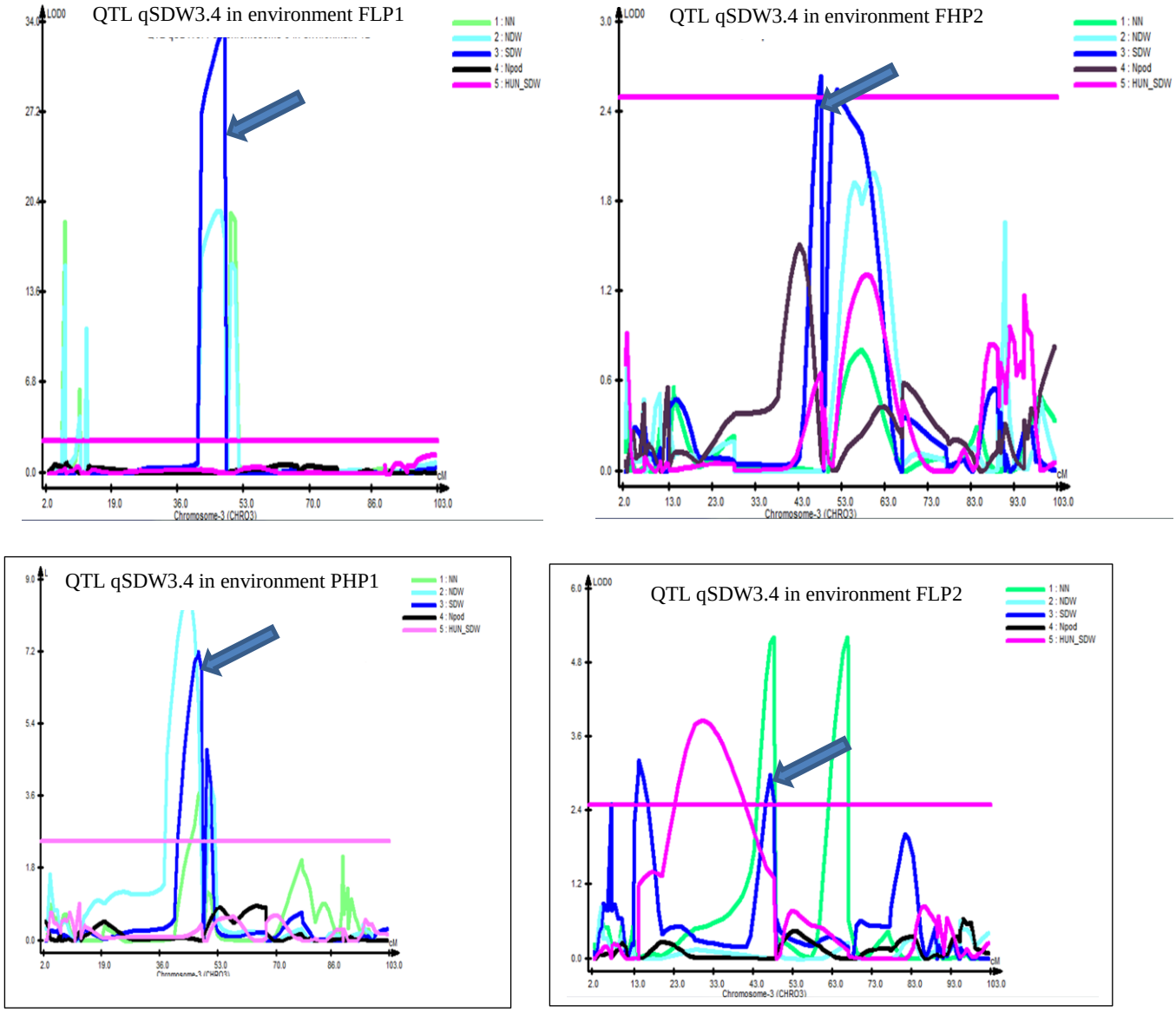

Figure 7. 2 Composite interval mapping output for constitutive qQTL SDW3.4 detected in four environments by Win Cartographer. Environment FLP1, PHP1, FHP2 and FLP2 represent Nkometou field low P, Nkometou pot high P, Nkoemvone field high P and Nkoemvone field low P, respectively. The Y-axis is the LOD score and the horizontal line is the significant LOD threshold based on 1000 permutations. The arrow points to the constitutive QTL.

7.3.3 Quantitative trait loci for nitrogen fixation and yield related traits identified by QTLnetwork across eight envoronments.

QTLnetwork identified the following 13 main QTL (M-QTL) for all traits across the eight environments (Table 7.8).

Table 7.8: Positions of Main QTL (M-QTL) identified by QTLnetwork in eight environments for all traits.

			Position				
Trait	**QTL**	**LG**	**interval**	**cM**	**range**	**h^2(a)**	**H^2(ae)**
	qNN1	1	1_1424-1_0238	39.5	36.3-40.5	0.005	0.013
NN	qNN10	10	1_0583-1_0960	50.5	50.3-51.8	0.006	0.010
	qNDW3	3	1_0938-1_0984	56.6	54.6-61.5	0.001	0.011
NDW	qNDW10.2	10	1_1018-1_0840	44.5	41.5-46.6	0.003	0.008
	qSDW1	1	1_0775-1_0333	24	23.4-24.2	0.004	0.035
	qSDW3	3	1_0199-1_0180	98.8	97.8-99.8	0.005	0.041
SDW	qSDW8	8	1_1101-1_1198	51.2	45.2-59.8	0.006	0.043
	qNpod2.1	2	1_1067-1_0113	5.2	4.5-6.2	0.015	0.070
	qNpod5	5	1_0032-1_0945	49.9	48.5-50.9	0.006	0.064
Npod	***qNpod8***	8	1_0762-1_1123	23.8	19.3-24.9	0.008	0.049
	qHUN_SDW6.1	6	1_0691-1_0307	2.9	2.9-4.0	0.068	0.052
	qHUN_SDW6.2	6	1_0015-1_0664	38.3	36.0-41.6	0.009	0.011
HUN_SDW	qHUN_SDW8	8	1_0451-1_0771	14.1	12.2-17.3	0.025	0.021

QTL with both detectable additive and epistasis effects are presented in bold italic form.LG= linkage group, h^2(a) = the heritability of additive effect, h^2(ae) = the heritability of additive by environment interaction effects. NN= nodule number, NDW= nodule dry weight, SDW = shoot dry weight, Npod = number of pods per plant, HUN_SDW= hundred seed dry weight.

Two QTL were detected for nodule number, qNN1 and qNN10 (Table 7.8, Figure 7.3), two for nodule dry weight, qNDW3 and qNDW10, three for shoot dry weight qSDW1, qSDW3 and qSDW8, three for number of pods per plant qNpod2.1, qNpod5 and qNpod8 and three for dry weight of hundred seeds qHUN_SDW6.1, qHUN_SDW6.2, and qHUN_SDW8 (Table 7.8). The positions of these QTL are indicated by the distance between the QTL and the first marker of the relevant linkage group. The interval refers to the flanking markers of the QTL while the range is the support interval of QTL position.

7.3.4 Analysis of QTL additive effects

Thirteen QTL with additive *[a]* main effect and/or additive by environment *[ae]* interaction effect are shown in Table 7.9. Both the *[a]* and *[ae]* effects were significant for 53.8% of the QTL while 46.2% of the QTL had only *[ae]* effect in one to six environments. There was no QTL with only the *[a]* effect, meaning that all the QTL had significant *[ae]* effects. All three QTL for Npod (qNpod2.1, qNpod5 and qNpod8) had no significant additive effects, while all three QTL for HUN_SDW (qHUN_SDW6.1, qHUN_SDW6.2, qHUN_SDW8) and two QTL for NN (qNN1, qNN10) had significant additive effects. The alleles increasing nodule number were from 58-77 (the better N_2-fixing parent), while those increasing nodule dry weight, shoot dry weight, number of pods per plant and weight of hundred seeds were from both parents. A major QTL qNDW10.2 flanked by markers 1_1018 and 1_0840 on linkage group 5 at position 44.5 cM, had significant additive main effect of 19.25 mg per plant (Table 7.9). Concerning QTL with *[ae]* effects, 12 out of 13 were detected in only one environment, while the constitutive QTL, qHun_SDW6.1 had opposite directions of *[ae]* effects in six environments. The additive main effect is the accumulative effect expressed in the same way across different environments, while the interaction effect is the deviation due to specific environment.

Table 7.9: Additive and /or additive x environment interaction effects of M-QTL across eight environments

Trait	QTL	*[a]*	*ae* FHP1	*ae* FLP1	*ae* PHP1	*aePLP1*	*aeFHP2*	*aeFLP2*	*aePHP2*	*aePLP2*
NN	qNN1	5.5474**	-1.526	-2.147	-5.317	-4.017	19.328**	-3.913	2.433	-4.552
	qNN10	5.463**	5.727	-1.5	-2.853	-3.072	11.116**	-3.131	-2.564	-3.896
NDW	qNDW3	-6.555	14.856	8.189	2.329	4.072	-47.139**	6.186	6.594	5.371
	qNDW10.2	19.255**	-4.865	-13.61	-16.005	-15.545	76.67**	1.318	-10.703	-16.281
SDW	qSDW1	-1.933	-10.113**	0.485	1.177	1.336	2.645	1.485	1.631	1.342
	qSDW3	2.625*	14.478**	-1.088	-2.104	-1.989	-2.985	-2.576	-1.73	-2.110
	qSDW8	-3.319	-15.643**	1.423	2.627	2.454	1.52	3.378	1.885	2.234
Npod	**qNpod2.1**	-0.734	0.122	0.654	0.576	0.478	-2.992**	0.091	0.538	0.561
	qNpod5	0.617	-0.845	-0.448	-0.424	-0.454	4.704**	-0.691	-1.017	-0.776
	qNpod8	-0.596	0.673	0.245	0.184	0.242	-1.721*	-0.114	0.243	0.242
HUN_SDW	qHUN_SDW6.1	0.782**	0.685**	0.577*	-0.405	-0.742	-0.642**	-0.547*	0.552*	0.540*
	qHUN_SDW6.2	-0.322**	-0.16	-0.140	-0.103	0.057	0.365*	0.217	-0.121	-0.109
	qHUN_SDW8	0.434**	0.240	0.245	-0.553**	-0.336	0.052	-0.158	0.244	0.265

[a], *ae* represent additive main effect and additive x environment interaction effect, respectively. Environments are defined as follows: FHP1 = Nkometou high P, FLP1 = Nkometou low P, FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low P in field experiments. PHP1 = Nkometou high P, PLP1 = Nkometou low P, PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low P in pot experiments. * and **represent the significance level of p=.05 and .01 respectively. NN= nodule number, NDW= nodule dry weight, SDW = shoot dry weight, Npod = number of pods per plant, HUN_SDW= hundred seed dry weight.

Given a specific environment, the total effect of a QTL includes the main effects plus the QTL by environment effect. The results of the present study indicate that additive by environment interactions were detected for all QTL even the major QTL qNDW10.2.

7.3.5 Analysis of QTL epistatic effects.

Digenic epistatic interactions with epistatic main effect *[aa]* and /or epistasis by environment interaction effect *[aae]* were detected for all five traits studied (Table 7.10). In total, the 15 digenic epistatic ineractions were spread as follows: three pairs were detected for NN (Figure 7.4), five pairs for NDW, one pair for SDW, three pairs for Npod and three pairs for Hun_SDW. Among them, one pair had only *[aa]* main effects, three pairs had only *[aae]* affects in one to two environments while 11 pairs had both *[aa]* and *[aae]* effects (Table 7.11). Considering the trait NDW with the highest number of digenic epistatic interactions, the absolute magnitude of the effects ranged from 3.88 -22.44 mg per plant for *[aa]* effect and from 0.73 -93.51 mg plant^{-1} for the *[aae]* effect (Table 7.11). The wide range of epistasis x environment interaction effects than that of epistasis main effects might suggest that although digenic interactions could have both main effects and environmental interaction effects, they are more easily subjected to environmental conditions.

Two M-QTL (qNpod2.1 and qNpod8) with *[ae]* effects, but without *[a]* effects (Table 7.8) were involved in digenic interactions (Table 7.10). The detected digenic interactions largely involved loci without detectable QTL *[a]* and/or *[ae]* effects. This result might mean that epistatic interactions are mostly induced by loci without detectable main effects leading to the conclusion that loci without detectable QTL main effect can also be putative QTL. The QTL qNN4 in position 4.4 cM of linkage group 4 interacted with two other non-allelic loci, one on linkage

group 6, qNN6.1 at position 5.6cM, and the other (qNN6.2) at position 19.8cM (Figure 7.5). This indicates the possibility of multi-loci associations for trait improvement.

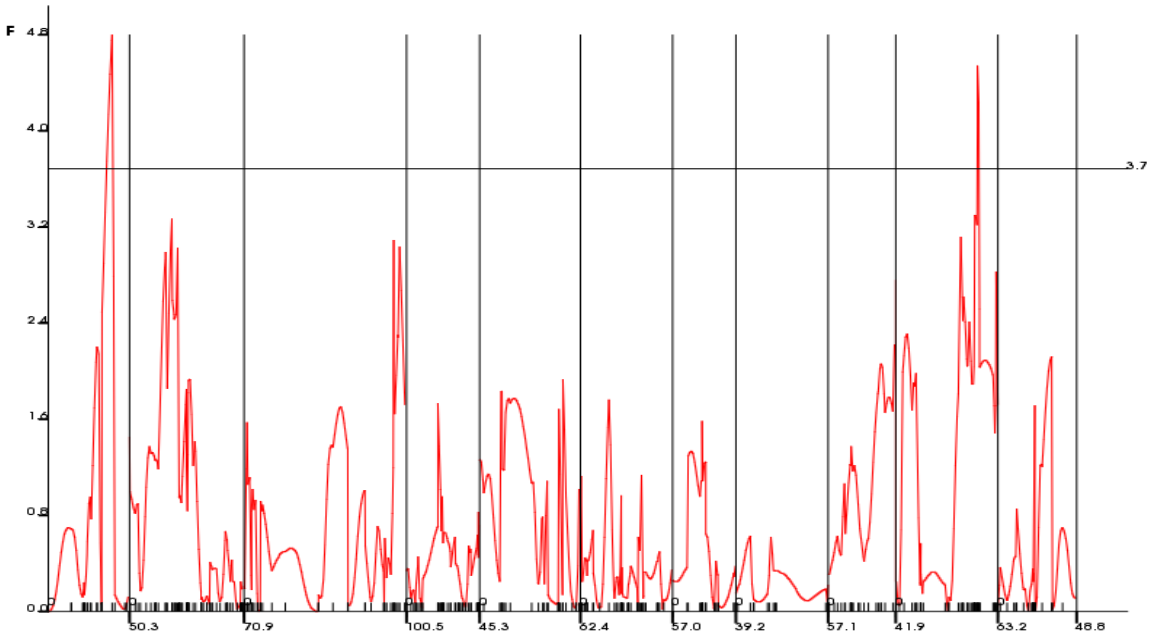

Figure 7. 3 F-statistic plots from one dimensional genome scan for QTL with individual effects for nodule number. Two peaks exceed the threshold F-value (3.7) calculated by permutation tests on chromosome 1 and 10, respectively.

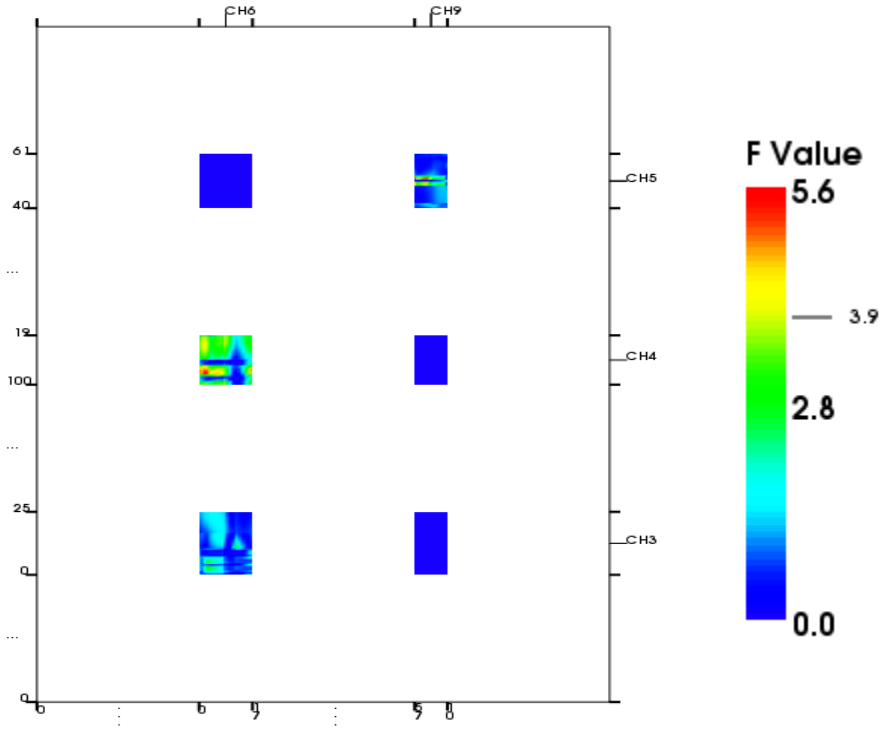

Figure 7. 4 Two dimensional genome scan for epistasis for nodule number in cowpea.

Table 7. 10: Positions of epistatic QTL (E-QTL) identifed by QTLNETWORK in eight environments for all traits.

Trait	QTL_i	interval_i	position_i	range_i	QTL_j	interval_j	position_j	range_j
NN	qNN4	1_0397-1_0976	4.4	3.7-6.0	qNN6.1	1_0690-1_0024	5.6	4.0-6.6
	qNN4	1_0397-1_0976	4.4	3.7-6.0	qNN6.2	1_0919-1_0224	19.8	18.8-19.8
	qNN5	1_0661-1_0226	51.5	51.3-52.5	qNN9	1_1016-1_1167	0.3	0.3-1.1
NDW	qNDW3	1_0853-1_1037	3.9	2.5-4.3	qNDW6	1_0690-1_0024	6.6	5.6-6.9
	qNDW4	1_1138-1_0027	14	11.0-18.0	qNDW10.3	1_0406-1_0583	50.5	49.3-50.5
	qNDW5	1_0684-1_0049	0	0.0-2.0	qNDW9.2	1_1188-1_0214	31.4	30.0-32.9
	qNDW6	1_0690-1_0024	6.6	5.6-6.9	qNDW10.1	1_1254-1_1225	10.1	7.1-11.1
	qNDW7	1_1026-1_0663	26.5	23.5-29.1	qNDW9.1	1_1167-1_0668	0.3	0.3-1.1
SDW	qSDW6	1_1271-1_0326	50.8	50.8-51.7	qSDW9	1_0246-1_0097	6.1	5.0-7.1
Npod	***qNpod2.1***	1_1067-1_0113	5.2	4.5-6.2	***Npod8***	1_0762-1_1123	23.8	19.3-24.9
	qNpod2.1	1_1067-1_0113	5.2	4.5-6.2	Npod2.4	1_0709-1_0513	63.8	62.4-64.8
	qNpod2.2	1_0062-1_0687	35.2	32.8-35.2	Npod2.3	1_0115-1_0885	59.4	58.1-60.2
HUN_SDW	qHUN_SDW2	1_0113-1_1523	8.7	6.2-10.3	qHUN_SDW5.1	1_0684-1_0049	0	0.0-1.0
	qHUN_SDW4	1_0699-1_0804	38.5	36.7-39.5	qHUN_SDW5.2	1_0127-1_1322	49	48.5-49.9
	qHUN_SDW9	1_0025-1_1217	20.1	18.9-22.1	qHUN_SDW11	1_0757-1_0414	13.4	12.3-14.4

QTL with both detectable additive and epistasis effects are presented in bold italic form. QTL_i and QTL_j are the two QTL involved in epistatic interaction. Interval_i = the flanking markers of QTL_i, LG= linkage group, range_i= the position of support interval of QTL_i in cM, interval_j = the flanking markers of QTL_j, range_j = the position support interval of QTL_j in cM. NN= nodule number, NDW= nodule dry weight, SDW = shoot dry weight, Npod = number of pods per plant, HUN_SDW= hundred seed dry weight.

Table 7.11: Additive x additive and /or additive x additive x environment interaction effects of E-QTL across eight environments.

Trait	QTL_i	QTL_j	*[aa]*	*aae* FHP1	*aae* FLP1	*aae* PHP1	*aaePLP1*	*aaeFHP2*	*aaeFLP2*	*aaePHP2*	*aaePLP2*
	qNN4	qNN6	2.759	11.766**	2.638	-3.192	-2.043	3.036	-1.434	-6.621	-4.256
	qNN4	qNN6	4.706**	2.242	-4.211	-5.280	-4.869	21.444**	-3.119	-1.391	-5.056
NN	qNN5	qNN9	6.728**	3.339	-5.223	-4.737	-6.188	28.05**	-5.079	-7.223	-2.956
	qNDW3	qNDW6	22.441**	-3.213	-18.877	-12.298	-17.351	93.508**	5.272	-25.129*	-22.182
	qNDW4	qNDW10.3	10.011*	2.579	-4.429	-10.109	-7.707	31.77**	1.559	-4.452	-9.808
	qNDW5	qNDW9.2	9.528**	0.731	-5.841	-2.328	-9.264	42.311**	-7.442	-11.683	-7.274
	qNDW6	qNDW10.1	-3.883	5.378	3.102	1.876	3.580	-26.185**	1.189	6.752	4.241
NDW	qNDW7	qNDW9.1	-5.309	19.676	8.955	2.864	7.528	-57.486**	13.350	2.572	2.660
SDW	qSDW6	qSDW9	4.653**	29.967**	-3.830	-4.365	-4.395	-2.443	-5.209	-4.776	-4.732
	qNpod2.1	**Npod8**	1.224**	0.439	-0.841	-0.660	-0.887	4.887**	-0.648	-1.322	-1.071
	qNpod2.1	Npod2.4	1.570**	-0.480	-1.728	-1.473	-1.618	9.676**	-1.022	-1.659	-1.673
Npod	qNpod2.2	Npod2.3	1.061**	-0.396	-0.603	-0.759	-0.901	4.187**	0.195	-0.828	-0.876
	qHUN_SDW2	qHUN_SDW5.1	0.442**	0.216	0.225	-0.208	-0.608**	-0.001	-0.073	0.212	0.219
	qHUN_SDW4	qHUN_SDW5.2	0.351**	0.077	0.085	0.024	-0.16	-0.103	-0.123	0.100	0.101
HUN_SDW	qHUN_SDW9	qHUN_SDW11	0.397**	0.367	0.293	-0.231	-0.495*	-0.071	-0.363	0.260	0.246

[aa],*aae* represent epistatic main effect and epistasis x environment interaction effect, respectively. Environments are defined as follows: FHP1 = Nkometou high P, FLP1 = Nkometou low P, FHP2 = Nkoemvone high P and FLP2 = Nkoemvone low P in field experiments. PHP1 = Nkometou high P, PLP1 = Nkometou low P, PHP2 = Nkoemvone high P and PLP2 = Nkoemvone low P in pot experiments. * and **represent the significance level of p=.05 and .01 respectively. NN= nodule number, NDW= nodule dry weight, SDW = shoot dry weight, Npod = number of pods per plant, HUN_SDW= hundred seed dry weight.

Legend

Graphic meta system	Line (Epistasis)	circle (QTL)
Green	with only epistasis × environment interaction effect *[aae]*	with both *[a]* and *[ae]*
Blue	with both *[aa]* and *[aae]*	with no *[aa]* effect
Dark	Not available	

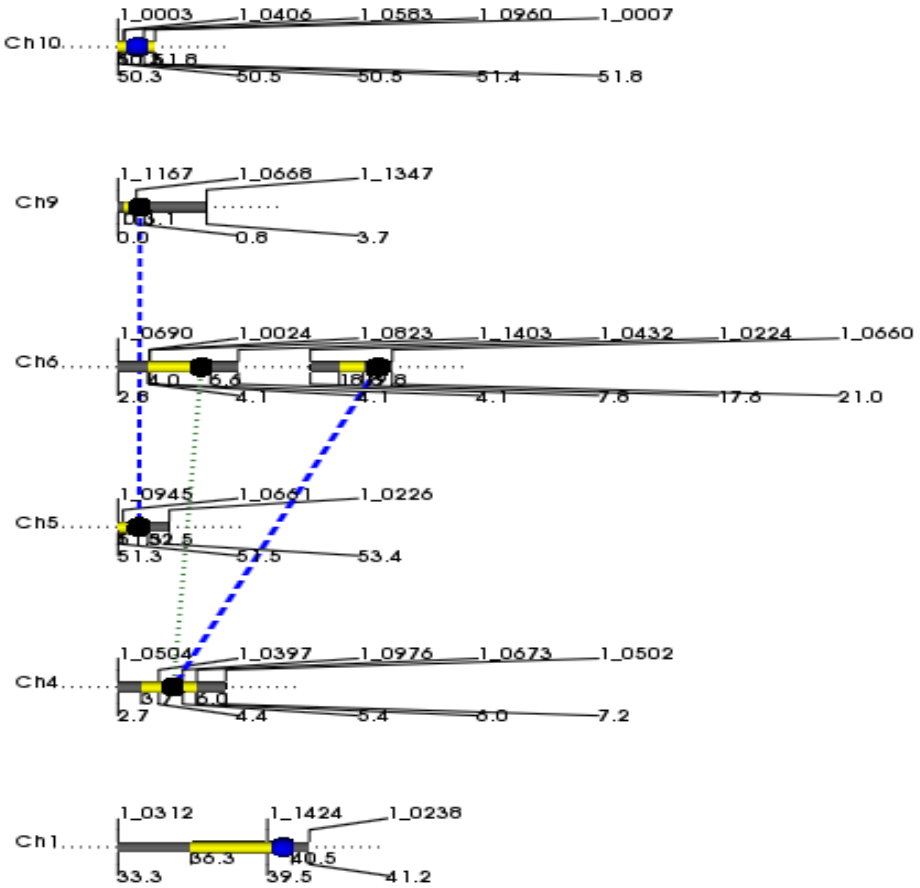

Figure 7. 5 The predicted genetic architecture for nodule number in cowpea.

The linkage group regions in yellow represent the support intervals of the QTL.

7.4 Discussion

The genetic control of nitrogen fixation and yield related traits of cowpea can be identified through QTL for the different traits studied and conducted under different environments. The use of multiple traits related at different P environments not only greatly facilitated the detection of QTL, but also allowed the identifcation of QTL by environment interactions. In summary, the majority of the identified M-QTL had low heritabilities, which indicated that the performance of the five traits might be affected by E-QTL or interaction between QTL and environments; therefore, E-QTL mapping or QTL by environment interaction analysis was very necessary. Both epistatic interaction effects and QTL by environment interactions effects were found to be very important genetic factors in this study. To infer epistasis between QTL, interaction effects between molecular markers were widely assayed by two-way analysis of variance (Lark *et al*, 1995). But his method usually cannot give unbiased estimation for QTL parameters. The possibility of QTL by environment interactions was also indicated simply by comparing results from different environments (Paterson *et al.,* 1991) as was identified by Win Cartograper in this study. The results of this study indicated comparing QTL from different environments to identify QTL by environment interactions leads to baised results. This was demonstrated by the "constitutive' QTL for SDW (qQTL SDW3.4) that was detected in four environments by Win Cartographer, but was not detected by QTLnetwork across the eight envronments. Quantitative trait loci (QTL) mapping has often been used to test for epistasis. But, numerous problems hinder estimation of QTL main effects, and these problems are exacerbated for QTL-by-QTL epistasis.

In this study, QTLnetwork program allowed the detection of QTL with epistasis and QE interactions and estimated their effects in multi-environments. QTLnetwork has also been used in other studies for similar purposes (Wang *et al.,* 2012; Ravi *et al.,* 2011). The dissection of

epistasis from other genetic components of variation is in no doubt helpful in obtaining reliable estimates of QTL effects. This can be seen in the difference in main QTL identified by Win Cartograper compared with QTLnetwork that estimates epistatic effects. In addition, considering epistasis in QTL analysis enhances the understanding of the inheritance of the traits under consideration. This study shows that, two QTL with epistasis effect were found to also have significant additive by environment effects. This means that the usual estimates of QTL effects could be confounded by epistatic interactions and result in biased estimation unless epistatic effect are isolated. The other loci involved in epistasis did not have any significant single effects of their own. These epistatic QTL have not been reported before as focus has always been on QTL with main effects. Epistatic interactions can occur between loci that have no significant main effects. Wade (2002) suggested caution when considering the importance of significant main effects, stating, "the existence of a statistical main effect is not an indication that a gene has any effect independent of its genetic background". Holland *et al.* (1997) detected several QTL for heading date and plant height in oat that were involved in epistatic interactions. They also found epistasis among loci that were not individually significant for trait effects and concluded that all pairs of loci should be tested for epistatic interactions, not merely the significant ones. Lecomte *et al.* (2004) also reported variability among fruit traits in tomato that were attributed to epistatic interactions between QTL and the genetic backgrounds.

The successful detection of significant epistasis effects resulting from QTL without additive and additive by environment main effects indicates that, many loci even without significantly affecting the trait on their own could still affect the trait in combination with other loci. Such loci may play the part of modifying agents which tend to activate other loci or modify the action of other loci (Reynolds and Tuberosa, 2008). At a specific environment, the total effect of a QTL

includes all the genetic main effects and QE interaction effects. In this study, the fact that 46.2% of M-QTL identified by QTLnetwork had only significant QE effects may imply that additive by environment interaction which is caused by gene expression in spatial pattern is an important component of genetic basis of quantitative traits. Two M-QTL, qNpod2.1 and qNpod8 were also found to have both epistasis and QE effects, implying that major gene or QTL could also interact with other genes under different environments. Prioul *et al.* (1999) reported that environmental or stress-specific gene regulation affects the detection rates and approximate genomic locations of QTL.

Epistasis is a form of non-additive gene action that results in a phenotype that cannot be predicted based on knowledge of the parental phenotypes. Epistatic effects can contribute to additive and dominance variances, breeding values (Cheverud and Routman, 1995), and heterosis (Holland, 2001). From the signs of the additive effects, it shows that seven QTL (with positive additive effects) out of the 13 QTL are from the better nodulating parent, 58-77. This suggests that alleles for improving these traits may be dispersed within the two parents. So pyramiding of all alleles increasing these traits from the two parents will produce segregants higher than the better parent. Pyramiding of all these minor QTL for the improvement of BNF in cowpea is not possible through marker-assisted backcrossing (MABC), since MABC involves the transfer of limited number of QTL from one genetic background to another (Ribaut *et al.*, 2010). Therefore, to improve these traits for BNF, alternative and more efficient approaches (genome wide marker approaches) like MARS (marker-assisted recurrent selection) and GWS (genome wide selection), which allows selection for several QTL with small effects (Varshney and Dubey, 2009) will have to be used in cowpea.

7.5 Conclusion

Through QTL analysis for epistasis and QTL by environment effects, this study has demonstrated the genetic basis of biological nitrogen fixation quantitative traits and revealed very important features of this phenomenon. Significant additive and epistatic interactions as well as QTL by environment were detected for all traits. Given the importance of QTL by environment interactions in this study, it goes a long way to confirm the fact that QTL detected are specific to the environments and comparison should only be done under similar environments. Those closely linked markers to the main QTL identified in this study will be useful for cowpea breeding by MAS for pyramiding of the main QTL involved in nitrogen fixation and yield related traits in low P soils. However, this study leads one to be concerned about the overestimation of performance predictions based solely on the pyramiding of additive effects of chromosomal regions of interest. Therefore, markers linked to epistatic QTL should also be considered in MAS bearing in mind that QTL effects are dependent not only on their own allelic makeup, but also on that of undetected QTL, or the background genome. For all the traits studied, the detection rates of significant additive and epistatic effects were higher within environments compared to the same effects averaged across environments. This indicates that the environment-specific gene effects were more common than environmentally stable gene effects.

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

The humid forest zone of Cameroon is one of the five agro-ecological zones in Cameroon. This zone is characterized by highly weathered soils with low phosphorus and nitrogen contents. Shifting cultivation has been the adapted farming system practiced in the humid forest zone (Bandy *et al.,* 1993), especially in areas of low population density (Nye et Greenland, 1960). Farmers will grow crops for a few years, until soil fertility, weed infestation and diseases reduce crop yield below an acceptable level (Akobundu, 1987). The plot will then be left for a period of restorative fallow (Seubert *et al.*, 1977; De Rouw, 1994). Due to pressure on lands, shifting cultivation can no longer be practiced and this has resulted into the soils being old and infertile. The challenge now is how to develop sustainable land use systems in the forest zone, taking into account the biophysical, socioeconomic and environmental constraints on natural resources. In this low-input cropping system of the HFZ of Cameroon, farmers usually do not apply fertilizers to their crops due to the cost and unavailability in the market (Jemo *et al.*, 2006b). Due to their innovative nature, farmers through experience have developed various techniques to detect and manage soil fertility problems in their fields. For example, some farmers use indigenous soil fertility management practices such as the integration of grain legumes in their cropping systems. However, adequate information on farmers' perception on the types and status of soils, the trend and constraints of integrating cowpea as a means to improve soil nitrogen fertility in the HFZ of Cameroon would be needed to facilitate breeding for specific traits needed to enhance soil fertility.

This piece of research started with a participatory rural appraisal (PRA) in the HFZ of Cameroon. PRA techniques through focus group discussions and questionnaire administration

were used to assess farmer's knowledge on role of grain legume in improving soil fertility in cropping systems; and to identify farmers' preferred cowpea traits in five sites in the HFZ of Cameroon. The farmers grew four varieties of cowpea, the white coated seed being the dominant (75%) and preferred variety. They also preferred the erect and early maturing cowpea type. Farmers in the HFZ were aware of the soil fertility decline on their farms and have indicators for poor and fertile soils. These farmers do not apply fertilizers but have other soil amendment strategies. Farmers in most non-benchmark villages lacked knowledge on the importance of legumes in cropping system. Ninety percent of the farmers considered legume root nodules as disease causing agents, which they called soil "cysts". However, after observing and distinguishing root nodules on legumes from root knots on tomatoes plants, all the farmers were ready to adopt cowpea for soil fertility improvement if crop yield improvements could be demonstrated on-farm as a result of residual effects of legumes. After critically examining the results from the PRA, the recommendation is that PRA should be conducted in the other four agro-ecological zones of Cameroon to diagnose cowpea farmers' production constraints and preferred cowpea traits. The farmers, especially those from non-bench mark villages should be trained on the importance of legume as an intercrop to improve soil nitrogen. Researchers should also demonstrate the residual effect of improved cowpea genotypes on farmers' fields to facilitate adoption.

In order to evaluate the ability of cowpea to fix nitrogen in the soil, the amount of rhizobia in the soil is paramount. Three cowpea (*Vigna unguiculata* L. Walp) varieties, (Dsch MMBr, Vya niebe and 58-77) were used as trap crops to estimate the population of indigenous *Bradyrhizobia spp* in two sites in the study area. The results of the Most Probable Number counts indicated that the total *Bradyrhizobia* population in Nkoemvone was between 1.0 x 10^5 and 5.8 × 10^5 cells per

gram of soil sample while Nkometou had population between 5.8 x 10^3 and 1.0 x 10^4. These sites had similar soil chemical properties but belong to different textural classes, clay for Nkoemvone and sandy clay loam for Nkometou. Nkoemvone recorded higher amounts of rhizobia because of its cropping history with cowpea-maize rotation while Nkometou field was dominated by cassava and groundnut cultivation. Interestingly, the results indicate that the population of *Bradyrhizobia* observed in the two sites were adequate to give satisfactory results on nodulation and nitrogen fixation. This suggests there is enough rhizobia in the soils of HFZ of Cameroon for natural inoculation.

For genetic studies, parents that are contrasting in the traits of interest were required to make crosses. More so, identification of related traits that correlate with complex traits like nitrogen fixed and yield was necessary. Fifty cowpea germplasm including ten Cameroon landraces were characterized in three environments: low phosphorus (LP; 0 mg P kg^{-1} soil), high phosphorus (HP; 30 mg P kg^{-1} soil) and high P and nitrogen (high P and N; 30 mg P and 90 mg N kg^{-1} soil). Data collection was done on twenty morphological traits comprising phenological, nitrogen fixation and yield related traits. Thirteen (LCS, NN, FN, NDW, SFW, SDW, RDW, Nsize, NI, δ^{15}N leg, %Ndfa, shoot N and N-fixed) out of these 20 traits were selected by the principal component analysis that explained 55.77% of the variation. Correlation studies indicated N-fixed and grain yield were positively correlated only in LP conditions, while shoot N was highly and positively correlated with N-fixed in all three environments. Among the five P efficiencies calculated, the index Phosphorus efficiency ratio (PER) correlated negatively with shoot N and N-fixed. In conclusion, shoot N can be used as a N_2 fixation related trait for the selection of high N_2-fixing cowpea genotypes while PER can be used to select for high N_2-fixing cowpea genotypes that are P-efficient. Among the 50 accessions, 58-77 and VYA niebe were cowpea

genotypes with high N-fixed and grain yield in low P soil. Cameroon landrace Lori-niebe and introduced genotype Yacine had poor grain yield and poor N-fixed in low P soil, while landrace Kodek had very high N-fixed but moderate grain yields. These results suggest that farmers in the humid forest zone of Cameroon could adopt VYA niebe for grain yield production and soil N replenishment. The farmers should also cultivate 58-77 and Kodek solely for soil N replenishment since the seed colours are not the preferred types. Given that morphoglogical markers are greatly affected by the environment, molecular characterization of Cameroon landraces should be done to compliment these results.

Selection of the appropriate breeding method for better exploiting the genetic potential of different traits depends on the type of genes controlling the trait and its inheritance (Akhtar and Chowdhry, 2006). There is a lot of scope for improvement of nitrogen fixation by gene manipulation. A study was done to determine the inheritance of nitrogen fixation related traits through generation mean analysis (GMA) in cowpea. The parents were one high N_2-fixing genotype, 58-77 and one low N_2-fixing type Lori-niebe. Data collected included nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), and shoot nitrogen (shoot N) content. All the traits had significant additive and/or additive x additive gene effects as was found by Sreekumar, (1995) working with cowpea. These results suggest that early generation selection for improved nitrogen fixation would be effective apart from shoot dry weight and shoot N content that had significant additive x domince effects. A negative estimate of dominance genetic variance was found for all traits. This suggested the presence of epistasis which could be due to a dominance bidirectional effect resulting in the cancellation between positive and negative effects of the alleles that controll the trait. Therefore, epistasis effects would seriously bias any attempt to partition the genetic variances of the segregating generations into additive or dominance

components. Besides gene effects, breeders would also like to know how much of the variation in a crop is genetic and to what extent this variation is heritable, because efficiency of selection mainly depends on additive genetic variance, influence of the environment and interaction between genotype and environment. Broad sense heritability ranged from 0 to 0.80 and the narrow sense from 0 to 1.48 for all traits. Nodule number and shoot N had a high narrow sense heritability of 1.48 and 1.04, respectively, while shoot N had moderate broad sense heritability value of 0.45. High magnitudes of dominance and additive variances in addition to presence of epistasis probably caused the narrow-sense heritability to be more than one. The high broad and narrow sense heritability estimates for Shoot N were consistent with the additive variance component estimates observed. Therefore selection for shoot N and nodule number may be highly effective.

Given that molecular markers are not available for these traits in cowpea, QTL were mapped using 372 polymorphic EST-SNPs on 88 F_{11} recombinant inbred lines (RILs) from 58-77 (high N_2-fixing cowpea) and Yacine (poor N_2-fixing cowpea) cross. The traits under study included: NN, NDW, SDW, number of pods per plant (Npod), and weight of hundred seeds (HUN_SDW). Win Cartographer identified a total of 31 QTL for NDW, 12 QTL for HUN_SDW, 26 QTL for NN, 32 QTL for SDW, and 14 QTL for Npod across the eight environments. QTLnetwork identified a total of 13 main QTL for all the traits across the eight environments. Fifteen pairs of epistatic QTL were also identified for all the traits. The results demonstrate the importance of epistasis as a genetic basis of the quantitaive traits studied. All the traits were involved in epistais with more that 90 percent of QTL found with significant additive x additive effects. Therefore, in marker assisted selection, markers linked to epistatic QTL should also be taken into consideration. QTL x environment interaction effects were detected and might indicate that

gene expression could be greatly influenced by environments. These results suggested that BNF in cowpea is governed by M-QTL and E-QTL each with a small phenotypic variation. Thus to improve upon these traits, gene pyramiding can be used. However, pyramiding of all these minor QTL for the improvement of BNF in cowpea is not possible through marker-assisted backcrossing (MABC), since MABC involves the transfer of limited number of QTL from one genetic background to another (Ribaut *et al.*, 2010). Therefore, to improve these traits for BNF, alternative and more efficient approaches (genome wide marker approaches) like MARS (marker-assisted recurrent selection) and GWS (genome wide selection), which allows selection for several QTL with small effects (Varshney and Dubey 2009) will have to be used in cowpea. The recommendations from this study are that QTL identified should be confirmed and validated for marker development. In Marker assisted selection, markers linked to epistasis QTL should also be taken into consideration and not only focus on QTL with single effects. The use of QTL in introgression should consider the genetic background of the receptor genotype to exploit epistasis. The cowpea backcross families and F_2 population from 58-77 x Lori-niebe cross should be selfed to advanced generations and evaluated in multi-location trials for nitrogen-fixing capacity and phosphorus efficiency. The 118 RILs from 58-77 x Yacine could be evaluated in the different agro-ecological zones of Cameroon for adaptability and release.

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APPENDIX

Appendix 1: PRA questionnaires

Section 1. Background information

Name of interviewer ---------- Name of interviewee----------

Questionaire no…… Location …….....

Date of interview..... Time of interview……

Section 2. Household information

1. Name of interviewee……

2. Gender: Male [ ] Female[ ]

3. Principal Occupation…….

4. Age range: 18-25 [ ] 26-35 [ ] 36-45 [ ] 46-55 [ ] 56-65 [ ] 65 plus [ ]

5. What is the role of the interviewee in the household? Mother [ ] child [ ] father [ ]

Sister/Brother-inlaw [ ] Other relatives [ ]

6. Family size: Number of male---------- Number of females----------

Section 3: General question on crops grown and family revenue

(1) What are the different crops you grow on your field? cassava [ ] maize [ ] ground nut [ ] plantain [ ] cowpea [ ] soybean [ ] yams [ ] cocoyam [ ] others----------

(2) Classify these crops in terms of surface area allocated for their cultivation : cassava [ ] maize [ ] ground nut [ ] plantain [ ] cowpea [ ] soybean [ ] yams [ ] cocoyam [ ] others----------

(3) What are the main sources of income for your family? Agriculture [ ] hunting [ ] business [ ] motor taxi driving [ ] civil service [ ] others [ ] ……..

(4) If agriculture, which are the main crops that bring in revenue? cassava [ ] maize [ ] ground nut [ ] plantain [ ] cowpea [ ] soybean [ ] yams [ ] cocoyam [ ] others----------

(5) Classify these crops in order of importance with regards to revenue contribution : cassava [ ] maize [ ] ground nut [ ] plantain [ ] cowpea [ ] soybean [ ] yams [ ] cocoyam [ ] others----------

Section 3: information on cowpea cultivation

1. Do you cultivate cowpea presently or in the past? cultivated in the past [ ] cultivate presently [ ]

2. For those who cultivated cowpea in the past, why did abandon the crop ? no market [ ] I don't eat cowpea [ ] low yields [ ] harvest difficulty [ ] poor soils [ ] insect attack [ ] diseases [ ] others [ ]

3. Why do you cultivate cowpea presently? for the market [ ] for consumption [ ] to transform [ ] to improve soil fertility [ ]

4. How do you call cowpea in your mother tongue? ----------….----------
5. Name the different types of cowpea varieties you cultivate? ----…----------
6. Among these varieties which is your best? ----------..----------
7. Why do you prefer this variety? Good taste [ ] high yielding [ ] resistant to insects and diseases [ ] easy to cook [ ] easy to harvest [ ] improves soil fertility [ ] others [ ]

Section 4: Sources of cowpea seeds

8. From where did you get the cowpea seeds you cultivated last season? Previous harvest [ ] IRAD [ ] IITA [ ] other farmers [ ] Family [ ] bought them [ ] others ----------

   If bought, from where? IRAD [ ] IITA [ ] market [ ] other farmers [ ] others ----------
9. What type of cowpea do you prefer to cultivate? Short and non-climbing [ ] climbing [ ]

Section 5 : Cowpea commercialization

1. Where do you sell your cowpea seeds? in the house [ ] village market [ ] urban market [ ] others ----------
2. Who buys your cowpea seeds? farmers [ ] grain dealers [ ] food dealers [ ] large companies
3. Which variety sells best in the market? big grains [ ] small grains [ ] white grains [ ] black grains [ ] red grains [ ]

Section 6. Cropping system practised

1. Which cropping system do you practise in cowpea cultivation? Sole cropping [ ] association [ ] rotation [ ]
2. If in association, with which other crops? cassava [ ] maize [ ] ground nut [ ] plantain [ ] soybean [ ] yam [ ] cocoyams [ ]
3. During which period/season(s) do you cultivate cowpea? first season [ ] Second season [ ] Both [ ]
4. Which season produces higher yields? first season [ ] second season [ ] Both [ ]

Section 7: Cowpea production constraints.

1. What are the problems you face when growing cowpea? poor soil [ ] low yields[ ] pest and diseases [ ] Harvesting difficulty [ ] poor marketing [ ] lack of seeds [ ] lack of rain [ ] lack of fertilizer [ ] weeds [ ] no constraints [ ]
2. How do you protect your cowpea against pest and diseases? abandon the field [ ] apply insecticide [ ] rotation[ ]

Section 8. Soil fertility diagnosis method

1. Can you identify poor and fertile soils? yes [ ] No[ ]
2. How do you identify a poor soil? It is hard [ ] it is red [ ] presence of soil "cyst"[ ] thin plants [ ] yellow plants[ ] poor vegetation [ ] no idea [ ]
3. How do you identify a fertile soil? It is black [ ] it is soft [ ] plants are green [ ]Plants are robust [ ]
4. Is the soil on which you grow cowpea poor or fertile? Poor [ ] fertile [ ]

4. Do you have names for different types of soil in your dialect? Yes [ ] No [ ] if yes, name them …….

5. What soil color predominant in your field? Black [ ] brown [ ] red [ ]
6. How do you make poor soil to become fertile? apply fertilizer [ ] addition of "Nkanwa" [ ] natural fallow [ ] fallow with grain legumes [ ]
   Burning plant debris [ ] apply chicken droppings [ ]
7. For how long do you leave your field on fallow? 1 year [ ] 2 years [ ] 3 years [ ] 4 years [ ] 5 years [ ] 5 years plus [ ]

Section 9 . Role of legumes in cropping systems

Have you observed root nodules on the crops you cultivate? yes [ ] No[ ]

If yes, name the crops? ----------

1. Which crop has many nodules? Ground nuts [ ] cowpea [ ] soybean [ ]
2. What is the role of these nodules? No function [ ] harbours disease causing agents [ ] improves soil fertility
3. Are you aware of role of grain legumes in soil fertility management? yes [ ] No [ ]
4. If you have clear evidence, will you cultivate grain legume for soil fertility restoration purpose? yes [ ] No [ ]

Section 10: SOIL FERTILITY STATUS

1. What causes soil infertility? Continuous production [ ] short fallow period [ ] lack of fertilization [ ] soil « cyst » [ ] bad climate [ ] lack of rains [ ]
2. Do you apply FERTILIZERS? yes [ ] No[ ]
3. On what crops do you apply fertilizer? Maize [ ] plantain [ ] cocoa [ ] coffee [ ] Tomate [ ] cassava [ ] others……..
4. Which type of fertilizers do you apply? organic [ ] chemical [ ]

5. If chemical, which of the following do you apply? N-P-K [ ] Triple superphosphate [ ] single super phosphate [ ] urea [ ]
6. What dose of fertilizer do you apply? 1 bag/ha [ ] 2 bags/ha [ ] 3 bags/ha [ ] 5 bags/ha [ ] others ----------
7. At what growth stage do you apply the fertilizer to your crops? At planting [ ] 1 month after planting [ ] 6 weeks after planting [ ] others ----------
8. How do you apply the fertilizer? In lines close to plants [ ] Circle round the plants [ ] spread everywhere [ ] spraying [ ]