

**UNIVERSITY OF GHANA**

**MORPHOLOGICAL AND GENETIC CHARACTERISATION OF  
SOME LIMA BEAN (*PHASEOLUS LUNATUS* L.) CULTIVARS AND  
THEIR NODULATING RHIZOBIA**

**BY**

**EDMUND TEYE MATE KOLE**

**10395988**



**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA,  
LEGON, IN PARTIAL FULFILLMENT OF THE REQUIREMENT  
FOR THE AWARD OF MPhil NUCLEAR AGRICULTURE  
DEGREE**

**JULY, 2014**

## Declaration

This thesis is the result of research work undertaken by Edmund Teye Mate Kole in the School of Nuclear and Allied Sciences of the Department of Nuclear Agriculture and Radiation Processing, University of Ghana (Legon), under the supervision of Dr. Samuel Amiteye and Mr. Emmanuel Ofori-Ayeh.

I hereby affirm that except for references which have been duly cited, this work is a result of my own research and that it has not been presented in part or whole for any other degree in this University or elsewhere.

..... Date.....  
EDMUND TEYE MATE KOLE  
(CANDIDATE)



..... Date.....  
DR. SAMUEL AMITEYE  
(PRINCIPAL SUPERVISOR)

..... Date .....

MR. EMMANUEL OFORI -AYEH  
(CO-SUPERVISOR)

## Dedication

This work is dedicated to God Almighty for the innumerable blessings meted to me and for a fruitful completion of this work. I will also dedicate it to my parents and siblings for their spiritual support and encouragement.



## Acknowledgment

My earnest appreciation to God Almighty for His tender mercies and for the opportunity granted me in furtherance and completion of my thesis work. I will also acknowledge the love, encouragement and financial support from my parents and siblings without whose help I might not have come thus far.

My profound gratitude to my supervisors, Dr. Samuel Amiteye and Mr. Emmanuel Ofori- Ayeh who painstakingly perused and corrected my thesis work. A special thanks to the Head of Department, Dr. Harry Amoatey for his good counsel and pursuance that a good work is attained.

I am also grateful to Mr. Addy and Mr. Bertrand Quaye, and to all the technical workers at Nuclear Agriculture Research Centre for their assistance in my field work. I am appreciative to Mr. Nettey, Mr. Benjamin Offei for granting me the opportunity to use facilities of the microbiological laboratory. I also acknowledge the support of Mr. Okona Frimpong and Mr. Asante for their help in data analysis and also to Mr. Nunno for inspiration in dire circumstances.

Special thanks to Mrs. Rosemary Kusi- Adjei, Mrs. Abigail Asare and most especially to Mr. Robert Appiah of the Biotechnology Centre without whose help, my molecular work would have been incomplete. I am also thankful to Mr. Benjamin Otu and Mr. Kweku Acquah for their assistance and contributions in my molecular studies.

Finally, my heartfelt gratitude to all lecturers of the Department of Nuclear Agriculture and Radiation Processing, friends and colleagues who all contributed in diverse ways to the successful completion of this work. God richly increase and bless you immensely.

## Table of Contents

Declaration .....	i
Dedication.....	ii
Acknowledgment.....	iii
Table of Contents .....	iv
List of Tables.....	xii
List of Abbreviations .....	xvii
Abstract.....	xxi
CHAPTER ONE.....	1
INTRODUCTION.....	1
1.1 Background.....	1
1.2 Problem Statement.....	5
1.3 Relevance and Justification .....	6
1.4 Hypothesis .....	7
1.5 Overall Objective.....	7
1.6 Specific Objectives.....	7
CHAPTER TWO.....	9
LITERATURE REVIEW.....	9
2.1 Origin and Distribution of Lima Bean.....	9
2.2 Botanical Classification .....	10
2.2.1 Botanical Description and Morphology .....	11
2.3 Lima Bean Production and Utilisation.....	12
2.3.1 Growth and Development.....	12
2.3.2 Climatic Adaptation.....	13
2.3.3 Production and Crop Yield.....	14
2.3.4 Utilisation of Lima Bean .....	15
2.3.4.1 Food for human consumption.....	15

2.3.4.2	Used for medicinal purposes.....	15
2.3.4.3	Used as fodder for livestock .....	16
2.4	Nutritional Value of Lima Bean .....	16
2.5	Potential Constraints to Lima Bean Production.....	17
2.5.1	Disease and Pest .....	17
2.5.2	Cyanogenic glucosides .....	18
2.5.3	Anti nutritional factors .....	18
2.6	Morphological Characterisation .....	18
2.7	Legumes and Nitrogen Fixation .....	20
2.7.1	Biological Nitrogen Fixation.....	21
2.7.2	Factors Affecting Biological Nitrogen Fixation.....	22
2.7.2.1	Soil water stress .....	22
2.7.2.2	Soil pH stress .....	23
2.7.2.3	Soil salinity .....	24
2.7.2.4	Temperature .....	25
2.7.2.5	Heavy metals effect .....	25
2.7.3	Rhizobia.....	27
2.7.3.1	Characteristics of rhizobia .....	27
2.7.3.2	Rhizobia contribution to nitrogen pool.....	27
2.7.3.3	Taxonomy of nodulating rhizobia.....	28
2.7.4	Nodule Formation.....	29
2.7.4.1	Types of Nodules .....	31
2.7.4.1.1	Determinate nodules.....	32
2.7.1.1.2	Indeterminate nodules .....	32
2.8	Molecular Characterisation of Rhizobia.....	33
REFERENCES	.....	35
CHAPTER THREE	.....	51

MORPHOLOGICAL CHARACTERISATION OF THE LIMA BEAN .....	51
3.1 INTRODUCTION .....	51
3.2 MATERIALS AND METHOD.....	52
3.2.1 Experimental Site.....	52
3.2.2 Plant Materials .....	52
3.2.2.1 Lima Bean Cultivars.....	52
3.2.2.2 Preparation of Potted Experiment.....	55
3.2.3 Experimental Design .....	55
3.2.4 Evaluation of Morphological Characters .....	55
3.2.4.1 Qualitative Traits .....	56
3.2.4.2 Quantitative traits .....	56
3.2.4.3 Data analysis of morphological data .....	57
3.3 RESULTS .....	58
3.3.1 Qualitative morphological traits analysis .....	58
3.3.2 Qualitative Principal component analysis .....	65
3.3.3 Cluster Analysis.....	67
3.3.4 Quantitative Traits .....	68
3.3.4.1 Quantitative Morphological Traits Analysis .....	68
3.3.4.2 Days of 50 % emergence .....	69
3.3.4.3 Leaflet length .....	70
3.3.4.4 Leaflet width.....	70
3.3.4.5 Pod length .....	70
3.3.4.6 Pod width .....	71
3.3.4.7 Seed length.....	71
3.3.4.8 Seed width.....	71
3.3.4.9 Seed weight.....	71

3.3.5	Quantitative Principal Component Analysis .....	71
3.4	Discussion.....	74
3.4.1	Qualitative Morphological Analysis.....	74
3.4.2	Quantitative Morphological Analysis.....	78
3.4.3	Qualitative and Quantitative Principal Component Analysis.....	80
3.4.4	Cluster Analysis.....	81
	REFERENCES .....	82
	CHAPTER FOUR .....	85
	NODULATION STUDIES OF THE LIMA BEAN .....	85
4.1	INTRODUCTION .....	85
4.2	MATERIALS AND METHOD .....	86
4.2.1	Experimental Setting.....	86
4.2.2	Lima Bean Cultivars .....	86
4.2.3	Nodule number per plant .....	87
4.2.4	Effective and Non- effective Nodules .....	87
4.2.5	Total Shoot Dry Matter (biomass).....	88
4.3	Data analysis.....	89
4.4	RESULTS .....	90
4.4.1	Germination Percentage .....	90
4.4.2	Mean Fresh and Dry Shoot Weight .....	91
4.4.3	Mean Fresh and Dry Root Weights .....	92
4.4.4	Total Shoot Dry Matter .....	93
4.4.5	Mean Number of Nodules per Plant.....	94

4.4.6	Mean number of Effective and Non-effective nodules .....	95
4.4.7	Mean Fresh and Dry Nodule Weight per Plant .....	97
4.4.8	Correlation between Nodulation and Growth Parameters in Lima Bean Cultivars.....	98
4.5	DISCUSSION .....	102
4.5.1	Germination Percentage .....	102
4.5.2	Fresh and Dry Shoot Weights per Plant .....	103
4.5.3	Mean Fresh and Dry Root Weights .....	104
4.5.4	Total Shoot Dry Matter .....	104
4.5.5	Mean Nodule Number per Plant.....	105
4.5.6	Mean Effective and Non-effective Nodule Numbers.....	106
4.5.7	Mean Fresh and Dry Nodule weight per Plant .....	107
4.5.8	Correlation among Nodulation and Growth Parameters .....	107
CHAPTER FIVE .....		112
MOLECULAR DIVERSITY STUDIES OF LIMA BEAN ROOT NODULE USING POLYMERASE CHAIN REACTION (PCR) .....		112
5.1	INTRODUCTION .....	112
5.2.1	Experimental Site.....	113
5.2.2	Lima bean collection .....	113
5.2.3	Nodule Harvesting, Sterilization and Preparation of Template DNA ....	114
5.2.5	Agarose Gel Electrophoresis .....	117
5.2.6	Scoring for Bacteria DNA Fingerprint Polymorphism and Statistical Analysis.....	118

5.3	Results.....	119
5.3.1	Specific nif-directed RPO1.....	119
5.3.2	The arbitrary primer RP04.....	121
5.3.3	The arbitrary primer ERIC 1 and 2 primers .....	123
5.3.4	Inter-accessional DNA amplification profile using the nif-directed primer RPO1.....	124
5.3.5	Inter-accessional DNA amplification profile using the arbitrary primer .	125
5.3.6	Genetic relationship within <i>Rhizobium</i> strains in lima bean cultivars.....	126
5.3.6.1	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar A1.....	126
5.3.6.2	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar A2.....	127
5.3.6.3	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar A3.....	128
5.3.6.4	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar A4.....	129
5.3.6.5	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar M1 .....	130
5.3.6.6	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar M2 .....	132
5.3.6.7	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar M3 .....	133

5.3.6.8	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar M4 .....	134
5.3.6.9	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar M5 .....	135
5.3.6.10	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar GH1714.....	136
5.3.6.11	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar GH 1719.....	137
5.3.6.12	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar GH 1726.....	138
5.3.6.13	Genetic relationship among <i>Rhizobium</i> bacteria DNA in lima bean cultivar GH 1737.....	139
5.3.7	Inter-accessional genetic relationship between and among <i>Rhizobium</i> bacteria.....	141
5.3.7.1	Inter-accessional genetic relationship between and among <i>Rhizobium</i> bacteria DNA in lima bean using RPO1 primer.....	141
5.3.7.2	Inter-accessional genetic relationship between and among <i>Rhizobium</i> bacteria DNA in lima bean using RPO4 primer.....	143
5.4	DISCUSSION.....	145
5.4.1	Differentiation of <i>Rhizobium</i> strains using three primers.....	145
5.4.2	Genetic relatedness and diversity within <i>Rhizobium</i> strains in thirteen lima bean cultivars .....	146
5.4.3	Inter-accessional diversity among <i>Rhizobium</i> strains.....	149

CHAPTER SIX .....	153
GENERAL CONCLUSIONS AND RECOMMENDATIONS .....	153
6.1    Conclusions.....	153
6.2    Recommendations.....	154
APPENDICES .....	155

### List of Tables

<b>Table</b>	<b>Page</b>
<b>Table 3.1</b>	Physical and chemical properties of the soil at the experimental site....53
<b>Table 3.2</b>	Morphological qualitative traits of 13 lima bean ( <i>Phaseolus lunatus</i> L.) cultivars.....61
<b>Table 3.3</b>	Seven (7) Principal components of sixteen (16) selected qualitative traits.....66
<b>Table 3.4</b>	Cluster distribution of thirteen (13) cultivars of lima bean based on sixteen qualitative (16) traits.....67
<b>Table 3.5</b>	Mean score of eight (8) quantitative morphological traits for thirteen lima bean cultivars.....69
<b>Table 3.6</b>	Seven (7) Principal components for eight (8) selected quantitative traits.....73
<b>Table 4.1</b>	Mean Fresh Nodule Weight (FNW) and Dry Nodule Weight (DNW) of thirteen lima bean cultivars.....98
<b>Table 4.2</b>	Correlation analysis of nodulation and growth parameters among thirteen cultivars of lima bean.....101
<b>Table 5.1</b>	Primers used for the amplification and detection of <i>Rhizobium</i> DNA obtained from root nodules of lima bean ( <i>Phaseolus lunatus</i> L.).....116
<b>Table 5.2</b>	PCR Reaction Mix for rhizobia work on thirteen (13) lima bean cultivars.....117

## List of Figures

<b>Figures</b>		<b>Page</b>
<b>Figure 3.1</b>	Seeds of thirteen cultivars of lima bean used in the experiment.....	54
<b>Figure 3.2</b>	Wing opening and colour of freshly opened flowers of some cultivars of the lima bean.....	60
<b>Figure 3.3</b>	Flower keel and standard of some cultivars of the lima bean.....	60
<b>Figure 3.4</b>	Dendrogram from complete linkage analysis showing relation between thirteen (13) lima bean cultivars based on 16 qualitative morphological data and Jaccard's distance.....	68
<b>Figure 4.1</b>	Fresh nodules harvested from a lima bean cultivar.....	88
<b>Figure 4.2</b>	Germination Percentage of thirteen lima bean cultivars.....	90
<b>Figure 4.3</b>	Mean fresh shoot and dry shoot weight of thirteen cultivars.....	92
<b>Figure 4.4</b>	Mean fresh and dry root weight per plant of thirteen lima bean cultivars.....	93
<b>Figure 4.5</b>	Total shoot dry matter (kg/ha) among thirteen cultivars of lima bean.....	94
<b>Figure 4.6</b>	Mean nodule number per plant of thirteen cultivars of lima bean.....	95
<b>Figure 4.7</b>	Mean effective and non-effective nodules of thirteen cultivars of lima bean.....	96
<b>Figure 5.1a</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> DNA	

	using the specific nif-directed RPO1.....	119
<b>Figure 5.1b</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> DNA using the specific nif-directed RPO1.....	120
<b>Figure 5.1c</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> using the specific nif-directed RPO1. ....	120
<b>Figure 5.2a</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> DNA using the arbitrary primer RPO4. ....	121
<b>Figure 5.2b</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> DNA using the arbitrary primer RPO4. ....	122
<b>Figure 5.2c</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> DNA using the arbitrary primer RPO4. ....	122
<b>Figure 5.3a</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> DNA using the arbitrary primer ERIC . ....	123
<b>Figure 5.3b</b>	Gel electrophoresis of PCR amplification of <i>Rhizobium</i> DNA using the arbitrary primer ERIC . ....	124
<b>Figure 5.4</b>	Representative DNA amplification profile using the nif-directed primer RPO1 from five nodules each of all thirteen cultivars of lima bean for dendrogram generation. ....	125
<b>Figure 5.5</b>	Representative DNA amplification profile using the arbitrary primer RPO4 from five nodules each of all thirteen cultivars of lima bean for dendrogram .....	126
<b>Figure 5.6</b>	Dendrogram showing the genetic relationship among 15 nodules of the A1 cultivar.....	127
<b>Figure 5.7</b>	Dendrogram showing the genetic relationship among 15 nodules	

	of the A2 cultivar.....	128
<b>Figure 5.8</b>	Dendrogram showing the genetic relationship among 15 nodules of the A3 cultivar.....	129
<b>Figure 5.9</b>	Dendrogram showing the genetic relationship among 15 nodules of the A4 cultivar.....	130
<b>Figure 5.10</b>	Dendrogram showing the genetic relationship among 15 nodules of the M1 cultivar.....	131
<b>Figure 5.11</b>	Dendrogram showing the genetic relationship among 15 nodules of the M2 cultivar.....	132
<b>Figure 5.12</b>	Dendrogram showing the genetic relationship among 15 nodules of the M3 cultivar.....	133
<b>Figure 5.13</b>	Dendrogram showing the genetic relationship among 15 nodules of the M4 cultivar. ....	134
<b>Figure 5.14</b>	Dendrogram showing the genetic relationship among 15 nodules of the M5 cultivar.....	136
<b>Figure 5.15</b>	Dendrogram showing the genetic relationship among 15 nodules of the GH 1714 cultivar.....	137
<b>Figure 5.16</b>	Dendrogram showing the genetic relationship among 15 nodules of the GH 1719 cultivar.....	138
<b>Figure 5.17</b>	Dendrogram showing the genetic relationship among 15 nodules of the GH 1726 cultivar .....	139
<b>Figure 5.18</b>	Dendrogram showing the genetic relationship among 15 nodules of the GH 1737 cultivar.....	140
<b>Figure 5.19</b>	Dendrogram showing the genetic relationship among 65	

	nodules of the thirteen lima bean cultivars using RPO1 primer. ....	142
<b>Figure 5.20</b>	Dendrogram showing the genetic relationship among 65 nodules of the lima bean cultivars using RPO4 primer.....	144

### List of Abbreviations

ANOVA	Analysis of Variance
AFLP	Amplified Fragment Length Polymorphism
BNARI	Biotechnology and Nuclear Agriculture Research Institute
BNF	Biological Nitrogen Fixation
bv	biovar
C	Carbon
°C	Degree Celsius
Cd	Cadmium
CIAT	Centro international de Agriculture Tropical
CIP	International Potato Center
CSIR	Centre for Scientific and Industrial Research
CTAB	Cethyltrimethylammonium bromide
cm	centimetre
cmol	centimole
Cu	Copper
DAP	Days after Planting

DMY	Dry matter yield
DMRT	Duncan Multiple Range Test
DNA	Deoxyribonucleic Acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
FAO	Food and Agriculture Organisation
GAEC	Ghana Atomic Energy Commission
g	Grams
H	hectare
HCN	Hydrogen Cyanide
IBPGRI	International Board for Plant Genetics Resource
K	Potassium
kg	kilogram
kg/ha	kilogram per hectare
LSD	Least Significance Difference
M	Molar
m	metres
mm	millimetre

N	Nitrogen
N <sub>2</sub>	Atmospheric Nitrogen
NaCl	Sodium Chloride
NF-	nod factors
NH <sub>3</sub>	Ammonia
NH <sub>4</sub>	Ammonium
P	Phosphorus
ppm	parts per million
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PGRRI	Plant Genetics Resource Research Institute
pH	Hydrogen ion concentration
Org	Organic
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomised Complete Block Design
RFLP	Restriction Fragment Length Polymorphism

rpm	revolution per second
SDS	Sodium dodecyl sulphate
SDW	Shoot dry weight
SFW	Shoot fresh weight
SSR	Simple Sequence Repeats
TAE	Tris Acetate Ethylene
TFW	Total fresh weight
TSDM	Total Shoot Dry Matter
t ha <sup>-1</sup>	tonnes per hectare
UNESCO	United Nations Educational, Scientific and Cultural Organisation
WAP	Weeks After Planting
Zn	Zinc
µg	micrograms

## Abstract

Three major investigations were carried out to assess the morphological traits and nodulation potential of thirteen lima bean cultivars as well as the genetic diversity of rhizobia nodulating these lima bean cultivars. Thirteen lima bean cultivars obtained from the CSIR-PGGRI and various market centres in Ghana were used. The experiment was conducted in pots filled with natural topsoil and arranged in a randomised complete block design (RCBD) with three replicates at the Biotechnology and Nuclear Agriculture Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC). The study aimed at obtaining some relevant information on the morphological traits of the lima bean to be improved upon, to evaluate their nodulation tendencies and determine similarities and differences of their nodulating rhizobia. Significant differences were obtained in quantitative characters (leaflet length, leaflet width, pod length, pod width, seed length, seed width, seed weight per 10 seeds and days to 50% emergence), contributing to divergence among the lima bean cultivars. Qualitative traits, however, were mostly similar, with few exceptions such as the flower wing colour, growth habit, leaf shape, main stem pigmentation, pod beak shape, seed secondary colour and seed pattern colour showing divergence among the lima bean cultivars. Two major clusters were joined at the similarity distance of 0.69. Majority of the lima bean cultivars were identified to be of the same morphotype. with exception in cultivars M4 and A2. There were no significant differences in mean nodule number, mean effective and non-effective nodule counts. The lima bean cultivar GH 1714 showed superior performance with respect to nodule number counts, effective nodules, fresh shoot weight and fresh root weight. Additionally lima bean cultivars, M5 and A2

indicated superior radiation use efficiency with total shoot dry matter of 731kg/ha and 704kg/ha respectively. A positive and high correlation existed between mean nodule number, and mean effective nodules, mean fresh nodule weight and mean fresh shoot weight. Molecular diversity studies indicated that primers RPO1 and RPO4 were effective in differentiating *Rhizobium* strains compared to the primer ERIC (1 and 2). Cluster analysis identified two major groups of *Rhizobium* strains at the similarity level of 49%. Inter-cultivar genetic studies identified similarities and differences within and between the lima bean cultivars with respect to nodule bacteria isolates. Possible isolates also existed among the *Rhizobium* isolates both within and between cultivars.

## CHAPTER ONE

### INTRODUCTION

#### 1.1 Background

The availability of crop plants that human beings depend on has reduced in these past years due to genetic diversity of crops being eroded. Such trends have come to the fore as human population is rapidly increasing and all available options will be needed to meet world food demand (Smith, 1998). Thus, it is imperative to safeguard and improve these crops and exploit the utilisation of underdeveloped crops to curb the problem of food shortage and malnutrition, especially in most developing countries. The role of food legumes in crop production cannot be downplayed due to their nutritional value as a primary source of proteins in human diets and most importantly, their ability to form associations with bacteria that fix nitrogen from the atmosphere.

Lima bean (*Phaseolus lunatus* L.), also known as 'Butter bean', 'Rangoon bean', 'Madagascar bean' or 'Sieva bean' is a legume of the *Leguminosae* family (Allen and Allen, 1981) with its origin in Tropical America, specifically from Peru. The lima bean has evolved over the century as adaptation and hybridization have led to development of local strains in America, Europe, Asia and Africa (Esquivel *et al.*, 1990; Nwokolo, 1996). It is the second most important economical species of the genus *Phaseolus* (Fofana *et al.*, 1999), which comprises other grain legumes such as common bean (*P. vulgaris*), scarlet runner bean (*P. coccineus*) and tepary bean (*P. acutifolius*), all of worldwide and regional economic importance (Delgado Salinas, 1985). In Ghana, it is

fourth in importance after cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogea*) and bambara groundnut (*Vigna subterranae*) (Doku, 1977). Countries noted largely for the production of the crop include the USA, Madagascar and Peru (Akande and Balogun, 2007). In Africa, however, the production of the crop is rather low which is attributable to it being produced for consumption purposes only. It is usually grown in backyard gardens mostly intercropped alongside other cereal and root and tuber crops (Broughton *et al.*, 2002). Saka *et al.* (2004) reported that the crop is grown in Ghana on a marginal scale, usually as a backyard crop, with 35% of the grain being sold.

Lima bean is an annual or perennial herb cultivated in the tropics and in some temperate regions. The crop can survive in marginal zones or areas that experience severe biotic stress like drought. The FAO (1984) reported that the crop is hardy and can withstand adverse conditions unlike other leguminous vegetables. It is highly productive in tropical rain forest zones which have low to medium soil fertility and is well adapted to tropical lowlands. The crop also thrives in soils with pH of 6-7.

Cultivars of the lima bean crop are varied and show some distinct features from each other. Wild cultivars show indeterminate climbing growth habit, with a prolonged flowering period and large production of pods (Zoro Bi *et al.*, 2003). The pole types usually exhibit twining features with large rootstocks and are perennial in nature (Purseglove, 1974). There are also annual bush forms which were developed in cultivation (Santos *et al.*, 2008). The seeds are distinguishable from each other with respect to shape, size, colour and appearance of eye. The flower colour is pale green, occasionally violet (Beyra and Artiles, 2004) with its size, smaller in comparison with the common bean (*Phaseolus vulgaris*).

Lima bean is cultivated primarily for its immature and dry seeds, usually eaten, boiled and fried. In some parts of West Africa, they are cooked with maize, rice or yam and used for making special soup and stews. Young pods and leaves are mostly taken as vegetables in Ghana and Malawi (Broughton *et al.*, 2002). The Yorubas in Nigeria have the seeds processed into porridge, puddings and cakes. In traditional Asian medicine, seeds and leaves are valued for their astringent qualities and used as diets in the treatment of fever (Roecklein, 1987).

A critical aspect of the crop is its ability to fix atmospheric nitrogen in association with some soil micro-organisms commonly referred to as rhizobia, into usable forms made available to plants. Nitrogen is an essential and often limiting plant nutrient in agricultural systems. Despite the vital role in ensuring plant growth, development and reproduction, it is deficient throughout the world, particularly in the tropics and has become a widespread problem in many soils used for crop production (Sanginga, 2003). Nitrogen loss can be attributable to denitrification, volatilization and bush burning. To compensate for the limited nitrogen in soils, modern agriculture has adopted usage of industrial nitrogenous fertilisers. Thus, the objective in most intensive agricultural practices is to use chemical fertilisers containing nitrogen to obtain maximum crop yields.

In recent years, prices of these chemical nitrogen fertilisers have increased making it difficult for peasant farmers in most developing countries to purchase. Despite the gains obtained through the use of these chemical fertilisers, serious environmental problems are created. Graham and Vance (2003) reported that application of chemical fertilisers was a largely inefficient process as 30% to 50% of applied fertilizer is lost to leaching

and eutrophication, which involves proliferation of microorganisms such as algae in fresh water and marine ecosystems. Thus, an alternative and cheaper source of nitrogen can be obtained naturally through Biological Nitrogen Fixation (BNF).

Biological Nitrogen Fixation (BNF) is a natural process through which several species of bacteria, normally of the genus *Rhizobium* convert atmospheric nitrogen into plant available nitrogen, usually ammonium (Galloway *et al.*, 2004). It is a cheaper and environmentally friendly procedure which contributes roughly 200 million tonnes of nitrogen annually (Peoples *et al.*, 2009). Brady (1990) intimated that BNF is probably the second most important biologically-mediated process on earth. Symbiotic nitrogen fixation between legumes and rhizobia occurs usually in two ways. One way is through the inoculation of legumes with native strains of *rhizobia* which are effective and competitive (Ampomah *et al.*, 2008) and the other, in plant-derived root organs called nodules of which competent nodulation is critical for efficient BNF.

Rhizobia strains have a defined group of legumes species, or host range, with which they can nodulate and in parallel, legumes select for specific rhizobia partner species (Denarie *et al.*, 1992). Although some rhizobia have a restricted host range, others can promiscuously enter symbiotic association with many species of legumes (Graham, 1976). Rhizobial populations or diversity in the soil is determinant on presence of a suitable host, inoculation practice, the physical and chemical environment of soil, fertility level of soil and presence and absence of pollutants. Characterisation and identification of rhizobia has been based on the ability to nodulate a range of legume hosts. There is a wide range of methods that have been used in ecological studies of *Rhizobium* species in soil which are in association with plants but attention is now

focused on molecular-based Polymerase Chain Reaction (PCR) techniques used with random sequence primers. The availability of several sensitive and accurate PCR-based genotyping method has enabled the differentiation among closely related bacterial strains and detection of a higher rhizobial diversity than previously considered (Jensen *et al.*, 1993). These methods have been shown to be effective ways of differentiating complex bacterial DNA genomes (De Bruijn., 1992 ; Laguerre *et al.*, 1994; Martinez-Romero and Caballero-Mellado, 1996 ; Sessitsch *et al.*, 1997;).

## 1.2 Problem Statement

Lima bean (*Phaseolus lunatus* L.) is available in the markets of Ghana, however, in small quantities and it is largely intercropped with plantain, cassava, cocoyam and vegetables by peasant farmers ( Aboagye, 2003). Though it has been cultivated and maintained by few traditional farmers over many decades, research findings on Lima bean are not readily available compared to other common legume species such as cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogea*) and bambara groundnut (*Vigna subterranae*) found in Ghana (Amoatey *et al.*, 2000, Aboagye *et al.*, 2007). It is an underdeveloped crop in most parts of the world due to preference by the larger community to the consumption of common bean, despite its diversity and yield potential. Tadawan *et al.* (2004) stated that lima bean has great potential for future development and needed to be conserved. Thus the genetic characterisation of the crop will be helpful in enhancing or prioritising materials for breeding programmes. The crop also has the potential to fix atmospheric nitrogen, improve soil fertility which is

key to food production and enhance environmental stability. Manipulation and full exploitation of BNF is, therefore, necessary (Hardarson, 1987). Nodulation and genetic diversity of *Rhizobium* strains are critical indicators for improvement of legume crop yields. Thus, characterisation and identification of bacteria associated with the lima bean crop using molecular-based Polymerase Chain Reaction (PCR) may provide valuable information on plant-bacteria interactions with different lima bean cultivars.

### **1.3 Relevance and Justification**

The lima bean is an underdeveloped crop in most parts of the world despite its diversity and yield potential. It is an important source of proteins and vitamin B in human nutrition and is beneficial to both man and animals. Expanded productivity and widespread utilization must be enhanced. Moulin *et al.* (2001) reported that legume biodiversity is concentrated in tropical regions, while most studies are on cultivated leguminous plants from temperate regions. It is therefore imperative that the morphological and genetic variability of rhizobia associated with lima bean cultivars be investigated.

Most West African soils are experiencing a decline in soil nitrogen status which is a major threat to food production (Sanginga, 2003). A hallmark trait of legumes is their ability to develop root nodules and fix nitrogen in symbiotic association with compatible rhizobia. (Peoples *et al.*, 1995, Postgate, 1998). Biological Nitrogen Fixation, has a great potential to contribute to the productivity and sustainability of the tropical agricultural systems by substituting for fertiliser inputs (Boddey *et al.*, 1997).

However, nodulation ability and specifically diversity of rhizobia studies have been done for a small proportion of legumes. Several methods and approaches are now available to generate information on microorganisms that reside in the soil. Molecular tools for the identification of microorganisms are now in common use for phylogenetic investigations.

#### **1.4 Hypothesis**

Different lima bean cultivars have distinct morphological attributes with different biological nitrogen fixations tendencies which show diversification in rhizobia nodulating them.

#### **1.5 Overall Objective**

To determine the morphological characteristics and nodulation potential of some local lima bean (*Phaseolus lunatus*) cultivars as well as the genetic diversity of *Rhizobium* nodulating the different cultivars of the crop.

#### **1.6 Specific Objectives**

1. To study the morphological characteristics and diversity of thirteen lima bean cultivars in potted soils.
2. To study the nodule occupancy and effectiveness of thirteen lima bean cultivars in potted soils.

3. To determine the fresh and dry shoot, root and total biomass yields of each cultivar under the prevailing conditions.
4. To determine the genetic similarity and diversity of *Rhizobium* nodulating the different cultivars of the crop, using PCR amplification profiles of *Rhizobium* DNA.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Origin And Distribution of Lima Bean

Lima bean (*Phaseolus lunatus* L.) is one of the five cultivated *Phaseolus spp*, with its origin in the Neotropics. It is believed to have originated from Guatemala, Mexico and Peru. Lima bean comprises of two botanical varieties; *Phaseolus lunatus* var. *Silvester* (wild types) and *Phaseolus lunatus* var. *lunatus* (domesticated types). The *Phaseolus lunatus* var. *Silvester* belongs to the Mesoamerica and Andean origin. The *Phaseolus lunatus* var. *lunatus* comprises of a smaller group of intermediate genotypes (Gutiérrez-Salgado *et al.*, 1995; Debouck, 1996; Caicedo *et al.*, 1999; Maquet *et al.*, 1999; Fofana *et al.*, 1997; Lioi and Gallasso, 2001). The wild lima bean is one of the few species for which multiple domestication events have been documented, giving rise to three culti-groups, namely the 'Sieva' and 'Potato' which are small- seeded and the large- seeded types called 'Big lima' (Gutiérrez-Salgado *et al.*, 1995, Maquet *et al.*, 1999; Fofana *et al.*, 2001). The distribution of both types were from the Pacific foothills of Mexico, through the Central and South Americas to Southern Canada (Gutiérrez-Salgado *et al.*, 1995; Rafael, 2001). It was later spread to Southern Asia and through the slave trade, extended to West and Central Africa. Van der Maeseen and Sadikin (1989) asserted that lima bean entered the African continent through the Portuguese explorers, particularly to the Central and Western parts of Africa from Brazil, during the slave trade era. Amoatey *et al.* (2000) reported that the crop was introduced into Ghana by the entry of early missionaries. Detailed information about the origin and distributions of the lima

bean have also been given by Kay (1979), Rachie *et al.* (1980), Lyman *et al.* (1985) and Smartt (1990).

Germplasm of lima bean is available at CIAT (Centro Internacional de Agricultura Tropical) at Cali, Columbia, with various cultivars collected from Central and South America, Western and Central Africa and some parts of Asia. Van der Maeseen and Sadikin (1989) intimated that there could be as many as 25000 cultivars available at CIAT. The PGRRI (Plant Genetics Research Resource Institute) in Ghana has germplasm collections of over forty cultivars of lima bean which have been documented since 1982 (Holloway, 1983; Bennett-Lartey, 1991).

## 2.2 Botanical Classification

The genus *Phaseolus* is strictly concentrated in tropical and warm temperate America. Lima bean is an important economic crop of the genus *Phaseolus* and one of the 12 primary grain legumes (Fofana *et al.*, 1999). The genus *Phaseolus* is made up of approximately 50 species of which five are domesticated (Delgado Salinas, 1985). These include common bean (*P. vulgaris* L.), lima bean (*P. lunatus* L.), scarlet runner bean (*P. coccineus* L.), tepary bean (*P. acutifolius* A. Gray) and year bean (*P. polyanthus* Greenm.). Lima bean belongs to the family *Leguminosae*, tribe *Phaseoleae* and Sub tribe *Phaseolinae*.

### 2.2.1 Botanical Description and Morphology

The lima bean plant is a warm season plant (Darbie *et al.*, 1999) propagated through its seed (Daisy, 1979; Van der Maeseen and Sadikin, 1989). They are twining vines or herbaceous plants that are either perennial or annual in nature, but usually grown as annuals (Floridata, 2012), and depending on the variety has a vegetative period within the of range, 80 days - 90 days or 120 days and beyond (Ibeawuchi, 2007). Baudoin *et al.* (1998) stated that it is an annual or short lived perennial species, with a mating system that is predominantly autogamous but without crossing levels up to 48%. It exhibits two kinds of growth habits. The annuals are pseudo determinate which appear in a bush form, having terminal and axillary flowers and thin roots (Baudoin, 2006). The perennial types are indeterminate and are mainly climbing and trailing in nature with axillary flowering and has swollen and fleshy roots (Ibeawuchi, 2007). The climbing types have vines or stems aided by stakes to provide support. The leaves are alternate and tri foliate in nature and have ovate to lanceolate leaflets. Inflorescences are 15 cm long and bear white to yellowish or violet bisexual flowers (Heuzé *et al.*, 2013) which are usually less than 1 cm in length (Floridata, 2012). Beyra and Artiles (2004) reported that the flower is pale green, occasionally violet in colour.

Lima bean pods are flat, oblong and generally curved. Depending on the cultivar type, they are of 5-15 cm in length and are either glabrous or sparsely pubescent (Baudoin, 2006) and 2-4 seeded (Ecoport, 2011, Ecocrop, 2011). Pods are uniformly green in colour for the immature bean and light brown in mature ones. Seeds vary in size, shape and colour. Baudoin (2006) inferred that cultivar groups have been distinguished according to seed differences; the large seeded (*Phaseolus limensis*) and the smaller

seeded 'baby Lima' or 'Sieva' bean (*Phaseolus lunatus*). Seeds have varied colouration ranging from white with black spots, green, brown, red, speckled and mottled, cream with speckled red or stripped red. The average size of these seeds range from 1 -1.5 cm in length and 0.8-1.5 cm in width and are flat, kidney- like to rhomboid in shape.

## **2.3 Lima Bean Production and Utilisation**

### **2.3.1 Growth and Development**

Lima bean germination and emergence take 4 -10 days after sowing usually under favourable rainfall. The seed undergoes epigeal germination (Ecoport, 2011).Vegetative phase of growth hastens usually after a month and its growth cycle is completed at about 100 days. Research work undertaken at the University of Delaware unveiled that the outer two flowers developed at the nodes simultaneously, with the middle structure lagging in development. The latter flower albeit continues to develop at a faster rate if one or both outer structures abscise and have to develop again (Kee *et al.*, 2004). The flowering period takes 35 - 70 days and pods ripe 80-120 days after sowing with short day length (Van der Maeseen and Sadikin, 1989). However, other lima bean cultivars have two cycles per year depending on rainfall distribution. Flowering and fruition occur throughout the rainy season.

The crop undergoes self and cross pollination processes. Pollen and stigma of the plant mature at the same time and due to closer proximity to unopened bud, self pollination ensues. Cross pollination, however, often occurs and 75-80 % of the flowers bud and

young pods are shed under field conditions (Ibeawuchi, 2007). Seeds of the crop are dispersed through explosive dehiscence of pods in the wild.

### **2.3.2 Climatic Adaptation**

Lima bean (*Phaseolus lunatus* L.) is generally a hardy species suitable to low altitude, humid and sub humid tropical climates, although it can be grown in a wide range of ecological conditions (Freitag *et al.*, 2002). Lima bean is found from sea level up to altitudes higher than 2000 m. It is usually found in warm temperate zones as well as arid and semi arid tropical regions. Ustimenko- Bakumovsky (1983) reported that the lima bean is more sensitive to temperature conditions than the common bean (*Phaseolus vulgaris*) and temperatures below 16-17°C and above 35°C are unfavourable for its growth. Average rainfall that supports the crop is 900-1500 mm per year. However, once the crop is established, it can tolerate rainfall as low as 500-600 mm (Baudoin, 1989). The crop thrive well in drained and aerated soils that are sandy loam. Despite its affinity for rainfall, lima bean can survive in marginal areas that experience severe biotic stress like drought (FAO,1984). The crop shows resistance to high salinity and can be short-day in its light requirement. Fofana *et al.* (2001) maintained that lima bean comprise photoperiod-insensitive types that flower in daylengths up to 18 hours, and short-day types that require a daylength as short as 11.0-12.5 hours to initiate flowering. The crop also thrives in soils with pH of 6-6.8 (Lyman *et al.*, 1985), although it is tolerable in acid soils with pH of 4.4 (Baudoin *et al.*, 2006).

### 2.3.3 Production and Crop Yield

Countries noted largely for production of the crop include the USA, Madagascar and Peru (Akande and Balogun, 2007). The USA is the largest producer and cultivation of the crop is undertaken in areas such as Delaware and Wisconsin. The state of Delaware planted more acreage annually for process purposes than any other state in the USA (Kee *et al.*, 2004). Production in Africa is rather low. In the past two decades, the total annual production was 50000-100000 tonnes in countries such as Ghana, Cote d'Ivoire, Nigeria and DR Congo altogether (Baudoin, 2006). The lima bean is grown mostly in gardens or backyards, usually intercropped with other crops (Broughton *et al.*, 2002).

Ecocrop (2011) reported that optimum yield of fresh seeds ranges from 2.0 t ha<sup>-1</sup> to 8.0 t ha<sup>-1</sup>. In the tropical regions, the average dry seed production is 1.0 t ha<sup>-1</sup> to 1.5 t ha<sup>-1</sup>. Yields of dry seed may reach 2 -2.5 t ha<sup>-1</sup> for annual lima bean types (bush types) and 3-4 t ha<sup>-1</sup> from climbing types (Baudoin, 2006). However, the type of cultivar and cultivation practices can affect yield of lima significantly (FAO, 2002). On the production yield of lima bean in Delaware, Kee *et al.* (2004) reported that weather conditions affected flower bud development, pollination and pod maturation. Under experimental conditions, Baudoin (2006) stated that climbing types may yield 3.0 t ha<sup>-1</sup> to 4.0 t ha<sup>-1</sup> when cultivated in the tropics. In Western Nigeria, under experimental conditions, yields over of 300 kg ha<sup>-1</sup> have been reported indicating potentials of the crop in the humid tropics (Ibeawuchi, 2007). When intercropped, a yield of 200-600 kg ha<sup>-1</sup> could be attained (Ecocrop, 2011).

### **2.3.4 Utilisation of Lima Bean**

#### **2.3.4.1 Food for human consumption**

Lima bean is a pulse crop usually grown for its enlarged seeds (IBPGRI, 2005) and cultivated primarily for its immature and dry seeds (Daisy, 1979, Van der Maeseen, 1989). The lima bean is an important source of protein and vitamin and has the potential to alleviate malnutrition in rural areas of developing countries. According to Oliveira *et al.* (2004), the lima bean is actually an alternative food source for human alimentation in the Northeast of Brazil. It can be dried and shelled, ground or as whole green beans and can be canned or freezed. It can also be baked, boiled and fried in oil. The young pods and leaves are consumed as vegetables in countries such as Ghana and Malawi (Broughton *et al.*, 2002). Seeds can be processed into porridges, puddings and cakes by the Yorubas, from Nigeria. The seeds and leaves can also be taken as beverages. In most parts of Asia, the young plants or leaves are consumed (Daisy, 1979, Van der Maeseen, 1989). It is also used in soups and stew and in a variety of dishes such as succotash (Floridata, 2012).

#### **2.3.4.2 Used for medicinal purposes**

Various parts of the crop have some medicinal properties. Juice from the leaves is used in nasal instillations against headache and as eardrops against otitis in Senegal and DR Congo (Baudoin, 2006). In traditional Asian medicine, both seeds and leaves are valued for their astringent qualities and consequently used as a diet for fever (Roecklein, 1987).

In other parts of Africa, seeds are processed into powder and applied into small cuts on tumours to promote suppuration (Baudoin, 2006).

#### **2.3.4.3 Used as fodder for livestock**

Lima bean straw (dried vines left after harvest) provides fodder to cattle and sheep. Dairy cows can be fed on young vines which are highly nutritive (Ishler and Adams, 2010). Vines cut at stage where leaves are still green promote high intake. The vines can easily be converted into silage (Ishler and Adams, 2010) and fed to growing and milking cows. Ajayi (2011) indicated that silage made from young vines before flowering, incorporated with fresh Napier grass (*Pennisetum purpureum*) and pineapple increased dietary protein content, nutrient digestibility, nitrogen absorption and retention and reduction in weight loss of goats during the dry season. In addition, silage made from vines of lima bean, as compared to other crops such as cajan pea or African yam bean, produced optimal growth rate and weight gain in goats (Ajayi *et al.*, 2012).

#### **2.4 Nutritional Value of Lima Bean**

Lima beans have excellent nutrition and health benefits .These include the following;

- They contain fibre and sodium that help to lower cholesterol levels ([www.whfoods.com](http://www.whfoods.com)).
- They contain magnesium and folate which are vital in preventing heart attacks and peripheral vascular diseases ([wiki-fitness.com](http://wiki-fitness.com)).
- They are iron-rich beans ([wiki-fitness.com](http://wiki-fitness.com)).

- Consumption of the beans aids in high metabolism and contains carbohydrate and energy production ([www.whfoods.com](http://www.whfoods.com)).
- They are fat-free and aid in weight reduction ([www.livestrong.com](http://www.livestrong.com)).
- They contain copper which supports proper function of the liver ([www.whfoods.com](http://www.whfoods.com)).
- They contain calcium and manganese for maintaining strong bones ([www.healthdiaries.com](http://www.healthdiaries.com)).
- They are relatively rich in proteins ([wiki-fitness.com](http://wiki-fitness.com)).
- They contain potassium that maintains and balances fluids in the body ([wiki-fitness.com](http://wiki-fitness.com)).

## **2.5 Potential Constraints to Lima Bean Production**

### **2.5.1 Disease and Pest**

One of the major diseases in lima bean is the stem anthracnose which affects the aboveground portions of the plant (Baudoin, 2006). Symptoms of the disease include severe pod blight and discolouration of the bloom. Other fungal diseases of notable interest are the damping off which hinders successful germination stands of plants and root rots which cause small sunken lesions and reddish brown colouration on roots (Long *et al*, 2014).

Pests also cause serious damage to yield and quality of the crop. Root knot nematodes tend to affect the underground portions of the plant and aphids suck fluids from the plant creating a honey dew substance behind which result in pale yellowish colour on leaves (Long *et al*, 2014). Other pests of importance include cut worms and beetles.

### **2.5.2 Cyanogenic glucosides**

Leaves and seeds of lima bean contain toxic substances such as cyanogenic glucosides and linamarase, which have the potential to cause rapid respiration, drop in blood pressure, headache, vomiting and convulsions (Heuzé *et al.*, 2013). Baudoin (2006) intimated that domesticated species contain a lower amount of cyanoglucosides (100-120 ppm) as compared to wild species (2000-2400 ppm). Cyanogenic potential varies with respect to maturity, stage of development, genotype and cultivation conditions. Young leaves and seeds have higher HCN (hydrogen cyanide) potential as compared to older leaves (Ballhorn *et al.*, 2005). It is important, therefore, to soak seeds overnight and cook lima beans properly to ensure most of the HCN is removed.

### **2.5.3 Anti nutritional factors**

Raw beans of lima bean contain anti nutritional factors mainly protease inhibitors and lectins which act negatively on the digestive tracts ( Ologhobo *et al.*, 1993) and affect growth and metabolism in broilers (Achi *et al.*, 2007). The foliage also contain factors such as phytic acid, saponins and tannins (Ajayi *et al.*, 2009). These factors can be eradicated using heat or moist treatment (Adeparusi, 2001).

## **2.6 Morphological Characterisation**

Morphological characterisation is the foundation of genetic diversity research in any taxonomical level (Chandran and Pandya, 2000). It is vital in management of crop germplasm collections (Ariyo, 1993; Polignano *et al.*, 1993; Annicchiarico and Pecetti, 1994) as it serves as a tool to investigate relationships between landraces and their wild

relatives and aids in choice of material for breeding programmes. It is also important in the identification of duplicate cultivars, detection of unique traits and also the structure of the population to be conserved, thus saving on the storage space and simplifying selection by plant breeders (Reed *et al.*, 2004). Assessment involves measuring variation in phenotypic traits such as shape of leaves, growth habit, pod colour and shape of beak. Sagayo (2006) undertook characterisation of eight lima bean cultivars in Benguet and evaluated on some morphological traits such as flower bud size, stem, leaves, pods and seeds. Phenotypic traits have been used to select crops that best suits the needs of farmers and has led to domestication of useful plants (Gepts, 2004).

Morphological characterisation of crops is facilitated by the use of internationally recognized descriptor lists. Erskine and Williams (1984) stated that the traditional approach of characterisation and evaluation involves cultivation of cultivar subsamples and their morphological and agronomic description; a procedure facilitated by the use of internationally recognised descriptor lists. However, use of morphological traits depends mostly on biochemical traits which are ambiguous descriptors and have limited use for cultivar identification (Zacarias, 1997) due to contribution of multiple genes which are subject to environmental modification and interaction. Liu and Furnier (1993) emphasized the fact that many of the morphological traits are also difficult to analyse because they do not have the simple genetic control assumed by many in genetic models (Tanksley *et al.*, 1989).

## 2.7 Legumes and Nitrogen Fixation

Members of the *Leguminosae* form the largest plant family on earth with around 19,000 species (Polhill *et al.*, 1981). They represent the third largest group of angiosperms and are the second largest group of food crops grown globally. According to the European Association for Grain Legume Research (2007), *Leguminosae* are cultivated on 12–15% of available arable land. They also contribute to more than 25 percent of global production, including food staples, fodder for livestock, cover crops, and emerging biofuels (Ferguson *et al.*, 2010). However, only a few are engaged on a large scale. Legumes are important both ecologically and agriculturally due to substantial role in global influx of nitrogen into useable forms by plants. Graham and Vance (2000) indicated that some researchers allude to its capacity of fixing atmospheric nitrogen although documentation about its nodulation potential and rhizobia association is not readily available.

Nitrogen is arguably one of the most essential nutrients required by plants for their growth and development. However, it is limited in many soils. Sanginga (2003) reported that most West African soils experienced decline in soil nitrogen status which was a major threat to food production. Thus, to compensate for this limited availability, modern agriculture has become highly reliant on industrial nitrogen fertilisers to achieve maximum crop productivity. In spite of the gains obtained through their use, they are quite expensive, and rarely available to peasant farmers in developing countries. In addition, Vitousek (1997) asserted that use of these chemicals has led to worldwide ecological problems as well as affecting human health. To buttress this point, Graham and Vance (2003) adjudged that applying chemical fertilisers is a largely

inefficient process as 30% to 50% of applied nitrogen fertiliser is lost through leaching, resulting in significant environmental problems such as the eutrophication of waterways. Nitrogen is also lost due to volatilization and denitrification. For these reasons, it is important that these nitrogenous fertilisers are used in the most efficient way, and only when necessary. Thus, to curb the impact on human health and the environment and yet optimize nitrogen inputs, interest has risen in environmental friendly sustainable practises such as the natural process of biological nitrogen fixation (Lee *et al.*, 2007).

### **2.7.1 Biological Nitrogen Fixation**

Biological Nitrogen Fixation (BNF) is a cheap and environmentally friendly alternative source of nitrogen fertiliser whereby atmospheric nitrogen ( $N_2$ ) is converted into biologically active source of nitrogen (ammonia) which is utilised by plants (Zahran, 1999). The reduction of atmospheric nitrogen to ammonia ( $NH_3$ ) is the second most important biological process on earth after photosynthesis (Sylvia, 2005). This procedure occurs during symbiotic interaction involving members of the *Leguminosae* family and nitrogen- fixing bacteria, commonly called rhizobia. The conversion process requires the use of the enzyme, nitrogenase (Keys *et al.*, 1984; Hardarson *et al.*, 1987; Zahran, 1999). It is an enzyme complex, containing metals such as iron and molybdenum, necessary in the atmospheric nitrogen reduction process. Reports by Hardarson *et al.* (1987) indicates that in spite of different interactions and organisms involved in biological nitrogen fixation, only rhizobia which possess the enzyme and are able to catalyse the biological reduction of  $N_2$  to  $NH_3$  (ammonia).

Symbiotic nitrogen fixation between legumes and rhizobia can also be introduced through artificial means. This involves the production of commercially produced rhizobia strains by researchers which are introduced to compatible leguminous plants to maximise nitrogen fixation (Györgypal *et al.*, 1988). Inoculation of legumes with rhizobial strains selected for their high nitrogen fixing capacities, can improve agriculture immensely, most especially, in the absence or ineffectiveness of indigenous rhizobial strains. Ampomah *et al.* (2008) attested to inoculation of legumes with native strains of rhizobia to be effective and competent. However, newly introduced strains of rhizobia in most cases are unable to compete with well adapted indigenous populations of the rhizobia. Additionally, a combination of flawed inoculants technology, substandard rhizobia inoculant strains and poor decision making are also key factors to their inefficiency (Brockwell and Botttomley, 1995).

## **2.7.2 Factors Affecting Biological Nitrogen Fixation**

### **2.7.2.1 Soil water stress**

Nitrogen fixation is affected by water content availability in soils. It poses a threat to nitrogen accumulation and yield potential of both tropical and temperate legumes (Sinclair *et al.*, 1987; Wery *et al.*, 1994). Soil water deficit has marked effect on nodule initiation, growth and development usually within the growth stage of the plant. Michiels *et al.* (1994) reported that water stress imposed during the vegetative stage of the plant was detrimental to nodulation and nitrogen fixation than that imposed during the reproductive stage. A reduction of soil water content from 5.5% to 3.5% decreased significantly infection threads number in root hairs completely inhibiting the nodulation

of *Trifolium subterraneum* (Worrall and Roughley, 1976). Soil water content below critical tolerance limits indirectly affects plant growth and populations of rhizobia in soils. Tate (1995) stressed that density populations of rhizobia tend to be lowest under desiccated conditions and increase as moisture stress is relieved. Low levels of water in soils was suggested to cause lack of success of soybean inoculation in soils with high indigenous population of *Rhizobium japonicum* (Hunt *et al.*, 1981).

Soil water processes such as diffusion and mass flow also contribute to the growth of rhizosphere soil micro organisms, including rhizobia. Soil aggregations, depending on size of pore spaces, also determine growth of rhizobia. Turco and Sadowsky (1995) asserted that soils with smaller internal pore spaces are more favourable environments for the growth of rhizobia.

#### **2.7.2.2 Soil pH stress**

Soil acidity contributes significantly to agricultural production in many areas of the world, consequently limiting legume productivity as well (Correa and Barneix, 1997, Bordeleau and Prevost, 1994). It is a constraint to symbiotic nitrogen fixation in tropical and temperate soils as it affects infection processes (Munns, 1986). Soil acidity limits rhizobia growth, survival, and nodule development. Brockwell *et al.* (1991) and Ibekwe *et al.* (1997) intimated that it is a limiting factor to *Rhizobium* survival and persistence in soils thereby reducing nodulation. Low pH caused reduction of the populations of *Rhizobium leguminosarum* bv *trifolii* cells in soils, which resulted in no or ineffective nodulation by clover plants (Ibekwe *et al.*, 1997). The number of nodules, the nitrogenase activity, the nodule ultra structure, and the fresh and dry weights of nodules

were affected to a greater extent at a low medium pH of less than 4.5 (Vassileva *et al.*, 1997).

Fast growing rhizobia are usually much more acid sensitive in comparison to *Bradyrhizobium*, although low pH-tolerant strains exist in many species. Thus, selection of strains capable of growing in acidified soils to enhance colonization of rhizosphere and nodulation of host plant has been proven to be a useful tool (Cooper, 1988).

### **2.7.2.3 Soil salinity**

Salinity of soils is characterized by the presence of high levels of neutral salts in the surface layers resulting from the capillary rise of water when evaporation exceeds precipitation (Al-Falih, 2002). Salt stress tends to affect nodule development and metabolism which eventually causes reduction in nodule number. Increase in the salinity of soils not only affects growth patterns of plant but also influence soil microbial populations as a result of direct toxicity (Zahran, 1999). Survival and proliferation of *Rhizobium spp* in the soil and rhizosphere is affected when plants grow in saline conditions. The response and adaptation of rhizobia to salt stress is a complex phenomenon implicating many physiological and biochemical processes that notably affect rhizobial colonization of roots and early infection (Nabizadeh *et al.*, 2011).

#### **2.7.2.4 Temperature**

High soil temperatures are a major problem for biological nitrogen fixation of legumes in tropical and subtropical regions (Michiels *et al.*, 1994). The effect of temperature affects survival and persistence of rhizobial strains in soils. Survival of these strains mostly are affected by higher temperatures than cold temperatures due to its deleterious nature (Al-Falih, 2002), and thus poses a threat to both symbiotic partners, steps in nodule formation and efficient fixation of nitrogen. The activity of rhizobia, however, can be altered by temperature changes. Most *Rhizobium spp* grow in culture with optimum temperature range of 28°C to 31° C (Graham, 1992). However, Eaglesham and Ayanaba (1984) reported that 90% of cowpea *Rhizobium* strains from the Sahel Savannah regions with hot, dry environments grew well at 40°C.

Triplett and Sadowsky (1992) reported that temperature also has marked influence on competition for nodulation, root hair infection, bacteroid differentiation, nodule structure and legume root nodule function. Rhizobial survival in soil exposed to high temperature is greater in soil aggregates than in nonaggregated soil and is favored by dry rather than moist conditions (Zahran, 1999). In addition, heat tolerant rhizobia form less effective symbiosis with their legume hosts (Moawad and Beck, 1991).

#### **2.7.2.5 Heavy metals effect**

The discharge of heavy metals from industrial operations and subsequent accumulation in varied ecological systems is a threat to agroecosystems (Cheung and Gu, 2007; Ceribasi and Yetis, 2001). Accumulation of heavy metals in soils to an abnormal level

causes dramatic changes to microbial composition and activities (Paudyal *et al.*, 2007; Wani *et al.*, 2008; Khan *et al.*, 2009; Krujatz *et al.*, 2011). Numerous reports of elevated amounts of heavy metals have been found to limit the rhizobial growth and their host legumes indirectly affecting yield (Broos *et al.* 2005). Hirsch *et al.* (1993) indicated that a single strain of *Rhizobium leguminosarum* could survive well in the metal contaminated plots, but this strain did not fix N with white clover (*Trifolium repens* L.), although it resulted in N formation with *Trifolium subterraneum*.

The deleterious nature of heavy metals on nodulation and N<sub>2</sub> fixation of *Rhizobium*–legume symbiosis are probably due to their inhibitory effects on the growth and activity of both symbionts. Metals such as Copper (Cu), Nickel (Ni), Zinc (Zn) and Cadmium (Cd) have been reported to inhibit the growth, morphology and activities of various groups of microorganisms (Khan and Scullion, 2002; Shi *et al.*, 2002; Lakzian *et al.*, 2002; Bondarenko *et al.*, 2010) including symbiotic N<sub>2</sub> fixers ( Santamaría *et al.*, 2003; Stan *et al.*, 2011) like *R. leguminosarum*, *Mesorhizobium ciceri*, *Rhizobium sp.* and *Bradyrhizobium sp.* (*Vigna*) and *Sinorhizobium* (Wani, 2008; Arora *et al.*, 2010; Bianucci *et al.*, 2011).

Other research works by Paudyal *et al.* (2007) in the determination of aluminium, iron and molybdenum on two strains of rhizobia isolated from root nodules of two tropical legume species *Mucuna pruriens* and *Trigonella foenum-graecum*, indicated that aluminium had adverse effect on rhizobia strains, invitro and invivo. Iron aided bacterial growth and nodulation but had negative effect thereafter, whereas molybdenum increased plant production and nodulation of test legumes.

### **2.7.3 Rhizobia**

Rhizobia are genetically diverse and physiologically heterogeneous group of bacteria (Somasegaran and Hoben, 1994) found in soils that are capable of eliciting nodule formation on leguminous plants. Rhizobia are ubiquitous part of the soil micro-flora in a free-living state in the rhizosphere of legumes (Allen and Allen, 1981; Somasegaran and Hoben, 1994) until the point where nodulation becomes possible (Rendig and Taylor, 1989).

#### **2.7.3.1 Characteristics of rhizobia**

Rhizobia are generally gram negative, motile rod shaped, approximately 0.5 $\mu$ m to 0.9 $\mu$ m in width and 1.2 $\mu$ m to 3.0 $\mu$ m in length and heterotrophic (Pepper and Upchurch, 1991; Somasegaran and Hoben, 1994). These rhizobia, also called root nodule bacteria, normally thrive under optimum temperatures of 25°C to 39°C in the pH range of 6-7 (Somasegaran and Hoben, 1994). They survive mostly in microaerophilic conditions (Somasegaran and Hoben, 1994) when fixing nitrogen, due to low levels of oxygen needed to protect the enzyme, nitrogenase (Rendig and Taylor, 1989). These bacteria are dual in nature as they exist as free living forms in soil and a symbiotic form inside of host legumes.

#### **2.7.3.2 Rhizobia contribution to nitrogen pool**

Rhizobia contribute to soil nitrogen pool through the symbiotic interaction with leguminous plants. Like other legumes, lima bean is able to establish nitrogen fixing

symbiotic relationship with rhizobia, which has potential to improve crop yield. Hatice *et al.*, (2008) alluded that *Rhizobium* symbiosis with legume species is of special importance producing 50% of 175 million tonnes of total BNF annually, providing nearly half of all N used in agriculture. The mutual benefit in this interaction is that plants supply the rhizobia with energy in the form of amino acids and the rhizobia in turn fix nitrogen from the atmosphere for plant uptake (Zahran, 1999). Rhizobia, however, can nodulate one woody plant, *Parasponia spp* from the family *Ulmaceae*, a non legume, which utilises nitrogen fixed by the bacteria (Doyle, 1998; Gualtieri and Bisseling, 2000).

### **2.7.3.3 Taxonomy of nodulating rhizobia**

The taxonomy of bacterial symbionts of leguminous plants has experienced profound series of extensions in the recent past (Young, 2003). Previously, classification of rhizobia was based on plant infection tests or ability to nodulate crops. Graham *et al.* (1991) proposed the use of phylogenetic and phenotypic (symbiotic, cultural, morphological, and physiological) traits as minimal criteria for description of new genera or species of rhizobia. This included the consideration of a number of strains, chosen from several geographic regions and original habitation of the host legumes. Presently, the use of molecular symbiotic data (nodulation and nitrogen fixation genes) supplemented with genomic and phenotypic data has increased knowledge in rhizobial diversity (Graham *et al.*, 1991; Vandamme *et al.*, 1996).

Currently, there are seven genera of rhizobia containing about 40 species as Alpha-

proteobacteria: *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* (Wei *et al.*, 2002) and a species in the genus *Methylobacterium* (Sy *et al.*, 2001). New lines that contain nitrogen-fixing legumes symbionts include *Ochrobactrum* (Ngom *et al.*, 2004; Trujillo *et al.*, 2005), *Devosia* (Rivas *et al.*, 2003), *Blastobacter* (Van Berkum and Eardly, 2002) and *Methylobacterium* (Jaftha *et al.*, 2002; Jourand *et al.*, 2004) in the alpha-Proteobacteria; *Burkholderia* (Moulin *et al.*, 2001), *Cupriavidus* (Vandamme and Coenye, 2004; Chen *et al.*, 2001) in the beta-Proteobacteria; and some unclassified strains in the gamma-Proteobacteria (Benhizia *et al.*, 2004) were recently described.

#### **2.7.4 Nodule Formation**

Nodulation process is a coordinated effort between legumes and rhizobia in the soil. It is a highly host specific interaction (CleyetMarel *et al.*, 1996) in which specific rhizobial strains infect limited range of plant hosts. Dénairé *et al.* (1992) asserted that rhizobia strains have a defined group of legume species, or host range, with which they can nodulate, and in parallel, legumes select for specific rhizobia partner species. However, specificity can be variable among legumes and rhizobia, with some legume species such as *Phaseolus vulgaris* known to associate with a wide range of rhizobia (Andrade *et al.*, 2002).

Symbiotic interaction between legumes and rhizobia occur in plant derived root organs called nodules and involves complex signalling processes. Nodule formation is initiated by the host plants root, which releases phenolic flavonoid compounds into the

rhizosphere (Redmond *et al.*, 1986). The flavonoid which partly determines the specificity of the *Rhizobium spp* attracts the bacteria by activating the bacterial NodD proteins that are members of the LysR family of transcriptional activators, which in turn induce the expression of the *nod* genes (Long, 2001). This results in the secretion by the bacteria of strain specific lipo-chito oligosaccharides, known as nod factors (NF) (Dénairé *et al.*, 1996; Spaink, 2000). Broughton *et al.* (2000) reported that the spectrum of flavonoids exuded by a legume, as well as the strain-specific chemical structures of the Nod factors, are primary determinants of host specificity. The presence of compatible *Rhizobium sp* and their corresponding NF is generally sufficient to trigger nodule development.

The bacteria then attach themselves to the root hairs which usually starts the infection process (Dénairé *et al.*, 1992; Prescott *et al.*, 1996). The attachment subsequently stimulates root hair deformation within 6 hours to 8 hours (Bhuvaneward and Solheim, 1985) and promotes cortical cell division (Matthews *et al.*, 1989).

Entry into root hairs by rhizobia is usually in two ways; either through cracks in intercellular cells of epidermal tissues or through the root hairs, which is the commonest route (Oldroyd and Downie, 2008). Root hair infection result in the formation of the infection thread, which traverse the root epidermis and cortex, penetrate primordial cells, and then invading bacteria are released into the host cells by an endocytosis-like mechanism (Ivanov *et al.*, 2010). This involves curling of root hairs, and subsequent degrading of root cell walls by specialised enzymes released by rhizobia, enabling their entry into the cytoplasm of the host cell (Salisbury and Ross, 1992). In the cytoplasm, bacterial cell multiplication takes place (Somasegran and Hoben, 1994). The movement

of rhizobia from the infection thread into the plant cell results in the rhizobia surrounded by a plant-derived peribacteroid membrane (Prescott *et al.*, 1996) which forms the symbiosomes (Udvardi and Day, 1997), which are vacuole-like structures. These symbiosomes ultimately completely fill the cytoplasm of infected cells. As the bacteria differentiate, infected cells undergo enlargement coupled to repeated endoreduplication cycles - genomic DNA replication without mitosis or cytokinesis and become large polyploid cells housing thousands of bacteroids (Jones *et al.*, 2007; Kondorosi *et al.*, 2000).

It is within these symbiosomes that nodules are formed and fixation of nitrogen occurs. In the cytosol of the bacteroid is where nitrogenase, the enzyme responsible for the reduction of atmospheric nitrogen into ammonia is synthesized (Somasegaran and Hoben, 1994). Active nitrogen fixation continues in mature nodules until senescence upon ageing or stress.

#### **2.7.4.1 Types of Nodules**

Nodules in legumes exist in two forms and are morphologically distinguished from each other (Mergaert *et al.*, 2006; Franssen *et al.*, 1992; Hirsch, 1992). Franssen *et al.* (1992) and Maunoury *et al.* (2008) asserted that nodules can be classified into two main groups according to their mode of development. The type of nodules developed depends on the host plant and are determined at the site of first internal cell division, maintenance of the meristematic region and the form of the mature nodules (Gresshoff and Delves, 1986; Rolfe and Gresshoff, 1988).

#### **2.7.4.1.1 Determinate nodules**

Determinate nodules are spherical or globular in shape and lack a permanent meristem (Mathews *et al.*, 1989). They occur in legumes that are predominantly tropical such as *Phaseolus vulgaris*, and *Glycine max* but may include temperate ones like *Lotus japonicus*. Most first cell division events occur in the sub epidermis in the outer cortex although with some few exceptions, like the *Lotus japonicus* (Wopereis *et al.*, 2000). Mature determinate nodules possess a relatively homogenous number of nitrogen fixing bacteroids as differentiation of infected cells occurs synchronously before senescence. Senescence in nodules occurs radially and has a life span of a few weeks.

#### **2.7.1.1.2 Indeterminate nodules**

Indeterminate nodules possess persistent meristems which elongate to become cylindrical. These nodules occur in legumes such as alfafa (*Medicago sativa*), pea (*Pisum sativum*) and clover (*Trifolium repens*) (Libbenga and Harkes, 1973; Newcomb, 1976; Newcomb *et al.*, 1979). First cell division events occur in the inner cortex and proceed to the periclinal regions in the endodermis and pericycle (Brewin, 1991; Hirsch, 1992). These divisions lead to formation of nodule primordia. Apical meristems divide continuously to produce new cells that become infected with bacteria. Mature nodules contain heterogeneous population of nitrogen fixing bacteroids due to actively dividing cells (Timmers *et al.*, 2000; Guinel, 2009). In contrast to bacteroids housed in determinate nodules, those from indeterminate nodules have lost their capacity to reproduce. Thus at the end of symbiosis, bacteria that are released from infection

threads can return to a free-living lifestyle and recolonize the rhizosphere (Mergaert *et al.*, 2006).

## 2.8 Molecular Characterisation of Rhizobia

The design of diversity of rhizobia is far from clear, particularly considering the large number of leguminous species available and their wide geographical distribution (Wei *et al.*, 2002). Characterisation of rhizobial strains naturally associated with roots of legumes has been recommended as an effective approach to successful management of the legume- *Rhizobium* symbiosis (Richardson *et al.*, 1995; Mpeperekki *et al.*, 1997). Since rhizobia are taxonomically very diverse (Wolde-Meskel *et al.*, 2004), efficient strain classification methods are needed to identify genotypes displaying superior nitrogen-fixation capacity (Sikora *et al.*, 2002). An array of methods has been used for the characterisation of rhizobia; they include serology, host range analysis, antibiotic resistance and biochemical analysis. Improved methods of characterisation have led to drastic changes in the classification of rhizobia and upgrade in the phylogenetic analysis of the family *Rhizobiaceae* and related genera (Martinez-Romero and Caballero-Mellado, 1996). They have helped to develop easy and quick methods to characterise microbes ; including works distinguish genera, species and even strains (Schneider and De Bruijn, 1996; Giongo *et al.*, 2008).

Presently, the use of PCR-based genomic fingerprinting techniques in the determination of their phylogenetic relationships has come to the fore. The polymerase chain reaction (PCR) used for microbes characterisation are very convenient due to their rapid, simple

and discriminative nature and it can create highly characteristic patterns when distinguished in agarose gels, providing well separation at strain level (Adiguzel, 2006).

## REFERENCES

- Aboagye, L.M. (2003). Evaluation of information on genetic resources activities of some legumes in Ghana. *Journal of Agricultural Science* 36: 31-40 Council for Scientific Industrial Research Plant Genetics Resources Research Institute, Bunso, Ghana.
- Aboagye, L.M., Owusu, S.K. and Badger, N.G. (2007). Evaluation of some lima (*Phaseolus lunatus*) Germplasm collected from Ghana. *Ghana Journal of Horticulture* volume 6 p 17-22.
- Achi, I., Adelanwa, M. and Ahmed, A. (2007). Performance of broiler chickens fed on lima bean, groundnut and soyabean diets. *Science World Journal*, 2 (2): 13-16.
- Adeparusi, E. (2001). Effect of processing on the nutrients and anti-nutrients of lima bean (*Phaseolus lunatus*) flour, *Nahrung*, 45 (2): 94-96.
- Adiguzel, A. (2006). Molecular Characterization of Thermophilic Bacteria Isolated From Water Samples Taken From Various Thermal Plants. PhD Thesis, Ataturk University, Graduate School at Natural and Applied Sciences, Erzurum, Turkey.
- Ajayi, F. T. (2011). Effects of feeding ensiled mixtures of elephant grass (*Pennisetum purpureum*) with three grain plants on digestibility and nitrogen balance of West African Dwarf goats. *Livestock Science*, 142 (1): 80-84.
- Ajayi, F.T., Funmi, O., Adewumi, A. and Sunday, D.E. (2012). Performance, hematology and serum biochemistry of West African dwarf goats fed ensiled mixtures of elephant grass ( *Pennisetum purpureum*) with lima bean, African yam bean and pigeon pea. *Kasetsart Journal, Natural Science*, 46 (5): 694-702.
- Ajayi, F.T., Akande, S.R., Adegbite, A. and Idowu, B. (2009). Assessment of seven under-utilised grain legume foliages as feed resources for ruminants. *Livestock Resource Rural Development*, 21 (9).
- Akande, S. R. and Balogun, M. O. (2007). Evaluation and heritability studies of local Lima bean (*Phaseolus lunatus L.*) cultivars from South- West Nigeria. . *Revista UDO Agrícola*, 7: 22-28.
- Al-Falih, A.M.K. ( 2002). Factors affecting the efficiency of symbiotic nitrogen fixation by *Rhizobium*, *Pakistan Journal of Biological Sciences*, 5 : 11, 1277 - 1293.
- Allen, C.N. and Allen, C.K. (1981). *The Leguminosae. A source book of characteristics, uses and nodulation*. Maxmillan Publishers Ltd, London. U.K. pp 5-29.
- Amoatey, H. M., Klu, G. Y. P., Bansa, D., Kumaga, F. K., Aboagye, L. M., Bennett-Lartey, S. O. and Gamedoagbao, D. K. (2000). The African Yam Bean (*Sphenostylis sternocarpa*): A neglected crop in Ghana. *West African Journal of Applied Ecology* 1: 53-60.

- Ampomah, O.Y., Ofori-Ayeh, E., Solheim, B. and Svenning, M.M. (2008) Host range symbiotic effectiveness and nodulation competitiveness of some indigenous cowpea Bradyrhizobia isolates from the transitional Savannah zone of Ghana. *African Journal of Biotechnology* 7(8): 988-996.
- Andrade, D.S., Murphy, P.J. and Giller, K.E. (2002) The diversity of *Phaseolus*-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. *Applied Environmental Microbiology*.
- Annicchiarico, P. and Pecetti, L. (1994). Morpho-physiological traits as descriptors for discrimination of durum wheat germplasm. *Genetics Resource Crop Evolution*. 41:47–54.
- Ariyo, O.J. (1993). Genetic diversity in West African okra (*Abelmoschus caillei* (A. Chev.) Stevels): multivariate analysis of morphological and agronomic characteristics. *Genetics. Resource Crop Evolution*. 40:25–32.
- Arora, N.K., Khare, E., Singh, S. and Maheshwari, D.K. (2010). Effect of Al and heavy metals on enzymes of nitrogen metabolism of fast and slow growing rhizobia under explanta conditions. *World Journal Microbiology Biotechnology* 26:811–816.
- Ballhorn, D.J., Lieberel, R. and Ganzhorn, J.U. (2005). Plant cyanogenesis of *Phaseolus lunatus* and its relevance for herbivore-plant interaction. The importance of quantitative data. *Journal Chemistry Ecology* 31. 1445-1473.
- Baudoin, J.P. (2006). *Phaseolus lunatus* L. In: Brink, M. & Belay, G. (Editors). PROTA 1: Cereals and pulses/Céréales et légumes secs. [CD-Rom]. PROTA, Wageningen, Netherlands.
- Baudoin, J. P. (2006). *Phaseolus lunatus* L.. Record from Protabase. Brink M. & Belay, G. (Editors). PROTA (Plant Resource of Tropical Africa/ Resource Vegetales de l' Afrique Tropicale), Wageningen, Netherlands.
- Baudoin, J.P., Degreef, J., Hardy, O., Janart, F. and Zoro Bi, I. (1998). Development of an *in situ* conservation strategy for wild Lima bean (*Phaseolus lunatus* L.) populations in the central valley of Costa Rica. In: Owens SJ, Rudall PJ (eds) *Reproduction Biology*, Royal Botanic Garden Press, England, pp 417-426.
- Baudoin, J.P. (1989). *Phaseolus lunatus* L. In: van der Maesen, L.J.G. & Somaatmadja, S. (Editors). *Plant Resources of South-East Asia No 1. Pulses*. Pudoc, Wageningen, Netherlands. pp. 57–60.
- Benhizia, Y., Benhizia, H., Benguedouar, A., Muresu, R., Giacomini, A. and Squartini, A. (2004). Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. *Systematic Applied Microbiology*, 27: 462-468.

- Bennett-Lartey, S. O. (1991). Exploration in Ghana 1987-1991. Plant Genetic Resources Unit, Crop Research Institute *Technical Report*.
- Beyra, A. and Artiles, G.R. (2004). Revisión taxonômica de los gêneros *Phaseolus* y *Vigna* (*Leguminosae* – *Papilionoideae*) en Cuba. *Anales Del Jardín Botánico de Madrid* 61, 135-154.
- Bhuvaneswari, T.V. and Solheim, B. (1985). Root hair deformation in the white clover/*Rhizobium trifolii* symbiosis. *Physiology. Plant.* 63, 25–34.
- Bianucci, E., Fabra, A. and Castro, S. (2011). Cadmium Accumulation and Tolerance in *Bradyrhizobium spp.* (Peanut Microsymbionts). *Current Microbiology* 62:96–100.
- Boddey, R.M., Sa, J.C.D., Alves, B.J.R. and Urquiaga S. (1997). The contribution of biological nitrogen fixation for sustainable agricultural systems in the tropics. *Soil Biology and Biochemistry* 29 (5/6) 287-799.
- Bondarenko, O., Rahman, P.K., Rahman, T.J., Kahru, A. and Ivask, A. (2010). Effects of rhamnolipids from *Pseudomonas aeruginosa* DS10-129 on luminescent bacteria: toxicity and modulation of cadmium bioavailability. *Microbiology Ecology* 59:588–600.
- Bordeleau, L. M. and Prevost, D. (1994). Nodulation and nitrogen fixation in extreme environments. *Plant Soil.* 161:115–124.
- Brady, N. (1990). *Nature and Properties of Soils*. Macmillan Publishing Company. New York.
- Brewin, N.J. (1991). Development of the legume nodule. *Annual Review. Cell Biology.* 7: 191-226.
- Brockwell, J., Pilka, A. and Holliday, R. A. (1991). Soil pH is a major determinant of the numbers of naturally-occurring *Rhizobium meliloti* in non-cultivated soils of New South Wales. *Australian Journal Experimental Agriculture.* 31:211–219.
- Brockwell, J. and Bottomley, P.J. (1995). Recent advances in inoculant technology and prospects for the future. *Soil Biology and Biochemistry.* 27:683–697.
- Broos, K., Beyens, H. and Smolders, E. (2005). Survival of rhizobia in soil is sensitive to elevated zinc in the absence of the host plant. *Soil Biology and Biochemistry* 37:573–579.
- Broughton, W. J., Hern´andez, G., Blair, M., Beebe, S., Gepts, P. and Vanderleyden, J. (2002). LBMPS, Universit´e de Gen`eve, ch. de l’Imp´eratrice, 1292 Chamb´esy, Gen`eve, Switzerland. CIFN, UNAM, Cuernavaca, Mexico. CIAT, Cali, Colombia. University of California, Davis, USA. CMPG, Katholieke Universiteit, Heverlee, Belgium.

Broughton, W. J., Jabbouri, S. and Perret, X. (2000). Keys to symbiotic harmony. *Journal Bacteriology* Vol. 182, No. 20, pp. 5641-5652.

Caicedo, A.L., Gaitán, E., Duque, M.C., Toro, O., Debouck, D.G. and Tohme, J. (1999) AFLP fingerprinting of *Phaseolus lunatus* L. and related wild species from South America *Crop Science* 39: 1497-1507.

Ceribasi, I.H. and Yetis, U. (2001). *Biosorption of Ni (II) and Pb (II) by Phanaerochaete chrysosporium from a binary metal system kinetics*. *Water SA* 24:15.

Chandran, K. and Pandya, S.M. (2000). Morphological characterization of *Arachis* species of section *Arachis*. *Plant Genetics Resources Newsletter*. 121:38–41.

Cheung, K.H. and Gu, J.D. (2007). Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: a review. *International Biodeterioration and Biodegradation* 59:8–15.

CleyetMarel, J.C., Rome, S., Salducci, X. and Wery, J. (1996). Host-specific regulation of nodulation mediated by flavonoid compounds present in plant. *Acta Botanica Gallica* 143, 521-528.

Cooper, J.E. (1988). Nodulation of legumes by rhizobia in acid soils. In *Developments in soil science 18* (Eds) Vancura V. and Kunc. F. eds.). Elsevier Science Publishers, Amsterdam, The Netherlands. pp 57-61.

Correa, O. S. and Barneix, A. J. (1997). Cellular mechanisms of pH tolerance in *Rhizobium loti*. *World Journal Microbiology and Biotechnology*. 13:153–157.

Daisy, E.K. (1979). Food legumes: TPI crop product digest. *Tropical Product Institute Circular*, pp 716.

Darbie, M., William, T. K. and George, A. (1999). *Lima bean: Commercial Vegetable Production*. *Georgia Extension Services Publication Circular*, pp: 716.

Debouck, D.G. (1996). Colombian common and lima beans: Views on their origin and evolutionary significance. *Revista Corpoica* 1: 7-15.

Delgado Salinas, A. (1985). Systematics of the genus *Phaseolus* (*Leguminosae*) in North and Central America. Ph.D. dissertation. University of Texas, Austin, TX.

Dénarié, J., Debelle, F. and Promé, J.C. (1996). *Rhizobium* lipochitooligosaccharide nodulation factors: Signalling molecules mediating recognition and morphogenesis. *Annual Review of Biochemistry*. 65, 503–535.

Dénarié, J., Debelle, F. and Rosenberg, C. (1992) Signaling and host range variation in nodulation. *Annual Review of Microbiology* 46:497-531.

Doku, E. V. (1977). Grain legume production in Ghana. *In Proceedings of the Joint University of Ghana Council for Scientific and Industrial Research Symposium on Grain Legumes in Ghana.* (E. V. Doku, ed.), pp. 1–7. The Institute of Adult Education, University of Ghana.

Doyle, J. J. (1998). Phylogenetic perspectives on nodulation: Evolving views of plants and symbiotic bacteria. *Trends Plant Science* 3, 473–478.

Eaglesham, A. R. J., and Ayanaba, A. (1984). Tropical stress ecology of rhizobia, root-nodulation and legume fixation. In: Subba Rao N S, editor. *Current developments in biological nitrogen fixation.* London, United Kingdom: Edward Arnold Publishers; pp. 1–35.

Ecocrop (2011). Ecocrop database. FAO.

Ecoport (2011). Ecoport database. Ecoport.

Erskin, N. and Williams, J.T. (1984). The principles, problems and responsibilities of the preliminary evaluation of genetic samples of seed-propagated crops. *Plant Genetics Resources Newsletter*, 41: 19-32.

Esquivel, M. L., Castineiras, A., and Hammer, K. (1990). Origin of lima bean (*Phaseolus lunatus* L.) in the light of Cuban Materials. *Euphytica* 49: 89-97.

European Association for Grain Legume Research (2007 ). [www.grainlegumes.com](http://www.grainlegumes.com).

FAO (2002). Legumes inoculates and their use. Food and Agriculture Organization of the United Nations, Rome.

FAO (1984). Legumes inoculates and their use. Food and Agriculture Organization of the United Nations, Rome.

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M., Lin, Y., Reid, D.E. and Gresshoff, P.M. (2010). Molecular analysis of legume nodule development and autoregulation. *Journal of Integrative Plant Biology* 52:61–76.

Floridata # 862 (2012). Floridata.com LC Tallahassee, Florida, USA (2012).

Fofana, B., du Jardin, P., Baudoin, J.P. (2001). Genetic diversity in the lima bean (*Phaseolus lunatus* L.) as revealed by chloroplast DNA (cpDNA) variations. *Genetics Resources and Crop Evolution* 48: 437-445.

Fofana, B., Baudoin J.P., Vekemans, X., Debouck, D.G. and Du Jardin, P. (1999). Molecular evidence for an Andean origin and a secondary gene pool for the Lima bean (*Phaseolus lunatus* L.) using chloroplast DNA. *Theoretical Applied Genetics* 98, 202-212.

- Fofana, B., Vekemans, X., du Jardin, P. and Baudoin, J.P. (1997). Genetic diversity in Lima bean (*Phaseolus lunatus* L.) as revealed by RAPD markers. *Euphytica* 95:157–165.
- Franssen, H. J., Vijn, I., Yang, W. C. and Bisseling, T. (1992). Developmental aspects of the *Rhizobium*-legume symbiosis. *Plant Molecular Biology*, Vol. 19, No. 1, pp. 89-107.
- Freytag, G. and Debouck, D. G. (2002). Taxonomy, distribution and ecology of the genus *Phaseolus* (*Leguminosae* - *Papilionoideae*) in North America, Mexico and Central America. *Botanical Institute of Texas*, Fort Worth, Texas, USA. pp 300.
- Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Cleveland, C.C., Green, P.A., Holland, E.A., Karl, D.M., Michaels, A.F., Porter, J.H., Townsend, A.R. and Vorosmarty, C.J. (2004). *Nitrogen cycles: past, present, and future. Biogeochemistry*, 70: 153-226.
- Gepts, P. (2004). Crop domestication as a long selection experiment. In Jules, J.(ed.). *Plant Breeding Reviews*, Vol 24:1-23. John Wiley & Sons, California.
- Giongo, A., Ambrosini, A., Vargas, L.K., Freire, J.R.J., Bodanese-Zanettini, M.H. and Passaglia, L.M.P. (2008). Evaluation of genetic diversity of bradyrhizobia strains nodulating soybean (*Glycine max* (L.) Merrill) isolated from South Brazilian fields. *Applied Soil Ecology*, 38: 261-269.
- Graham, P.H. and Vance, C.P. (2003). Legumes: Importance and Constraints to Greater Use. *Plant Physiology* 131: 872-877.
- Graham, P. H. and Vance, C. P. (2000). Nitrogen fixation in perspective: an overview of research and extension needs. *Field Crop Research* 65: 93–106.
- Graham, P. H. (1992). Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. *Canadian Journal of Microbiology* 38:475–484.
- Graham, P.H., Sadowsky, M.J., Keyser, H.H., Barnett, Y.M., Bradley, R.S., Cooper, J.E., Deley, D.J., Jarvis, B.D.W., Roslycky, E.B., Strijdom, B.W. and Young, J.P.W. (1991). Proposed minimal standards for the description of new genera and species of root- nodulating and stem nodulating bacteria. *International Journal of Systematic Bacteriology* 41:582-587.
- Graham, P.H. (1976). Identification and classification of root nodule bacteria. In P.S. Nutman (ed.), *Symbiotic nitrogen fixation*. Cambridge Press, Cambridge. p. 99-112.
- Gresshoff, P.M. and Delves, A.C. (1986). Plant genetic approaches to symbiotic nodulation and nitrogen fixation in legumes. In: Blonstein AD, King PJ, eds. *Plant*

*Gene Research III. A Genetical Approach to Plant Biochemistry*. Springer Verlag, Wien. pp. 159–206.

Gualtieri, G. and Bisseling, T. (2000). The evolution of nodulation. *Plant Molecular Biology*. 42, 181–194.

Guinel, F.C. (2009). Getting around the legume nodule: II Molecular biology of its peripheral zone and approaches to study its vasculature. *Botany* 87: 1139-1166.

Gutiérrez Salgado, A., Gepts, P. and Debouck, D.G. (1995). Evidence for two gene pools of the lima bean, *Phaseolus lunatus* L., in The Americas. *Genetic Resources and Crop Evolution* 42: 15-28.

Györgypal, Z., Iyer, N. and Kondorosi, A. (1988) Three regulatory *nodD* Alleles of Diverged flavonoid-specificity are involved in host-dependent Nodulation by *Rhizobium meliloti*. *Molecular and General Genetics MGG*, 212, 85-92.

Hardarson, G., Danso, S. K. A. and Zapata, F. (1987). Biological Nitrogen Fixation in Field Crops.

Hatice, O., Ömer, F., Erdal, E. and Faik, K. (2008). The Determination of Symbiotic Effectiveness of *Rhizobium* Strains Isolated from Wild Chickpeas Collected from High Altitudes in Erzurum. *Turkish Journal Agriculture Forestry Science*, 32(4): 241-248.

Heuzé, V., Sauvant, D., Tran, G., Bastianelli, D. and Lebas, F. (2013). Lima bean (*Phaseolus lunatus*). Feedipedia.org.

Hirsch, P.R., Jones, M.J., McGrath, S.P. and Giller, K.E. (1993) Heavy metals from past applications of sewage sludge decrease the genetic diversity of *Rhizobium leguminosarum biovar trifolii* populations. *Soil Biology and Biochemistry* 25:1485–1490.

Hirsch, A.M. (1992). Developmental biology of legume nodulation. *New Phytologist* 122: 211-237.

Holloway, H. L. O. (1983). Germplasm collection in Ghana. November, 1982 to February, 1983. *AGBP/IBPGR* 83/10.

<http://www.healthdiaries.com/eatthis/7-health-benefit-of-lima-beans.html>.

<http://www.whfoods.com/genpage.php?tname=foodspice&dbid=59>.

Hunt, P. J, Wollum, A. G. and Matheny, T. A. (1981). Effects of soil water on *Rhizobium japonicum*. Infection nitrogen accumulation and yield in bragg soybean. *Agriculture Journal* 73:501–505.

IBPGR (2005). Descriptors list of *Phaseolus lunatus*. FAO, Rome, Italy.

Ibeawuchi, I. I. (2007). Landrace legumes: Synopsis of the culture, importance, potentials and roles in Agricultural Production Systems, Department of Crop Science and Technology, Federal University of Technology, Owerri, Nigeria. *Journal of Biological Sciences* 7(3): 464-474, 2007.

Ibekwe, A. M., Angle, J. S., Chaney, R. L., and Vonberkum, P. (1997). Enumeration and nitrogen fixation potential of *Rhizobium leguminosarum* biovar *trifolii* grown in soil with varying pH values and heavy metal concentrations. *Agriculture Ecosystems and Environment*. 61:103–111.

Ishler, V. A. and Adams, R. S. (2010). Drought-related issues in dairy cattle nutrition. Pennsylvania State University, College Agriculture Science Cooperative Extension.

Ivanov, S., Fedorova, E. and Bisseling, T. (2010). Intracellular plant microbe associations: secretory pathways and the formation of perimicrobial compartments. *Current Opinion in Plant Biology*, Vol. 13, No. 4, pp. 372-377.

Jaftha, J., Strijdom, B.W. and Steyn, P.L. (2002). Characterization of pigmented methylotrophic bacteria which nodulate *Lotononis bainesii*. *Systematics and Applied Microbiology*, 25: 440-449.

Jensen, M.A., Webster, J.A. and Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction- amplified ribosomal DNA spacer polymorphism. *Applied and Environmental Microbiology*. 59: 945-952.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E. and Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology*, Vol. 5, No. 8, pp. 619-633.

Jourand, P., Giraud, E., Béna, G., Sy, A., Willems, A., Gillis, M., Dreyfus, B. and De Lajudie, P. (2004). *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria. *International Journal of Systematic and Evolutionary Microbiology*, 54: 2269-2273.

Kay, D. E. (1979). *Food Legumes. Crop and Products Digest* No. 3. Tropical Products Institute, Ministry of Oversea Development, London, p. 225-243.

Kee, E., Glancey, J. and Wooten, T. (2004). Successful Lima Bean Production in Delaware. *Cooperative Extension* Vf-6. 1-15.

Keys, S. O. and Ssali, H. (1984). Proceedings of the first conference of the African Association for Biological Nitrogen Fixation, 23-27 July, 1987, Nairobi, Kenya.

- Khan, M.S., Zaidi, A., Wani, P.A. and Oves, M. (2009). Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environment Chemistry Letters* 7:1–19.
- Khan, M. and Scullion, J. (2002). Effects of metal (Cd, Cu, Ni, Pb or Zn) enrichment of sewage-sludge on soil micro-organisms and their activities. *Applied Soil Ecology* 20:145–155.
- Kondorosi, E., Roudier, F. and Gendreau, E. (2000). Plant cell-size control: growing by ploidy? *Current Opinion in Plant Biology*, Vol. 3, No. 6, pp. 488-492.
- Krujatz, F., Haarstrick, A., Nörtemann, B. and Greis, T. (2011). Assessing the toxic effects of nickel, cadmium and EDTA on growth of the plant growth-promoting rhizobacterium *Pseudomonas brassicacearum*. *Water Air Soil Pollution*.
- Laguerre, G., Allard, M.R. and Amarger, N. (1994). Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR- amplified 16S rRNA genes. *Applied and Environmental Microbiology*. 60:56-63.
- Lakzian, A., Murphy, P., Turner, A., Beynon, J.L. and Giller, K.E. (2002). *Rhizobium leguminosarum* bv. *viciae* populations in soils with increasing heavy metal contamination: abundance, plasmid profiles, diversity and metal tolerance. *Soil Biology and Biochemistry* 34:519–529.
- Lee, J.Y. and Song, S.H. (2007). Evaluation of groundwater quality in coastal areas: implications for sustainable agriculture. *Environmental Geology*, 52: 1231-1242.
- Libbenga, K.A. and Harkes, P.A.A. (1973). Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. *Planta* 114. 17-28.
- Lioi, L. and Galasso, I. (2002). Oligonucleotide DNA fingerprinting revealing polymorphism in *Phaseolus lunatus* L. *Genetics Resources and Crop Evolution*. 49:53–58.
- Liu, Z. and Furnier, G.R. (1993). Comparison of allozyme, RFLP and RAPD for revealing genetic variation within and between trembling aspen and bigtooth aspen. *Theoretical and Applied Genetics* 87: 97-105.
- Long, S. R. (2001). Genes and signals in the rhizobium-legume symbiosis. *Plant Physiology* Vol. 125, No. 1, pp. 69-72.
- Long, R., Temple, S., Meyer, R., Schwankl, L., Godfrey, L., Canevari, M., Roberts, P. and Gepts, P. (2014). Lima bean Production in California. University of California, a Division of Agriculture and Natural Resources Publication 8505. p. 1-24.

- Lyman, S. M., Baudoin, J. P. and Hidalgo, R. (1985). Lima bean (*Phaseolus lunatus*), in: *Grain Legume Crops*. Edited by Summer R.J and E. H. Roberts. Williams Collins Sons & Co. Ltd London United Kingdom. p. 477-519.
- Maquet, A., Vekemans, X. and Baudoin, J.P. (1999). Phylogenetic study on wild allies of lima bean, *Phaseolus lunatus* (*fabaceae*), and implications on its origin. *Plant Systematics and Evolution* 218: 43-54.
- Martinez-Romero, E. and Caballero-Mellado, J. (1996). *Rhizobium* phylogenies and bacterial genetic diversity. *Critical Reviews in Plant Science*. 15:113-140.
- Martinez-Romero, E. and Caballero-Mellado, J. (1996). *Rhizobium* phylogenies an bacterial genetic diversity. *Critical Review in Plant Science* 15: 113140.
- Matthews, A., Carroll, B.J. and Gresshoff, P.M. (1989). Development of *Bradyrhizobium* infection in supernodulating and non-nodulating mutants of soybean (*Glycine max* [L.] Merrill). *Protoplasma* 150, 40–47.
- Maunoury, N., Kondorosi, A., Kondorosi, E. and Mergaert, P. (2008). Cell biology of nodule infection and development. In *Nitrogen-fixing Leguminous Symbioses* James, E.K., Sprent, J.I., Dilworth, W.E. and N. W.E., pp. 153–189, Springer, the Netherlands
- Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., Mausset, A. E., Barloy-Hubler, F., Galibert, F., Kondorosi, A. and Kondorosi, E. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis. *Proceedings of the Nationall Academy of Science U S A*, Vol. 103, No. 13, pp. 5230-5235.
- Michiels, J., Verreth, C. and Vanderleyden, J. (1994). Effects of temperature stress on bean nodulating *Rhizobium* strains. *Applied Environmental Microbiology*. 60:1206–1212.
- Moawad, H. and Beck, D. (1991). Some characteristics of *Rhizobium leguminosarum* isolates from uninoculated field-grown lentil. *Soil Biology and Biochemistry*. 23:917–925.
- Moulin, L., Munive, A., Dreyfus, B. and Boivin, M.C. (2001). Nodulation of legumes by members of the beta-subclass of Proteobacteria. *Nature*, 411: 948–950.
- Mpeperekki, S., Makonese, F. and Wollum, A.G. (1997). Physiological characterisation of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. *Symbiosis* 22: 275-292.
- Munns, D. N. (1986). Acid soils tolerance in legumes and rhizobia. *Advanced Plant Nutrition*. 2:63–91.

- Nabizadeh, E., Jalilnejad, N. and Armakani, M. (2011). Effect of salinity on growth and nitrogen fixation of alfalfa (*Medicago sativa*), *World Applied Sciences Journal*, 13 : 8, 1895 - 1900.
- Newcomb, W. (1976). A correlated light and electron microscopic study of the symbiotic growth and differentiation In *Pisum sativum* root nodules. *Canadian Journal of Botany* 54, 2163-2186.
- Newcomb, W., Sippel, D. and Peterson, R.L. (1979). The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. *Canadian Journal of Botany* 57. 2603-2616.
- Ngom, A., Nakagawa, Y., Sawada, H., Tsukahara, J., Wakabayashi, S., Uchiumi, T., Nuntagij, A., Kotepong, S., Suzuki, A., Higashi, S. and Abe, M. (2004). A novel symbiotic nitrogen-fixing member of the *Ochrobactrum* clade isolated from root nodules of *Acacia mangium*. *Journal of General and Applied Microbiology*, 50: 17–27.
- Nwokolo, E. (1996). Lima beans (*Phaseolus lunatus* L.), in: Food and Feed from Legumes and oil Seed Edited by Nwokolo E. and J. Smartt, Chapman and Hall p 144-155.
- Oldroyd, G.E.D. and Downie, J.A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annual Review Plant Biology* 59, 519–546.
- Oliveira, A.P., Alves, E.U., Alves, A.U., Dornelas, C.S.M., Silva, J.A., Porto, M.L. and Alves, A.V. (2004) *Produção de feijão-fava em função do uso de doses de fósforo em um Neossolo Regolítico*. *Horticultura Brasileira* 22, 543-546.
- Ologhobo, A., Apata, D., Oyejide, A. and Akinpelu, R.O. (1993) A comparison of Protein fractions prepared from lima beans (*Phaseolus lunatus*) in Starter diets for broiler chicks. *Journal of Applied Animal Research*, 4(1): 13-30.
- Paudyal, S.P., Aryal, R.R., Chauhan, S.V.S. and Maheshwari, D.K. (2007). Effect of heavy metals on growth of *Rhizobium* strains and symbiotic efficiency of two species of tropical legumes. *Science World* 5:27–32.
- Peoples, B., Brockwell, J., Herridge, D.F., Rochester, I.J., Alves, B.J.R., Urquiaga, S., Boddey, R.M., Dakora, F.D., Bhattari, S., Maskey, S.L., Sampet, C., Rerkasem, B., Khan, D.F., Hauggaard-Nielson, D.F.H. and Jensen, E.S. (2009). The contributions of nitrogen fixing crop legumes to the productivity of agricultural systems. *Symbiosis* 48, 1-17.
- Peoples, M.B., Herridge, D.F. and Ladha, J.K. (1995). Biological nitrogen Fixation: An efficient source of nitrogen for sustainable agricultural production. *Plant Soil* 174: 3-28.

- Pepper, I.L. and Upchurch, R.P. (1991). Nitrogen fixation by desert legumes associated with rhizobia, In: *Semiarid lands and deserts: Soil resource and reclamation*, Editor: Skujins, J., Marcel Dekker Inc., New York.
- Polhill, R.M., Raven, P.H. and Stirton, C.H. (1981). Evolution and systematics of *Leguminosae*. In: Polhill R.M., Raven, P.H., (eds) *Advances in legume systematics* Part 1, Royal Botanic Gardens, Kew, UK, pp 1-26.
- Polignano, G.B., Ugenti P. and Scippa, G. (1993). The patterns of genetic diversity in faba bean collections from Ethiopia and Afghanistan. *Genetics Resources and Crop Evolution* 40:71–75.
- Postgate, J. (1998). Nitrogen fixation. Cambridge University Press, Cambridge, UK Pp 112.
- Prescott, L.M., Harley, J.P. and Klein, D.A. (1996). *Microbiology*. 3rd edition. United States of America: WCB Publishers. Chicago, IL. Pp 811-877.
- Purseglove, J.W. (1974). *Phaseolus lunatus* In: *Tropical Crops: Dicotyledons*, Longman, London, pp 296-301.
- Rachie, K. O., Song, L. and Lyman, S. (1980). Lima beans (*Phaseolus lunatus* L.) and its potential in the Tropics. In: *Advances in Legumes Science*. Edited by Summerfield R. J. and A. H. Bunting Kew: Royal Botanic Gardens. P.375-381.
- Rafael, L. H. (2001). *Los Mochicas*. Museo Arqueológico Rafael Larco Herrera. Lima 2001. [ISBN 9972-934-10-1](https://www.isbn-international.org/product/9789972934101)
- Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L. and Rolfe, B.G. (1986). Flavones induce expression of nodulation genes in *Rhizobium*. *Nature* 323, 632–635.
- Reed, B., Engelmann, F., Dullo, M. and Engels, J. (eds) (2004). Technical guidelines for the management of field and in vitro germplasm collection. IPGRI Handbooks for Gene banks No. 7. International Plant Genetic Resources Institute, Rome, Italy.
- Rendig, V.V. and Taylor, H.M. (1989). Principles of Soil-Plant Interrelationships, McGraw-Hill Publishing Company, New York pp 227.
- Richardson, A.E., Viccars, L.A., Watson, J.M. and Gibson, A.H. (1995). Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biology and Biochemistry* 27: 515-524.
- Rivas, R., Willems, A., Subba-Rao, N.S., Mateos, P.F., Dazzo, F.B., Kroppenstedt, R.M., Martínez-Molina, E., Gillis, M. and Velázquez, E. (2003). Description of *Devosia*

*neptuniae* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. *Systematics and Applied Microbiology*, 26: 47-53.

Roecklein, J. (1987). Rhizobia. *Current Opinion in Microbiology*, 3: 613-617.

Rolfe, B.G. and Gresshoff, P.M. (1988). Genetic analysis of legume nodule initiation. *Annual Review of Plant Physiology and Plant Molecular Biology*. 39, 297–319.

Sagayo, F. (2006). Morphological characterisation and evaluation of lima bean (*Phaseolus lunatus* L.) collected from Benguet. Benguet State University, La Trinidad, Benguet.

Saka, J.O., Ajibade, S.R., Adeniyi, O.N., Olowoyo, R.B. and Ogunbodede, B.A. (2004). Survey of under-utilized grain legume production system in south-west Agricultural zone of Nigeria. *Journal of Agriculture and Food Information* 6 (2/3): 93-108.

Salisbury, F.B., and Ross, C.W., (1992). *Plant Physiology*. 4<sup>th</sup> edn. California: Wadsworth Publishing Company.

Sanginga, N. (2003). Role of biological nitrogen fixation in legume based cropping systems; A case study of West Africa farming systems. *Plant Soil* 252: 25-39.

Santamaría, M.M., Marrero, A.R.D., Hernaández, J., Navarro, A.M.G. and Corzo, J. (2003). Effect of thorium on the growth and capsule morphology of *Bradyrhizobium*. *Environmental Microbiology* 5:916–924.

Santos, J.O., Araújo, A.S.F., Gomes, R.L.F., Lopes, Â.C.A., and Figueiredo, M.V.B. (2008). Rhizobia- *Phaseolus lunatus* Symbiosis: Importance and Diversity in Tropical Soils- A Review. *Dynamic Soils, Dynamic Plant2* (2). Global Science Books. Pp 56-60.

Schneider, M. and De Bruijn, F.J. (1996). Rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic patterns analysis. *World Journal of Microbiology and Biotechnology*, 12: 163-174.

Sessitsch, A., Hardarson, G., Akkermans, A.D.L. and de Vos, W.M. (1997). Characterisation of *Rhizobium elti* and other *Rhizobium spp.* that nodulate *Phaseolus vulgaris* in an Austrian soil. *Molecular Ecology* 6: 601-608.

Shi, W., Bischoff, M., Turco, R. and Konopka, A. (2002). Long-term effects of chromium and lead upon the activity of soil microbial communities. *Applied Soil Ecology* 21 : 169 177.

Sikora, S., Dedzepovic, S. and Bradic, M. (2002). Genomic fingerprinting of *Bradyrhizobium japonicum* isolates by RAPD and rep-PCR. *Microbiological Research*, 157: 213-219.

Sinclair, T.R., Muchow, R.C., Bennet, J.M. and Hammnd, L.C. (1987). Relative sensitivity of nitrogen and biomass accumulation to drought in field grown soyabean. *Agronomy Journal* 79: 986-991.

Smartt, J. (1990). The New World Pulses: *Phaseolus* species. In: *Grain Legumes, Evolution and Genetic Resources*. Cambridge University Press Cambridge, p. 85-139.

Smith, I.F. (1998). *Foods of West Africa, their origin and use*. National library of Canada, Legal Deposit number, D702971. Ottawa Ont. KIVOP 6.

Somasegaran, P. and Hoben, H.J. (1994). *Handbook for Rhizobia: Methods in Legume- Rhizobium Technology*, New York: Springer-Verlag.

Spaink, H.P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. *Annual Review of Microbiology*, 54, 257–288.

Stan, V., Gament, E., Corena, C.P., Voaides, C., Dusa, M. and Plopeanu, G. (2011). Effects of heavy metal from polluted soils on the *Rhizobium* diversity. *Notulae Botanicae Horti Agrobotanici, Cluj* 39:88–95.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., De Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C. and Dreyfus, B. (2001). Methylo-trophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology*, 183: 214-220.

Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G. and Zuberer, D.A. ( 2005). *Principles and Applications of Soil Microbiology*. 2nd Edition. 373 - 404.

Tadawan, B., Padua, D., Tianza, G. and Sagalla, E.J. (2004). Germplasm collection of indigenous agronomic crops in Benguet. Paper presentation during the sectors review held at ATI, NTC on July 16, 2004.

Tanksley, S.D., Young, N.D., Paterson, A.H. and Bonierval, M.W. (1989). RFLP mapping in plant breeding: new tools for an old science. *Biotechnology* 7: 257-264.

Tate, R. L. (1995). *Soil microbiology (symbiotic nitrogen fixation)* New York, N.Y: John Wiley & Sons, Inc.. pp. 307–333.

Timmers, A.C.J., Scupene, E., Auriac, M.C., de Billy, F., Vasse, J., Boistard, P. and Truchet, G. (2000). Saprophyte Intercellular rhizobia in alfafa nodules. *Molecular Plant Microbe Interaction*. 13: 1204-1213.

Triplett, E.W. and Sadowsky, M.J. (1992). Genetics of competition for nodulation. *Annual Review Microbiology*, 46: 399-428.

- Trujillo, M.E., Willems, A., Abril, A., Planchuelo, A.M., Rivas, R., Ludena, D., Mateos, P.F. and Martinez-Molina, E. (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Applied Environmental Microbiology*, 71: 1318–1327.
- Turco, R.F. and Sadowsky, M.J. (1995). Understanding the micro flora of bioremediation. In H. D. Skipper and R. F. Turco (Eds.), *Bioremediation: Science and Applications*. Soil Science (Special Publication). Madison, WI: *Soil Science Society American Journal*, 43: 87-103.
- Udvardi, M. and Day, D. (1997). Metabolite transport across symbiotic membranes of legume nodules. *Annual Review of Plant Physiology and Plant Molecular Biology*. 48, 493–523.
- Ustimenko-Bakumovsky, G.V. (1983). *Plant growing the Tropics and Sub Tropics*. MIR Publishers, Moscow, pp: 28-264.
- Van Berkum, P. and Eardly, B.D. (2002). The Aquatic Budding Bacterium *Blastobacter denitrificans* is a Nitrogen-Fixing Symbiont of *Aeschynomene indica*. *Applied Environmental Microbiology*, 68: 1132-1136.
- Vandamme, P. and Coenye, T. (2004). Taxonomy of the genus *Cupriavidus*: a tale of lost and found. *International Journal of Systematic and Evolutionary Microbiology*, 54: 2285–2289.
- Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kerster, K. and Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiology Review* 60: 407-438.
- Van der Maeseen, L. J. G. and Sadikin, S. (1989). *Plant Resources of South East Asia. Pulses production*. Wageningen, pp: 56-60.
- Vassileva, V., Milanov, G., Ignatov, G. and Nikolov, B. (1997). Effect of low pH on nitrogen fixation of common bean grown at various calcium and nitrate levels. *Journal of Plant Nutrition*. 20: 279–294.
- Vitousek, P.M. (1997). Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications* 7: 737-750.
- Wani, P.A. (2008). Heavy metal toxicity to plant growth promoting rhizobacteria (PGPR) and certain legume crops. Ph.D. Thesis, Aligarh Muslim University, Aligarh, India.
- Wani, P.A., Khan, M.S. and Zaidi, A. (2008). Effect of metal-tolerant plant growth-promoting *Rhizobium* on the performance of pea grown in metal-amended soil. *Archives of Environmental Contamination and Toxicology* 55:33–42.

Wei, G.H., Wang, E.T., Tan, Z.Y., Zhu, M.E. and Chen, W.X. (2002). *Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov. respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea*. *International Journal of Systematic and Evolutionary Microbiology*, 52: 2231-2239.

Wery, J., Silim, S.N., Knight, E.J., Malhotra, R.S. and Cousin, R. (1994). Screening techniques and sources of tolerance to extremes of moisture and air temperature in cool season food legumes. *Euphytica* 73: 73-83.

Wolde-Meskel, E., Terefework, Z., Lindstrom, K. and Frostegard, A. (2004). Metabolic and Genomic Diversity of Rhizobia Isolated from Field Standing Native and Exotic Woody Legumes in Southern Ethiopia. *Systematics and Applied Microbiology*, 27: 603-611.

Wopereis, J., Pajuelo, E., Dazzo, F.B., Jiang, Q., Gresshoff, P.M., De Bruijn, F.J., Stougaard, J. and Szczyglowski, K. (2000). Shoot root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype. *Plant Journal* 23, 97–114.

Worrall, V. S. and Roughley, R. J. (1976). The effect of moisture stress on infection of *Trifolium subterraneum* L. by *Rhizobium trifolii* Dang. *Journal of Experimental Botany*. 27:1233–1241.

[wiki-fitness.com/health-benefits-lima-beans/](http://wiki-fitness.com/health-benefits-lima-beans/).

[www.livestrong.com/article\(42153-limabeans-nutrition-information/](http://www.livestrong.com/article(42153-limabeans-nutrition-information/).

Young, J.M. (2003). The genus name *Ensifer Casida* 1982 takes priority over *Sinorhizobium* Chen *et al.* 1988, and *Sinorhizobium morelense* Wang *et al.* 2002 is a later synonym of *Ensifer adhaerens* Casida 1982. Is the combination “*Sinorhizobium adhaerens*” (Casida 1982) Willems *et al.* 2003 legitimate, Request for an Opinion. *International Journal of Systematic and Evolutionary Microbiology*, 53: 2107–2110.

Zacarias, A.M. (1997). Identification and genetic distance analysis of cassava (*Manihot esculenta* Crantz) cultivars using RAPD fingerprinting. MSc thesis. University of the Free State, Bloemfontein, South Africa.

Zahran, H.H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews*, 63: 968–89.

Zoro Bi, I., Maquet, A. and Baudoin, J.P. (2003). Population genetic structure of wild *Phaseolus lunatus* (*Fabaceae*), with special reference to population sizes. *American Journal of Botany* 90, 897-904.

## CHAPTER THREE

### MORPHOLOGICAL CHARACTERISATION OF THE LIMA BEAN

#### 3.1 INTRODUCTION

Variations in germplasm collection with respect to morphological phenological traits can be studied when characterisation is done. Morphological characterisation is the foundation of genetic diversity research in any taxonomical level (Chandran and Pandya, 2000) and is a vital tool in the management of crop germplasm collections (Ariyo, 1993; Polignano *et al.*, 1993). It has been used for various purposes including identification of duplicates, studies of genetic diversity patterns and correlation with characteristics of agronomic importance (CIAT, 2007).

Lima bean cultivars can be distinguished on the basis of morphological traits and possess a wide variability of botanical characteristics. Assessment of morphological characters involve measuring of phenotypic traits in plants which is an attribute of the interaction of genetic and environmental factors.

Morphological characterisation of crops is facilitated by the use of standard descriptors which provides an international format, for producing a universally understood language for plants genetics resource data (CIP/AVDRC/IBPGR, 1991). These descriptors serve as characteristics by which germplasm can be known and its potential usefulness determined. In addition, these characterisation descriptors aid in relatively easy discrimination among phenotypes.

## **OBJECTIVE**

To evaluate the divergence and similarities among lima bean cultivars, as well as characterise them by their morphological traits.

## **3.2 MATERIALS AND METHOD**

### **3.2.1 Experimental Site**

A pot experiment was conducted from August, 2013 through to December, 2013 at the research farm of the Biotechnology and Nuclear Agriculture Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC) at Kwabenya, Accra. The site is situated on latitude 05° 40'N and longitude 0° 30'W in the Coastal Savannah zone of Ghana. It lies 76 m above sea level and located 20 km north of Accra. Annual rainfall ranges from 700 mm to 1000 mm (Morris *et al.*, 1999). The predominant soil type in the locality is the well drained Savannah ochrosol (Ferric Acrisol, locally called Haatso series, (sandy loam), derived from quartzite schist (FAO/UNESCO, 1994). Indicated in Table 3.1 are some of the physical and chemical properties of the soil at the experimental site.

### **3.2.2 Plant Materials**

#### **3.2.2.1 Lima Bean Cultivars**

Thirteen (13) cultivars of the lima bean were collected and used as test plants. Four of the cultivars were obtained from the CSIR- PGRRI at Bunso, Eastern Region and the other nine cultivars obtained from markets such as 'Makola', 'Kaneshie', 'Nima',

'Newtown' and 'Madina'. These were sources mainly from some villages in the Northern parts of Ghana and from the Eastern parts of Ghana. The cultivars collected were GH 1714, GH 1719, GH 1726 and GH 1736 (all from CSIR-PGRRI) and A1 (BNARI, brown), A2 (BNARI, black eyed), A3 (Kaneshie, brown), A4 (Madina), M1 (Nima), M2 (Makola), M3 (Newtown), M4 (Kaneshie) and M5 (Kaneshie).

**Table 3.1 Physical and chemical properties of the soil at the experimental site**

Soil property	Value
<sup>a</sup> pH (H <sub>2</sub> O) (1:2)	7.8
<sup>b</sup> Org. C. /Org. matter (%)	1.83/3.2
<sup>c</sup> NH <sub>4</sub> N (mg/Kg/μg/g)	56 (0.0224 % N)
<sup>c</sup> NO <sub>3</sub> N (mg/Kg/μg/g)	476 (0.1904 % N)
<sup>d</sup> P (mg/Kg/μg/g)	84
<sup>e</sup> K (cmol/Kg/me/100g)	4.35
Sand (%)	41.41
Silt (%)	43.17
Clay (%)	15.42
<b>Textural class</b>	Sandy loam

a = Electrical method b = Walkey and Black (Allison *et al.*, 1965) c = Kjeldahl procedure (Jackson, 1956) d = (Olsen *et al.*, 1954, Bray and Kurtz, 1945) e = Flame photometric method.

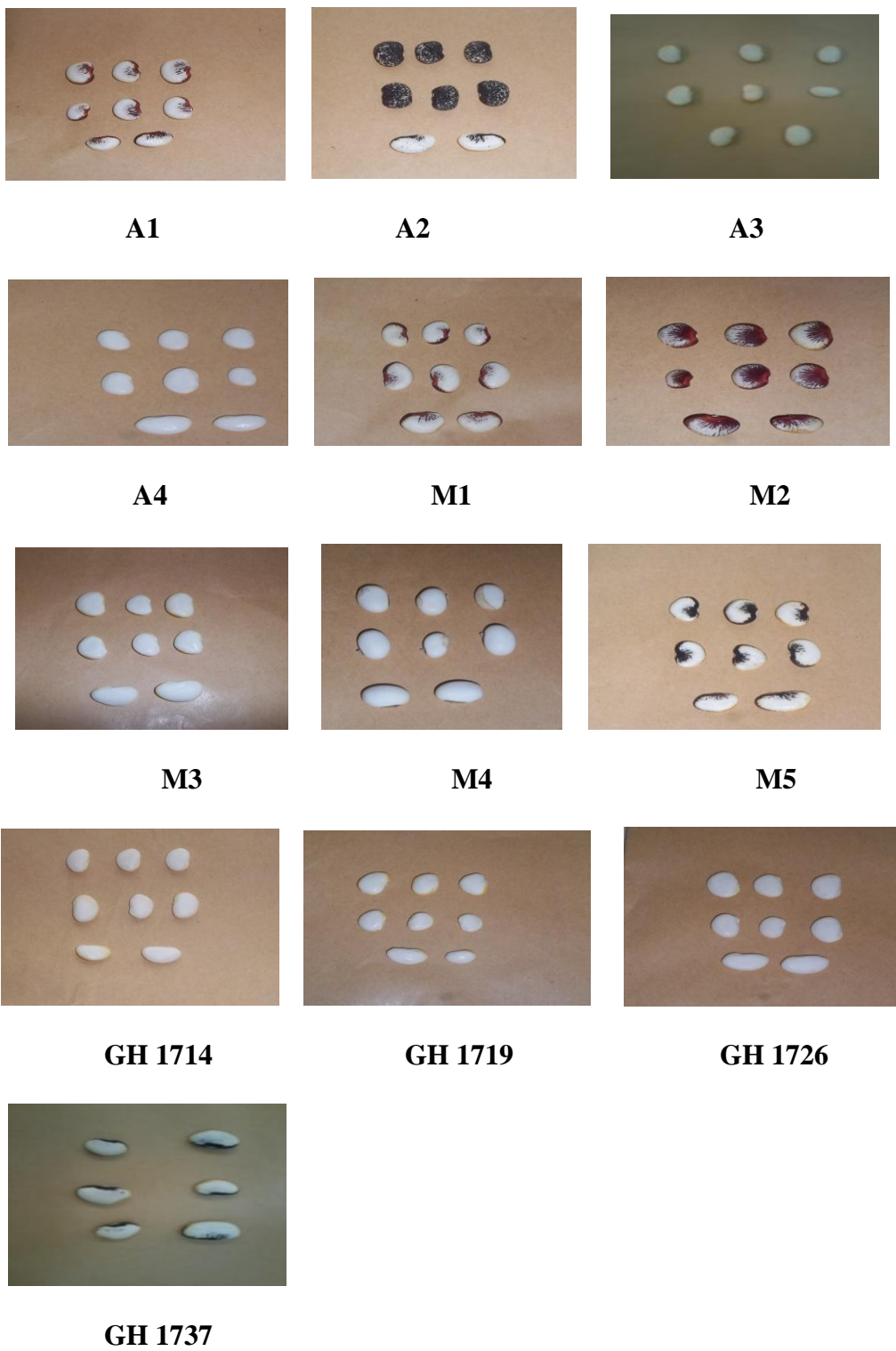


Figure 3.1 Seeds of thirteen cultivars of lima bean used in the experiment.

### **3.2.2.2 Preparation Of Potted Experiment**

Prior to sowing, top soil from two different sources were collected and homogenously mixed thoroughly in a ratio of 1:1. Poly bags were filled with 36 kilograms (kg) of soil and perforations made underneath to enhance air circulation and drainage.

### **3.2.3 Experimental Design**

The experiment was arranged in a Randomised Complete Block Design (RCBD) with thirteen treatments (lima bean cultivars) which were replicated three times. Each replicate was made up of six (6) poly bags per cultivar. The seeds were planted on the 10th August, 2013. The total field size for the pot experiment was 1.33 m<sup>2</sup> with inter row and intra row spacing of 0.09 m × 0.07 m respectively. Four seeds were sown per pot and later thinned to one seedling per pot. The experiment relied solely on irrigation to maintain adequate moisture. Weeds were removed manually from pots throughout the experiment. Neither pesticides nor fertilizers were applied during the period of the experiment. Plants, mostly creeping, were all staked and trained to climb the stakes.

### **3.2.4 Evaluation of Morphological Characters**

Morphological characters were evaluated for each of the thirteen cultivars of the lima bean. Assessment of ( 16 ) qualitative and ( 8 ) quantitative traits was done using lima bean descriptors (IBPGR, 1982) at 90-120 days after planting (DAP). Samples of six plants from each of the 13 lima bean cultivars, two from each replicate were used for morphological characterisation.

### 3.2.4.1 Qualitative Traits

The qualitative traits were evaluated by visual observation and scored using the scale of 1- 7 (IBPGR, 1982). Traits include the following:

---

a. Growth habit	i. Hairiness of standard
b. Main stem pigmentation	j. Pod colour
c. Leaflet shape	k. Pod beak shape
d. Vein colour of fully developed primary leaves	l. Pod curvature
e. Colour of flowering wings.	m. Seed background colour
f. Colour of flower standard	n. Seed pattern colour
g. Colour of keel	o. Second pattern colour (seed)
h. Wing opening	p. Seed coat pattern

---

### 3.2.4.2 Quantitative traits

Quantitative traits were evaluated using assessments such as the average score of the length or width, largest score of their width or either the range of length or breadth (IBPGR, 1982). Measurements were done using a 30 cm ruler. Quantitative traits observed include the following:

---

a. Leaflet length	e. Seed length
b. leaflet width	f. Seed width
c. Pod length	g. Seed weight per 10 seeds
d. Pod width	h. Days to 50% emergence

---

### **3.2.4.3 Data analysis of morphological data**

Data recorded on qualitative morphological traits were used to construct a dendrogram. Genetic similarity and cluster analysis were performed by calculating Jaccard's similarity with the aid of GENSTAT software 12<sup>th</sup> edition. For quantitative morphological traits, an ANOVA was performed with the aid of GenStat software 12<sup>th</sup> edition. Means were separated using Least Significant Difference (LSD) procedure when differences showed significance at  $P \leq 0.05$ .

### 3.3 RESULTS

#### 3.3.1 Qualitative morphological traits analysis

Data on sixteen (16) qualitative morphological traits of the thirteen cultivars of lima bean were studied and their scoring are presented in APPENDIX (I).

Twelve of the lima bean cultivars had indeterminate growth habit with the exception of M4 which showed determinate growth habit.

Twelve lima bean cultivars had no pigmentation on the main stem. Cultivar M4, however, had an almost extensive pigmentation.

Three types of leaf shapes were observed in the lima bean cultivars. Nine cultivars had roundish leaf shape with A4, M4 and GH 1719 having ovate lanceolate leaf shape. Cultivar M1, however, had ovate leaf shape.

All the thirteen lima bean cultivars had greenish vein colour along fully developed primary leaves.

Two flower wing colours were observed for the thirteen lima bean cultivars studied. Twelve lima bean cultivars had white flower wings with M4 being an exception with a light pink colouration (Figure 3.2).

All the thirteen lima bean cultivars exhibited white flower standard (Figure 3.3).

Greenish keel colour was observed in all the thirteen lima bean cultivars of the lima bean (Figure 3.3).

Freshly opened flowers observed in all the thirteen lima bean cultivars showed a parallel and closed wing openings (Figure 3.2). Also, all thirteen lima bean cultivars had no hairs on their flower standard.

The pods, on reaching maturity, were brown with red spotting and mottling colour in all the thirteen lima bean cultivars.

In terms of pod beak shape, three shapes were observed; long, medium and thick beaks. Ten lima bean cultivars had pod beak that were long in shape, with cultivars M2 and M3 having a medium length beak. Cultivar M4, however, had a thick beak shape. Also, twelve lima bean cultivars had slightly curved pods with the exception of M4 which had straight curved pods.

Additionally, seeds of the thirteen lima bean cultivars had a white background colour. The lightest colour pattern including that around the eye of seeds of A1, M1, M2 and M4 were light brown in colour. Cultivar M5 had a purple red seed colour pattern. Seven other cultivars had no pattern on their seeds.

The darkest colour pattern observed on the seeds were of three types; monocoloured, dark red and dark patterns. Monocoloured pattern on seeds were observed in lima bean cultivars A3, A4, M3, M4, GH 1719, GH 1726 and GH 1737. Cultivars A1, M1, M2 and GH 1714 had dark red pattern on their seeds, while cultivars A2 and M5 had a dark pattern on their seeds.

The seed coat of six cultivars A3, A4, M3 GH 1719. GH 1726, GH 1737 were monocoloured in pattern, while cultivars A1, M1 and M5 had eye linked to pattern, with blotch covering hilar region and front with specks present. Cultivars A2 and GH 1714 had eye linked to pattern with blotch in hilar region with some specks whereas M4 had pattern around eye only.



Figure 3.2 Wing opening and colour of freshly opened flowers of some cultivars of the lima bean



Figure 3.3 Flower keel and standard of some cultivars of the lima bean.

**Table 3.2 Morphological qualitative traits of 13 lima bean (*Phaseolus lunatus* L.) cultivars.**

<b>Cultivars</b>	<b>Growth pattern</b>	<b>Main stem pigmentation</b>	<b>Vein colour of developed primary leaves</b>	<b>Leaf shape</b>	<b>Flower wings (colour)</b>	<b>Flower standard (colour)</b>	<b>Keel (colour)</b>	<b>Wing opening</b>	<b>Standard (hairiness)</b>
<b>A1</b>	Indeterminate	No pigmentation	Green	Round	White	white	Greenish	Parallel, closed	Absent
<b>A2</b>	Indeterminate	No pigmentation	Green	Round	White	white	Greenish	Parallel, closed	Absent
<b>A3</b>	Indeterminate	No pigmentation	Green	Round	White	White	Greenish	Parallel, closed	Absent
<b>A4</b>	Indeterminate	No pigmentation	Green	Ovate lanceolate	White	White	Greenish	Parallel, closed	Absent
<b>M1</b>	Indeterminate	No pigmentation	Green	Ovate	White	White	Greenish	Parallel, closed	Absent

**Table 3.2 CONT'D Morphological qualitative traits of 13 lima bean (*Phaseolus lunatus* L.) cultivars.**

<b>M2</b>	Indeterminate	No	Green	Round	White	White	Greenish	Parallel,	Absent
		pigmentation						closed	
<b>M3</b>	Indeterminate	No	Green	Round	White	White	Greenish	Parallel,	Absent
		pigmentation						closed	
<b>M4</b>	Determinate	Almost solid	Green	Ovate	Light pink	White	Greenish	Parallel,	Absent
				lanceolate				closed	
<b>M5</b>	Indeterminate	No	Green	Round	White	White	Greenish	Parallel,	Absent
		pigmentation						closed	
<b>GH 1714</b>	Indeterminate	No	Green	Round	White	White	Greenish	Parallel,	Absent
		pigmentation						closed	
<b>GH 1719</b>	Indeterminate	No	Green	Ovate	White	White	Greenish	Parallel,	Absent
		pigmentation		lanceolate				closed	
<b>GH 1726</b>	Indeterminate	No	Green	Round	White	White	Greenish	Parallel,	Absent
		pigmentation						closed	
<b>GH 1737</b>	Indeterminate	No	Green	Round	White	white	Greenish	Parallel,	Absent
		pigmentation						closed	

**Table 3.2** CONT'D Morphological qualitative traits of 13 lima bean (*Phaseolus lunatus* L.) cultivars.

<b>Cultivars</b>	<b>Mature Pod</b>	<b>Pod</b>	<b>Pod beak</b>	<b>Seed background</b>	<b>Seed pattern</b>	<b>Seed secondary</b>	<b>Seed coat pattern</b>
	<b>Colour</b>	<b>curvature</b>	<b>shape</b>	<b>colour</b>	<b>colour</b>	<b>pattern</b>	
<b>A1</b>	Brown with red spots mottle	Slightly curved	Long	White	Light brown	Dark red	Eye linked to pattern, hilar and front (blotch), specks
<b>A2</b>	Brown with red spots mottle	Slightly curved	Long	White	No pattern	Black	Eye linked to pattern, hilar region (blotch ), specks
<b>A3</b>	Brown with red spots mottle	Slightly curved	Long	White	No pattern	Monocoloured	No pattern
<b>A4</b>	Brown with red spots mottle	Slightly curved	Long	White	No pattern	Monocoloured	No pattern
<b>M1</b>	Brown with red spots mottle	Slightly curved	Long	White	Light brown	Dark red	Eye linked to pattern, hilar and front (blotch), specks
<b>M2</b>	Brown with red spots mottle	Slightly curved	Medium length	White	Light brown	Dark red	Eye to pattern, hilar region (blotch), bands to hilar region

**Table 3.2 CONT'D Morphological qualitative traits of 13 lima bean (*Phaseolus lunatus* L.) cultivars.**

<b>M3</b>	Brown with red spots mottle	Slightly curved	Medium length	White	No pattern	Monocoloured	No pattern
<b>M4</b>	Brown with red spots mottle	Straight	Thick	White	Light brown	Monocoloured	Pattern around eye
<b>M5</b>	Brown with red spots mottle	Slightly curved	Long	White	Purple red	Black	Eye linked to pattern, hilar and front (blotch), specks
<b>GH 1714</b>	Brown with red spots mottle	Slightly curved	Long	White	Dark brown	Dark red	Eye linked to pattern, hilar region (blotch ), specks
<b>GH 1719</b>	Brown with red spots mottle	Slightly curved	Long	White	No pattern	Monocoloured	No pattern
<b>GH 1726</b>	Brown with red spots mottle	Slightly curved	Long	White	No pattern	Monocoloured	No pattern
<b>GH 1737</b>	Brown with red spots mottle	Slightly curved	long	White	No pattern	Monocoloured	No pattern

### 3.3.2 Qualitative Principal component analysis

The first two principal components had latent roots greater than 1.0 and a combined total variation of 80.85 % (Table 3.3 ). Scores on the first principal component (PC1) accounting for 55.39 % of the total variation, correlated to characters relating to flower wings colour, main stem pigmentation and pod beak shape. The second principal component (PC2) and third principal component (PC3) accounting for 25.46 % and 7.31 % of total variation respectively, did not have any particular qualitative traits which contributed positively to the divergence among the cultivars. The fourth principal component (PC4) accounted for 5.89% of percentage variation correlating to traits, pod beak shape and secondary pattern. The traits, leaf shape and secondary pattern characterised the fifth principal component (PC5) with percentage variation of 4.65 %. The sixth principal component (PC6) was associated with secondary pattern and seed pattern colour.

**Table 3.3 Seven (7) Principal Components of sixteen (16) selected qualitative traits**

PC	PC1	PC2	PC3	PC4	PC5	PC6	PC7
LR	4.985	2.291	0.658	0.530	0.419	0.116	0.00
PV	55.39	25.46	7.31	5.89	4.65	1.29	0.00
CPV	55.39	80.85	88.16	94.05	98.70	99.9	99.9
Latent Vectors (loadings)							
FWC	0.435	-0.106	0.193	0.0360	0.102	0.014	<b>0.385</b>
GH	-0.435	0.106	-0.193	-0.036	-0.102	-0.014	-0.031
KC	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LS	0.288	0.190	-0.688	-0.466	<b>0.420</b>	0.108	0.00
MSP	<b>0.435</b>	-0.106	0.193	0.036	0.102	0.014	-0.190
PBS	<b>0.332</b>	-0.082	-0.551	<b>0.409</b>	-0.560	-0.310	0.00
PC	-0.435	0.106	-0.193	-0.360	-0.102	-0.014	0.227
PMC	0.00	0.00	0.00	0.00	0.00	0.00	0.013
SP	-0.155	-0.510	-0.265	<b>0.534</b>	<b>0.376</b>	<b>0.465</b>	0.00
SBC	0.00	0.00	0.00	0.00	0.00	0.00	-0.873
SCP	-0.154	-0.578	-0.343	-0.187	0.288	-0.722	0.00
SPC	0.019	-0.562	-0.022	-0.536	-0.491	<b>0.390</b>	0.00
SC	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SH	0.00	0.00	0.00	0.00	0.00	0.00	0.00
VC	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WO	0.00	0.00	0.00	0.00	0.00	0.00	0.00

PC- Principal Component, LR- Latent Roots, PV- Percentage Variation, CPV- Cumulative Percentage Variation, FWC- Flower Wings Colour, GH- Growth Habit, KC- Keel Colour, LS- Leaf shape, MSP- Main Stem Pigmentation, PBS- Pod beak shape, PC- Pod Curvature, PMC- Pod mature colour, SP- Secondary pattern, SBC- Seed background colour, SCP- Seed coat pattern, SPC- Seed pattern colour, SC- Standard colour, SH- Standard hairiness, VC-Vein colour, WO- Wing opening.

### 3.3.3 Cluster Analysis

The dendrogram constructed on the basis of the data generated from the 16 qualitative traits divided the 13 lima bean cultivars into two (2) major clusters (A and B), at a similarity level of 0.69. The major cluster B is further grouped into two sub-clusters (I and II) as summarised in Table ( 3.4 )

**Table 3.4 Cluster distribution of thirteen (13) cultivars of lima bean based on sixteen (16) qualitative traits.**

Cluster	Number of cultivars	Name of cultivars
Cluster A I	1	M4
Cluster B I	6	A3, A4, M3, GH 1719, GH 1726, GH 1737
II	6	a- A2 b- A1, M1, M2, M5, GH 1714

---

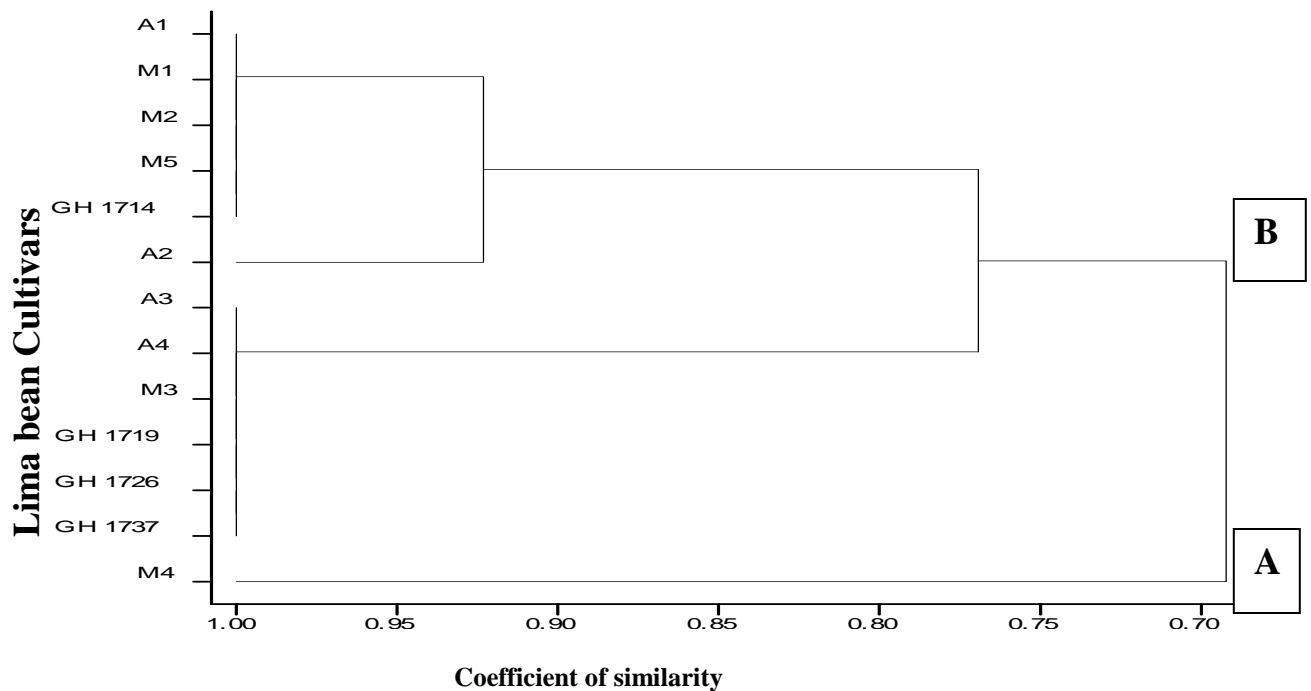


Figure 3.4 Dendrogram from complete linkage analysis showing relationship between thirteen (13) lima bean cultivars based on 16 qualitative morphological data and Jaccard distance.

### 3.3.4 Quantitative Traits

#### 3.3.4.1 Quantitative Morphological Traits Analysis

Table (3.5) shows a summary of results obtained from analysis of eight (8) quantitative morphological traits and parameter assessment in APPENDIX (2). The ANOVA result indicates that there are significant differences ( $p \leq 0.05$ ) among cultivars in all studied characters.

**Table 3.5 Mean scores of eight (8) quantitative morphological traits for thirteen lima bean cultivars**

Cultivars	Leaflet length (cm)	Leaflet width (cm)	Pod length (cm)	Pod width (cm)	Seed length (cm)	Seed width (cm)	Seed weight/10seeds (g)	Days to 50% emergence
A1	5.2 de	4.6 bc	7.8 b	2.2 cd	1.7 ab	1.2 b	0.86 bcd	0.402 bc
A2	5.7 de	4.5 c	7.1 b	2.2 cd	1.9 a	1.2 b	0.89 bcd	0.596 abc
A3	5.6 de	4.7 bc	8.1 b	2.5 b	1.6 ab	1.3 ab	0.90 bc	0.666 abc
A4	7.1 b	4.4 c	6.8 b	2.0 e	1.8 ab	1.2 b	0.83 cd	0.555 abc
M1	6.9 bc	4.9 bc	7.7 b	2.3 c	1.9 a	1.4 a	0.87 bcd	0.500 abc
M2	5.4 de	5.0 bc	7.2 b	2.6 b	1.9 a	1.4 a	0.80 de	0.805 b
M3	4.9 e	5.1 bc	7.0 b	2.1 de	1.9 a	1.2 b	0.95 b	0.587 abc
M4	11.3 a	8.5 a	20.7 a	2.8 a	1.7 ab	1.3 ab	1.19 a	0.903 a
M5	6.0 cd	5.3 bc	6.7 b	2.2 cd	1.9 a	1.3 ab	0.87 bcd	0.475 abc
GH 1714	5.6 de	4.6 bc	8.2 b	2.2 cd	1.8 ab	1.2 b	0.59 f	0.472 abc
GH 1719	6.9 bc	4.7 bc	7.6 b	2.2 cd	1.8 ab	1.2 b	0.61 f	0.694 abc
GH 1726	5.5 de	4.9 bc	7.0 b	2.1 de	1.5 b	1.2 b	0.52 f	0.309 bc
GH 1737	5.6 de	4.7 bc	7.7 b	2.1 de	1.7 ab	1.2 b	0.71 e	0.303 bc
LSD	1.02	0.78	2.27	0.11	0.31	0.17	0.09	0.44
CV%	4.3	2.7	7.2	0.4	6.8	4.6	3.9	22.5

#### **3.3.4.2 Days of 50 % emergence**

Cultivars of lima bean seedlings took between 4 and 7 days after sowing to emerge. Two lima bean cultivars namely M3 and A4, showed 50 % emergence in 4 days, with cultivar A1 emerging in 7 days. Majority of cultivars showed 50 % emergence in 5 days and 6 days (A3, M1, M2, M4, M5 and cultivars A2, GH 1714, GH 1719, GH 1726, GH 1737, respectively).

#### **3.3.4.3 Leaflet length**

The terminal leaflet length of the thirteen lima bean cultivars ranged from 4.9 cm to 11.3 cm with a mean of 6.3 cm. The longest terminal leaflet length was recorded for cultivar M4 with 11.3 cm. However, the smallest terminal leaflet length was for M3 with 4.9 cm.

#### **3.3.4.4 Leaflet width**

The mean terminal leaflet width was 5.1 cm. Cultivar M4 had the broadest terminal leaflet width of 8.5 cm while the cultivar with the lowest terminal width of 4.4 cm was for A4.

#### **3.3.4.5 Pod length**

The overall average length of dry mature pods of all thirteen lima bean cultivars was 8.4 cm. M4 had the longest pods of 20.7 cm while M5 had the least pod length of 6.7 cm.

#### **3.3.4.6 Pod width**

The average pod width of all lima bean cultivars was 2.3 cm. M4 had the widest pod width of 2.8 cm and the least pod width of 2.0 cm in A4. Five lima bean cultivars had pod width of 2.2 cm.

#### **3.3.4.7 Seed length**

The seed length of the thirteen lima bean cultivars averaged 1.8 cm. Cultivars M1, M2, M3 and M5 had the same seed length of 1.9 cm. GH 1726 had the smallest seed length of 1.5 cm.

#### **3.3.4.8 Seed width**

Two cultivars, M1 and M2, had the widest seed width of 1.4 cm. Eight cultivars, however had the least width of 1.2 cm. The average seed width was 1.25 cm.

#### **3.3.4.9 Seed weight**

The average weight of 10 seeds was 8.14 g. The cultivar M4 had the heaviest seed weight of 11.2 g and cultivars GH 1726 and GH 1714 recorded the least seed weight of 5.2 g and 5.9 g, respectively.

### **3.3.5 Quantitative Principal Component Analysis**

The first principal component (PC1) showed leaflet length, leaflet width, pod length, pod width, seed weight and days of 50 % emergence mostly contributed to the divergence among cultivars at 57.99 % of total percentage variation. The second principal

component (PC2) with 19.20 % as total variation has been correlated with seed length and seed width. For the third principal component (PC3), seed length and seed weight dominated at 10.22 % of total variation. The trait, days to 50 % emergence characterised the fourth principal component (PC4) with 5.37 % of variation. Principal component five (PC5) was correlated to seed length and seed width with a variation of 4.59 %. Also, PC6 correlated with leaflet length while PC7 was dominated by characters that include pod length and pod width.

**Table 3.6 Seven (7) Principal components for eight (8) selected quantitative traits**

Principal components	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Latent roots	4.639	1.536	0.817	0.429	0.368	0.152	0.053
Percentage variation	57.99	19.20	10.22	5.37	4.59	1.90	0.66
Cumulative percentage variation	57.99	77.19	87.41	92.78	97.37	99.27	99.93
<b>Latent vector (loadings)</b>							
Leaflet length	<b>0.395</b>	-0.234	0.204	0.091	0.518	0.573	0.168
Leaflet width	<b>0.424</b>	-0.223	0.103	-0.154	0.181	-0.476	-0.663
Pod length	<b>0.419</b>	-0.312	0.099	-0.010	0.099	-0.263	<b>0.412</b>
Pod width	<b>0.408</b>	0.097	-0.439	0.137	-0.223	-0.329	<b>0.446</b>
Seed length	0.020	<b>0.677</b>	<b>0.536</b>	0.079	<b>0.320</b>	-0.323	0.187
Seed weight	<b>0.356</b>	0.156	<b>0.356</b>	-0.570	-0.569	0.263	0.029
Seed width	0.236	<b>0.491</b>	-0.572	-0.348	<b>0.337</b>	0.198	-0.151
Days to 50% emergence	<b>0.371</b>	0.251	0.027	<b>0.704</b>	-0.312	0.229	-0.320

### 3.4 Discussion

Morphological measurements have been widely used to study crop diversity, including that of *Phaseolus lunatus* (Vargas *et al.*, 2003). Previous works done reiterate that the study of morphological traits (qualitative and quantitative) is still a relevant tool for lima bean diversity studies.

#### 3.4.1 Qualitative Morphological Analysis

The observed growth habit of the lima bean cultivars used in this study, showed twelve cultivars with 92.3 % indeterminate growth and cultivar M4, 7.7 % determinate growth. Work done by Nienhuis *et al.* (1995) on lima bean indicated that cultivar G25267 had a determinate (bush) growth habit, a characteristic similar to the Fordhook type of lima bean, by virtue of its phenotypic trait. Also, Zoro Bi *et al.* (2003) intimated that wild individuals of lima bean are characterised by determinate climbing growth. Thus, it is possible that cultivar M4 could be of the wild gene pool of lima bean cultivars, due to its determinate (bush) growth. Results from this study, correspond to the two growth habits, that is, determinate and indeterminate, documented by the morphological descriptors of lima bean in (IBPGR, 1982).

The main stem pigmentation of most of the lima bean had no pigmentation. Some cultivars however, had some pigmentation, but less predominate. Cultivar M4, however, possessed an almost solid pigmentation. In a similar study conducted on thirteen lima bean genotypes, Melo (2005) observed one genotype having purple pigmentation, others pigmented generalised, and most genotypes with no pigmentation.

For the leaf shape, three types were identified with 69 % roundish, 23 % ovate lanceolate and 8 % ovate. In reference to the lima bean descriptor (IBPGR, 1982), the leaf shapes not identified in this study were that of lanceolate and linear lanceolate. This result differs from observations made by Melo (2005) who had one out of thirteen genotypes of lima bean with leaf shapes classified as round. There were little differences among the thirteen (13) cultivars with regard to vein colour of fully developed primary leaves; they were greenish in colour.

Phenotypic observation of the flower wings colour showed primarily two colours. Generally, 92.3 % of the lima bean cultivars exhibited white flower wings with 7.7 % being light pink. This agreed with studies done by Asante *et al.* (2008) on lima bean in which white flower wings were predominant over light pink flower wings. However, an additional violet colour was also exhibited in their studies. Further studies by Yagui *et al.* (2003) showed flower wings in lima bean having mostly whitish colouration. There were little differences between the thirteen lima bean cultivars with regard to colour of flower standard and keel which were white and greenish, respectively.

The observed keel colour agrees with the results obtained by Asante *et al.* (2008), who observed predominantly greenish keel and a few with pink colouration. Similarly, Melo (2005) observed greenish keel colour in thirteen lima bean genotypes.

Observations made with respect to wing opening of freshly opened flowers showed that all thirteen cultivars were parallel and closed which corresponded to observations made by Asante *et al.* (2008), although other wing openings were intermediate in nature in that study. The additional observations made by Asante *et al.* (2008) can be attributed to the

numerous cultivars worked on. Similar results from present study are confirmed by Melo (2005) who had parallel wings in lima bean genotypes.

Phenotypic observation made on hairiness of standard indicated absence of hairs on flower standard in all cultivars, quite in contrast to results by Asante *et al.* (2008) in which moderate and sparse hairs on tips were observed.

In accordance to the lima bean descriptor IBPGR (1982), colour of matured pod could be either brown, brown with red spots or red, purple and black pigments. However, this present study showed the presence of matured pods with brown, red spotting or mottling pigmented patches.

The long, medium and thick beak shapes of pods were observed in this study. This is in contrast with results obtained by Asante *et al.*, (2008), which showed rather short, medium and long pod beak shapes. There were little differences between cultivars with regard to pod curvature, as 92.3% of the cultivars had slightly curved and 7.7 % with straight curvature.

Seed morphology is a basis of genetic diversity in lima bean cultivars. The seed background colour was white in all cultivars which were in contrast to results obtained by Yagui *et al.* (2003) in which diverse background colours were observed. This could be attributed to the few cultivars used in this study and also to the wide assembly or collection of lima bean accesses from Brazil, Tropical America, which is the centre of genetic diversity and origin. In addition, work done by Nienhuis *et al.* (1995) indicated that background colour of majority of lima bean seeds used were predominantly white although few were brown, green, grey and red. Melo (2005), working on the variability in

thirteen genotypes of lima bean, noted that the most frequent background colour was white, with four genotypes being an exception.

There were variability in seed pattern colour (lightest colour of pattern) and secondary colour (darkest colour of pattern) among the lima bean cultivars. The seed colour pattern (lightest colour pattern) of the thirteen lima bean cultivars had 53.8 % being monocoloured pattern, 30.8 %, light brown pattern and 7.7 %, with purple red pattern. Studies by Asante *et al.* (2008) had varied seed coat patterns of brown, cream, purple brown and others. Lioi and Gallasso (2002) intimated that *Phaseolus lunatus* exhibits variation in testa patterns in both shape and colour. Reasons for low amounts of seed colour patterns in this present study could be due to low number of cultivars worked on.

The seed secondary colour showed 53.8 % of the lima bean cultivars being monocoloured, 23.1 % dark red and 15.4 % with dark pattern. This result corresponds to results by Melo (2005) who observed low variability in seed secondary colour in thirteen lima bean genotypes. Colours observed included five seeds (red), three seeds (black) four seeds (missing) and one seed (light brown). Seed coat colour of the lima bean plays a role in crop yields as it may be a determinant factor in marketability of the lima bean depending on preference of consumers in a particular region (Lyman, 1983).

In accordance to the lima bean descriptor used (IBPGR, 1982), the seed coat pattern indicated were of thirteen pattern types. The present study showed only four types of seed coat patterns showing high variability in the lima bean seeds. Six cultivars contributing 46.2 % had no pattern, 38.5 %, eye linked to other parts of pattern, hilar and front with

blotches and few specks, and 7.7 %, with eye linked to other parts of pattern, hilar region with blotch and has bands around it.

### 3.4.2 Quantitative Morphological Analysis

The number of days for the lima bean cultivars to germinate was between 4 days (A3 and M4) and 7 days (A1). Lima beans usually take 5 days to 8 days to germinate. The difference in germination can be attributable to some soil conditions prevailing as at the time of sowing. Lima bean seeds fail to germinate in wet and compacted soils. In addition, attacks from soil borne pathogens can be a factor in low percentage emergence. The depth of soil in which seeds are sown can go a long way in determining early emergence as seeds experience difficulty in breaking loose from the soil.

The terminal leaflet length of the thirteen lima bean cultivars ranged from 4.9 cm to 11.9 cm. The longer leaflet length was associated with M4 and the shorter leaflet length observed in M3. Studies by Asante *et al.* (2008) on 31 lima bean cultivars had terminal leaflet length ranging from 6.5 cm to 10.6 cm. Majority of the cultivars collected by Asante *et al.* (2008) had leaflet length above 6cm which differed from results obtained in this study.

With respect to leaflet width, the cultivar with broadest width was cultivar M4 with 8.5 cm. The narrowest leaflet width was for A4 with 4.4 cm. This result was in contrast with results by Asante *et al.* (2008), in which leaflet width ranged from 2.4 cm to 7.6 cm. However, most of the cultivars had almost similar leaflet widths in both studies.

Leaflet length and width contribute to the size of the leaf whose effect is controlled by a combination of two or more genes. Guimarães (2005) stressed that the size of leaves is polygenic inheritance and may vary according to phenology phase, weather, location and mainly with soil fertility.

There was little variability among cultivars with regard to pod length. Cultivar M4 had the longest pod length of 20.7 cm while M5 had the least with 6.7 cm. This differed quite slightly from the study by Akande and Balogun (2007), in which pod lengths ranged from 5.0 cm to 7.1 in 2005 cm and 4.0 cm to 10.0 cm in 2006. Also majority of cultivars in the study by Akande and Balogun (2007) had pod lengths less than 7.0 cm, in contrast to results obtained in this study. Also, Santos *et al.* (2002) and Melo (2005) indicated that longest pod in their studies were 9.0 cm and 8.5 cm respectively, which differed from values in this study. Pod length is an important factor that affects seed yield thus, invariably, the longer the pod the higher the seed yield.

There was not much variability among the thirteen lima bean cultivars in this study with respect to pod width, which ranged between 2.0 cm to 2.8 cm. These results fell within the range of lima bean investigated by Melo (2005) and Guimarães (2005) with pod widths of 2.04 cm and 2.14 cm, respectively. The cultivar GH 1726 recorded the lowest seed length, seed width and seed weight of 1.5 cm, 1.2 cm and 0.52 g, respectively. Five lima bean cultivars (A2, M1, M2, M3 and M5) had seed lengths of 1.9 cm. Also, lima bean cultivars, A1, A2, A4, M3, GH 1714, GH 1719 and GH 1737 had similar seed widths of 1.2 cm. Lima bean cultivars M1 and M2 had the largest seed width of 1.4 cm with M4 having 1.20 g for seed weight. Nienhuis *et al.* (1995) observed larger seed lengths and weights which contrast values in the present study with exception of M4 that

agreed with some cultivars and landrace lima bean in their studies. With regard to seed width in the study of Nienhuis *et al.* (1995), lower seed widths were observed in most lima bean cultivars, which agreed with results in this present study.

In addition, studies by Guimarães (2005) on fourteen genotypes of lima bean originating from Ceará and Pernambuco, showed that seeds were 16.9 mm long and 11.7 mm wide, which corresponded to results observed in cultivars, A1, M4, GH 1737 and A1, A2, A4, GH 1714, GH 1719, GH 1726 and GH 1737, respectively, in present study. Santos *et al.* (2002) also indicated length variations in seeds from eight lima bean genotypes ranging from 7.8 mm to 17.5 mm of which cultivars, A1, M4 and GH 1737, in this study, alludes to. Loi (1994) and Esquivel (1990) identified five different morphotypes of lima bean based on seed length and seed width; baby lima (25 mm long, 14 mm wide), Sieva ( 11 mm long), potato (9 mm long, 8 mm wide), Potato Sieva (11 mm long, 8 mm wide) and Sieva big (17 mm long, 11 mm wide). From their investigations, we can infer that cultivars, A1, M4 and GH 1737, in this study fell within the range stipulated for Sieva Big.

### **3.4.3 Qualitative and Quantitative Principal Component Analysis**

Principal component analysis (PCA) is perhaps the most useful tool for screening multivariate data with significant high correlations (Johnson, 1998). From the PCA of the sixteen (16) qualitative traits studied, seven (7) traits contributed to divergence between the thirteen lima bean cultivars. These traits for distinguishing among the lima bean cultivars include flower wings colour, growth habit, leaf shape, main stem pigmentation,

pod beak shape, seed secondary colour and seed pattern colour. All eight (8) quantitative traits studied contributed highly for divergence among the cultivars studied.

#### **3.4.4 Cluster Analysis**

Cluster analysis aids in the decreasing of a number of individual variable units by classifying such variation into groups, which are translated into a dendrogram using the coefficient of similarity (Sneath and Sokal, 1973; Tatineni *et al.*, 1996).

The cluster analysis for qualitative traits separated the thirteen cultivars as different genotypes with Jaccard similarity distance ranging from 0.69 to 1.00. The dendrogram at the similarity distance 0.69 identified two major clusters according to the morphological character associated with them. Cluster A had M4 which had distinct determinate growth habit, almost solid pigmentation, light pink flower wing colour and straight pod curvature. For cluster B, majority of cultivars had similar attributes however with some slight variations. At 0.77 similarity coefficient, cluster B was subdivided into two subclusters I and II.

## REFERENCES

- Akande, S. R. and Balogun, M. O. (2007). Evaluation and heritability studies of local Lima bean (*Phaseolus lunatus* L.) cultivars from South- West Nigeria. *Revista UDO Agrícola*, 7: 22-28.
- Allison, L.E., Bollen, W.B. and Moodie, C.D. (1965). Organic carbon. In Methods of soil analysis, Part 2 Chemical and microbiological properties. Ed C A Black. *American Society of Agronomy Monograph* No.9. Madison, Wisconsin, USA pp 1346- 1378.
- Ariyo, O.J. (1993). Genetic diversity in West African okra (*Abelmoschus caillei*) (A. Chev.) Stevels): multivariate analysis of morphological and agronomic characteristics. *Genetics Resource Crop Evolution*. 40:25–32.
- Asante, K., Offei, S.K., Addy, R. and Carson, A.G. ( 2008). Phenotypic and seed protein analysis in 31 lima bean (*Phaseolus Lunatus*) cultivars in Ghana. *West African Journal of Applied Ecology* Vol. 12 . pp 129-138.
- Bray, R . H . and Kurtz, L.T . (1945). Determination of total , organic, and available forms o f phosphorus in soils . *Soil Science* . 59 : 39-45 .
- Chandran, K. and Pandya, S.M. (2000). Morphological characterization of *Arachis* species of section *Arachis*. *Plant Genetics Resource Newsletter*. 121:38–41.
- CIAT (2007). Germplasm characterisation. In: Pineda B. and Hidalgo R.. Multi-Institutional distance learning course on the ex-situ conservation of plant genetic resources. (eds). pp 171-184.
- CIP, AVRDC, IBPGR, and Huaman, Z. (ed). (1991). Descriptors for sweet potato. International Board for Plant Genetic Resources. Rome, Italy.
- FAO/UNESCO (1994). Soil map of the world, revised legend. *World Resources Report* 60, FAO, Rome, 146.
- Guimarães, W.N.R. (2005). Morphological and molecular characterisation of hits lima bean (*Phaseolus lunatus* L., Fabaceae) Collection germplasm Department of Agronomy UFRPE Master Thesis Recife, PE., Federal Rural University of Pernambuco. p. 73.
- IBPGR (1982). *Lima bean Descriptors*, IBPGR Secretariat, Rome.
- Jackson , M. L . (1956). Soil chemical analysis - advanced course .Pub . by the Author . Department of Soils , University of Wisconsin, Madison , Wisconsin . pp 991.
- Johnson, D.E. (1998). *Applied multivariate methods for data analysis*. New York: *Duxbury Press*.

Lioi, L. and Galasso, I. (2002). Oligonucleotide DNA fingerprinting revealing polymorphism in *Phaseolus lunatus* L. *Genetic Resources and Crop Evolution* 49: 53–58.

Loi, L. (1994). Morphotype relationships in Lima bean (*Phaseolus lunatus* L.) deduced from variation of the evolutionary marker phaseolin. *Genetics Resources Crop Evolution*. 41: 81–85.

Lyman, J.M. (1983). Adaptation studies on the lima bean cultivars in Colombia. *Journal of the American Society for Horticultural Science*, Alexandria. v. 108 n.3 pp 369-373.

Melo, L.J. (2005). Morphophysiology and yield of lima bean under different conditions of cultural management. Doctoral thesis. Meadow Grande, Paraiba. Federal University of Campina Grande. pp 184.

Morris, M.L., Tripp, R. and Dankyi, A.A. (1999). Adoption and impacts of improved maize production technology: *A case study of the Ghana Grains Development Project*. CIMMYT. Mexico, D.F.

Lyman, J.M. (1983). Adaptation studies on the lima bean cultivars in Colombia. *Journal of the American Society for Horticultural Science*, Alexandria. v. 108 n.3 pp 369-373.

Nienhuis, J., Tivang, J. and Skroch, P. (1995). Genetic Relationships among cultivars and landraces of lima bean (*Phaseolus lunatus* L.) as measured by RAPD markers. *Journal American Society Horticultural Science*. 120 (2): 300-306.

Olsen, S. R., Cole, C.V., Watanabe, F.S. and Dean, L.A. (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate. *USDA Circular 939*, Washington, D. C. p 19.

Polignano, G.B., Ugenti, P. and Scippa, G. (1993). The patterns of genetic diversity in faba bean collections from Ethiopia and Afghanistan. *Genetics Resources Crop Evolution*. 40:71–75.

Santos, D., Corlett, F.M., Mendes, J.E.F. and Wanderley jr, J.S.A. (2002). Productivity and morphology of pods and seeds of varieties of bean in the state of Paraiba. *Brazilian Agricultural Research*, Brasilia, D.F. V. 37, pp 1407- 1412.

Sneath, P.H.A. and Sokal, R.R. (1973). *Numerical taxonomy, the principles and practice of numerical classification*. (W. H. Freeman: San Francisco). p 573.

Tatineni, V., Cantrell, R.G. and Davis, D. (1996). Genetic diversity in elite cotton germplasm determined by morphological characteristics and RAPD. *Crop Science*. 36: 136-196.

Vargas, E.M., Castro, E., Macaya, G. and Rocha, O.J. (2003). Variation in fruit and seed size from 38 wild populations of *Phaseolus lunatus* (Fabaceae) from Central Valley, Costa Rica. *Revista de Biología Tropical* 51: 707–724.

Yagui, A., Machado- Neto, N.B. and Cardoso, J.M. (2003). Grouping of Brazilian accesses of lima bean (*Phaseolus lunatus* L.) according to SDS-PAGE patterns and morphological characters. *Acta Scientiarum*. V.25, n.1 p 7-12 Maringa.

Zoro, Bi. I., Maquet, A. and Baudoin, J.P. (2003). Population genetic structure of wild *Phaseolus lunatus* (Fabaceae), with special reference to population sizes. *American Journal of Botany* 90, 897-904.

## CHAPTER FOUR

### NODULATION STUDIES OF THE LIMA BEAN

#### 4.1 INTRODUCTION

Requirements for nitrogen exceed any other major nutrients and rarely do soils have enough of this nutrient to produce high sustainable yields (Woldeyohannes *et al.*, 2007; Mkandawire *et al.*, 1998). Studies have also shown that despite availability of other nutrient sources to enhance nitrogen in soil for improved crop yield, chemical fertilisers have been prioritised as a solution to nutrient deficiencies in soil (Otieno *et al.*, 2009; Gentili *et al.*, 2006). Although there have been massive gains through the usage of inorganic nitrogenous fertilisers in agricultural production, they do come at a cost. Pearson *et al.* (2010) intimated that their demerits when excessively used, contributed to effects such as greenhouse gas emissions, reduction in water quality, biodiversity reduction and often potential health hazards. Consequently, there has recently been an interest in environmental friendly sustainable agricultural practices and organic farming systems (Rigby and Caceres, 2001; Lee *et al.*, 2007). Improved nitrogen management is needed to optimize economic returns to farmers and minimise environmental concerns associated with nitrogen use (Bundy and Andraski, 2005).

A major benefit derived from using legumes in cropping systems is the fixation of atmospheric nitrogen through the formation of nodules on roots and root hairs (Ennin *et al.*, 2004). The lima bean (*Phaseolus lunatus*), like other leguminous plants, establishes a symbiotic association with soil rhizobia which convert atmospheric nitrogen into useable forms made available to the plant (Martinez- Romero *et al.*, 1991; Souza *et al.*, 1994; Martinez-Romero, 2003). The potential of lima bean with respect to diversity and yield

(Tadawan *et al.*, 2004) is of great relevance, and evaluation of its biological nitrogen fixation tendencies will enhance the identification and selection of suitable cultivars for increased lima bean production in a Coastal Savannah environment of Ghana. The objective of this study was to evaluate the nodulation potential of thirteen lima bean cultivars and the effect on growth and development of the plant.

## **4.2 MATERIALS AND METHOD**

### **4.2.1 Experimental Setting**

The pot experiment was carried out at the research farm of the Biotechnology and Nuclear Agricultural Research Institute of the Ghana Atomic Energy Commission (GAEC) as previously described.

### **4.2.2 Lima Bean Cultivars**

Thirteen cultivars of lima bean were used for the experiment. They were obtained from market centres and CSIR-PGRRI. Four seeds were sown per pot and later thinned to one seed per pot. The experiment relied solely on manual irrigation to maintain adequate moisture. Weeds were removed manually throughout the experiment. Neither pesticides nor fertilizers were applied during the period of the experiment. Plants, mostly creeping, were all staked and trained to climb the stakes. The seeds were planted on the 10th August, 2013 and nodules harvested on the 11th December, 2013.

#### **4.2.3 Nodule number per plant**

Four pots representing each of the thirteen cultivars were randomly selected from each replication for the nodule count and determination of fresh and dry nodule weight at eight weeks after planting (8WAP). The process required removal of the plant from the soil gently and carefully loosening the soil around the plant to ensure roots were not disturbed. The plants were then put in polyethylene bags and taken to the laboratory. The roots were gently washed under running water to remove all soil particles. The nodules were then removed, counted and weighed.

#### **4.2.4 Effective and Non- effective Nodules**

The nodules counted were separated and classified into effective and non- effective nodules, based on their size and interior colour, when sliced. Nodules of size less than 2 mm with creamy whitish colour were referred to as non effective while those of size 2 mm - 5 mm with bright or dark pink colour due to the presence of high levels of leghaemoglobin were considered effective. Effective and non effective nodules were combined to obtain total fresh nodule weight, after which it was kept in small brown envelopes and oven-dried at 65 °C for 72 hours. They were weighed again at the end of the drying period to determine the dry nodule weight for each sampled pot.

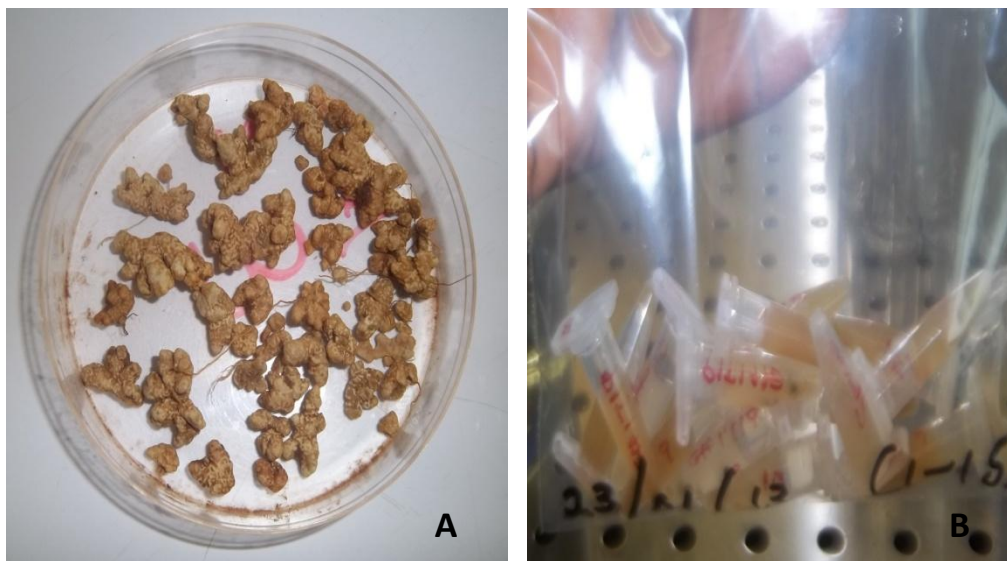


Figure 4.1 Fresh nodules harvested from a lima bean cultivar (A) and crushed nodule suspension in eppendorf tubes (B).

#### 4.2.5 Total Shoot Dry Matter (biomass)

Total shoot dry matter (TSDM) was determined at eight weeks after planting (8WAP). The sampling involved four pots selected randomly for all the cultivars in each replication. The roots were removed from the shoots and their fresh weights determined. Similarly, subsamples of the roots and shoots were taken. The weighed samples were then kept in large brown envelopes and oven dried at a constant temperature of 65 °C for 72 hours. The dried samples were again weighed to determine the dry weight. The shoot dry weight per hectare was estimated using the formula below:

$$\text{DMY (kg/ha)} = \text{TFW (kg)} \times \frac{1000 \text{ (m}^2\text{/ha)}}{\text{H (m}^2\text{)}} \times \frac{\text{SDW (kg)}}{\text{SFW (kg)}}$$

Where:

DMY is the dry matter yield

TFW is the total fresh weight

SFW is the subsample fresh weight

SDW is the subsample dry weight

### **4.3 Data analysis**

Data collected on yield and nodulation parameters were subjected to analysis of variance (ANOVA) based on the Randomized Complete Block Design (RCBD) and where the means were significantly different ( $p \leq 0.05$ ), the Duncan Multiple Range Test (DMRT) was used to separate them. The GENSTATS statistical package version 12<sup>th</sup> edition was employed in the analysis of the data. Correlation analysis using Pearson correlation matrix was also employed to determine the relationship between the growth parameters and the nodule parameters.

## 4.4 RESULTS

### 4.4.1 Germination Percentage

The germination percentages of lima bean cultivars A1, A2, A3, A4, GH 1714, GH 1719, M1, M2, M3 and M5 were not significantly different from each other. Germination percentages ranged from 40.2 % to 80.5 %. However, they were significantly lower ( $p \leq 0.05$ ) in comparison with cultivar M4, which had the highest germination percentage of 90.3 %. The least percentage germination were observed in cultivars GH 1726 and GH 1737 with 30.9 % and 30.3 %, respectively (Figure 4.2).

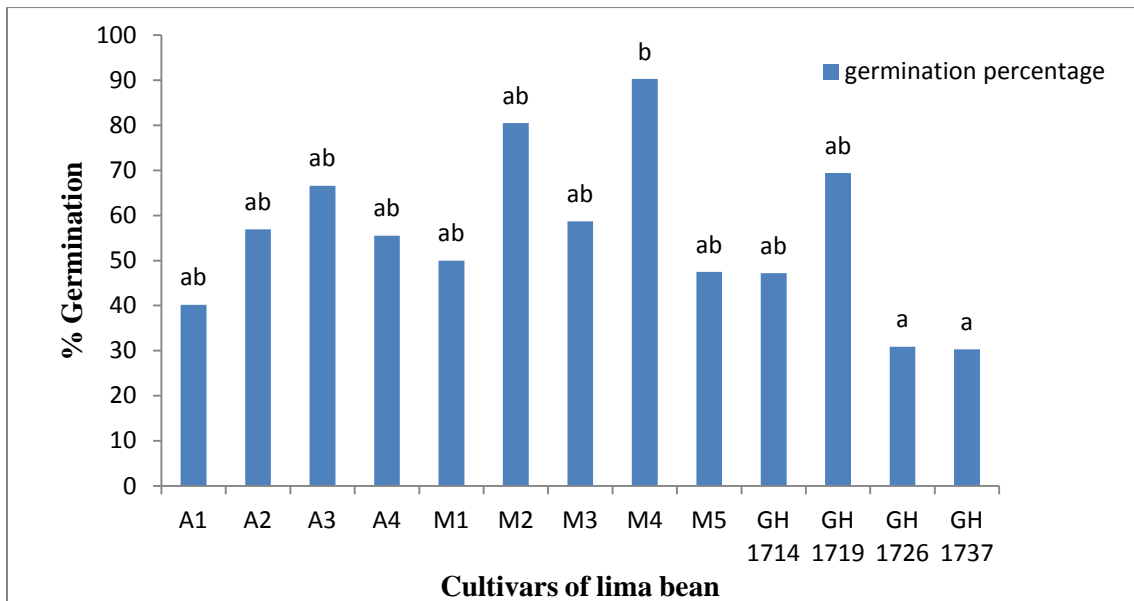


Figure 4.2 Germination Percentage of thirteen lima bean cultivars.

Bars with identical letters are not significantly different at 5 % significance level by Duncan's Multiple Range Test.

#### 4.4.2 Mean Fresh and Dry Shoot Weight

Mean fresh and dry shoot weights of all the lima bean cultivars were taken at 8WAP. Estimates of fresh shoot weights indicated some significant differences ( $p \leq 0.05$ ) as well as similarities among the cultivars. The lima bean cultivars A3, M1, M3 and M5 recorded statistically similar mean fresh shoot weight values, ranging from 31.2 g to 37.8 g that were statistically lower ( $p \leq 0.05$ ) than those of A2, A4, GH1714 and M4 which showed no significant differences among themselves. Lima bean cultivars GH 1719 and GH 1726 recorded the highest values of 47.8.0 g and 45.0 g respectively. However, the lowest mean fresh shoot weight of 20.5 g was recorded in M2 (Figure 4.3).

For the mean dry shoot weight, the lima bean cultivar M4 had the highest value of 25.09 g, which was significantly higher ( $p \leq 0.05$ ) in comparison with the other remaining cultivars. Cultivars GH 1719, GH 1726 and M3 showed no significant differences ( $p \geq 0.05$ ) among themselves with values that ranged from 15.75 g and 16.63 g. The remaining cultivars indicated statistically similar values ranging between 7.32 g and 14.35 g. Cultivar M2, however produced the lowest mean dry shoot weight of 7.32 g (Figure 4.3).

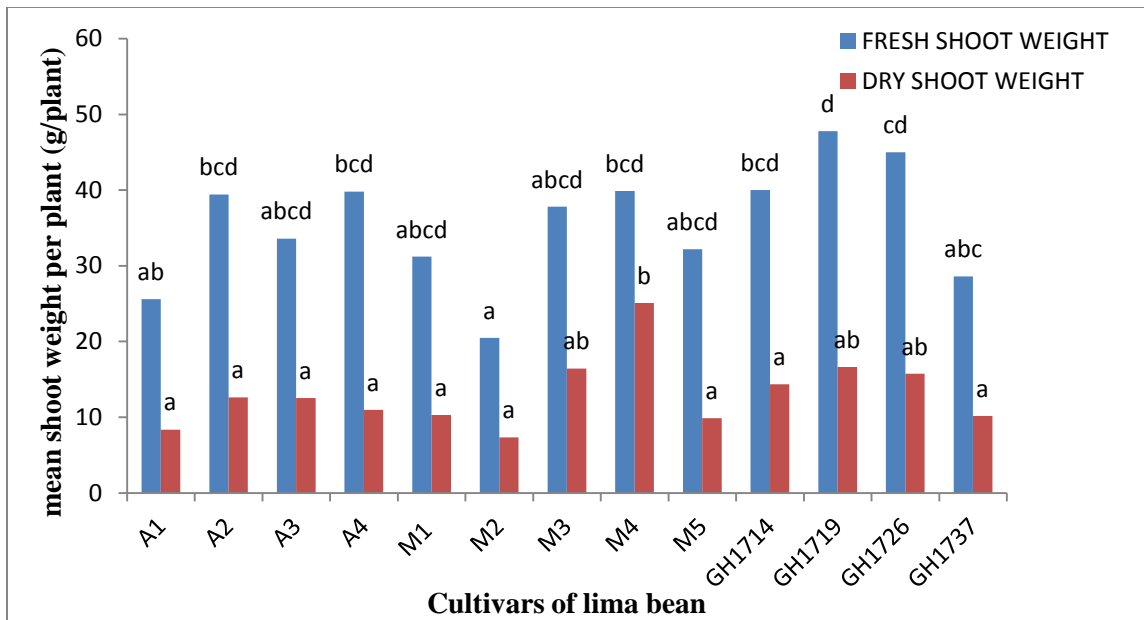


Figure 4.3. Mean fresh shoot and dry shoot weight of thirteen cultivars of lima bean.

Bars with identical letters in the same bars are not significantly different at 5 % significance level by Duncan's Multiple Range Test.

#### 4.4.3 Mean Fresh and Dry Root Weights

Mean fresh and dry root weights were also taken at 8WAP. The lima bean cultivar GH 1714 had the highest mean fresh root weight of 8.81 g which was significantly higher ( $p \leq 0.05$ ) than the fresh weight of other cultivars. Lima bean cultivars A3, A4 and M4 were statistical similar to each other, ranging from 2.29 g to 2.96 g. They were however, significantly lower ( $p \leq 0.05$ ) to nine other cultivars (A1, A2, GH 1719, GH 1726, GH 1737, M1, M2, M3 and M5) which ranged from 4.75 g to 8.16 g. The lowest fresh root weight of 2.29 g was observed in A4. (Figure 4.4 ). With respect to the mean dry root weight, cultivar M3 recorded the highest value of 4.07 g which was statistically greater ( $p \leq 0.05$ ) than values recorded for other cultivars. Lima bean cultivar A4 had the lowest mean dry root weight value of 0.92 g which was significantly lower ( $p \leq 0.05$ ) than the

values for other eleven cultivars (A1, A2, A3, GH 1714, GH 1719, GH 1726, GH 1737, M1, M2, M4 and M5) (figure 4.4 ).

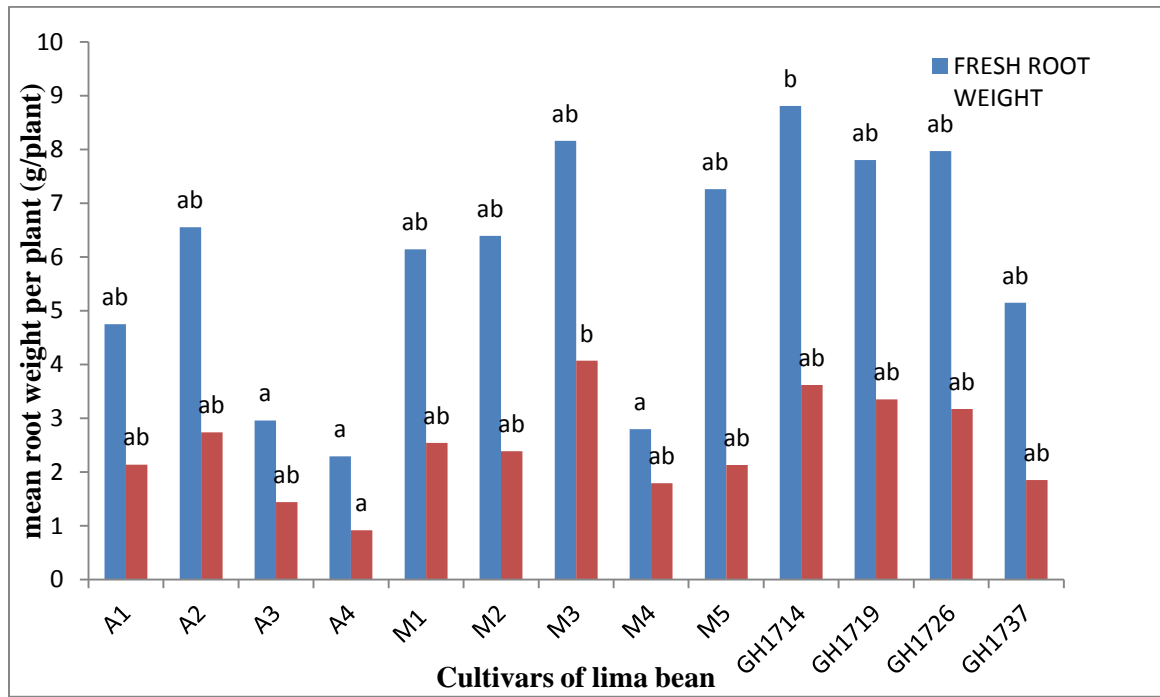


Figure 4.4 Mean fresh and dry root weight per plant of thirteen lima bean cultivars.

Bars with identical letters in the same bars are not significantly different at 5 % significance level by Duncan's Multiple Range Test.

#### 4.4.4 Total Shoot Dry Matter

The total shoot dry matter among the thirteen lima bean cultivars showed highly significant differences ( $p \leq 0.05$ ) with M5 and A2 recording the highest values of 731 kg/ha and 704 kg/ha, respectively. The total shoot dry matter weights for the lima bean cultivars A1, A3, M1, M2, M3 and GH 1719 were similar ( $P \leq 0.05$ ). The lima bean cultivar GH 1714 had the least total shoot dry weight (Figure 4.5).

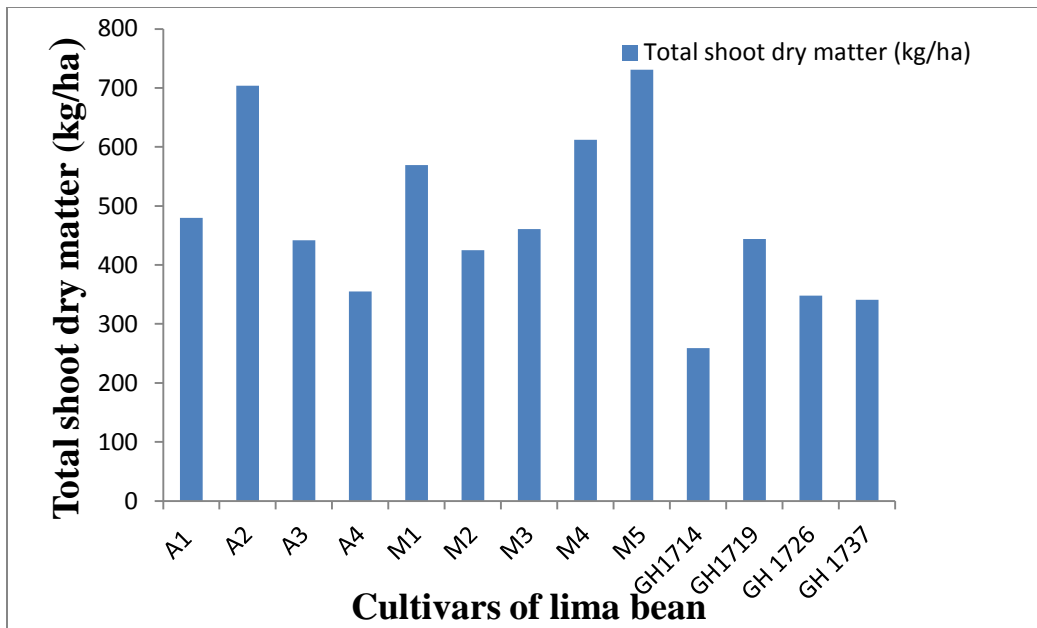


Figure 4.5 Total shoot dry matter (kg/ha) among thirteen cultivars of lima bean.

Bars with identical letters in the same bars are not significantly different at 5 % significance level by Duncan's Multiple Range Test.

#### 4.4.5 Mean Number of Nodules per Plant

There were no significant differences ( $p \geq 0.05$ ) in the mean nodule number among the 13 cultivars of the lima bean at 8WAP (Figure 4.6). The lima bean cultivar GH 1714 however, produced the highest mean nodule number of 205.0 while M5 had the lowest value of 36.0. The other remaining cultivars (A1, A2, A3, A4, GH1719, GH 1726, GH 1737, M1, M2, M3 and M4) recorded mean nodule numbers ranging from 45.0 to 168.0.

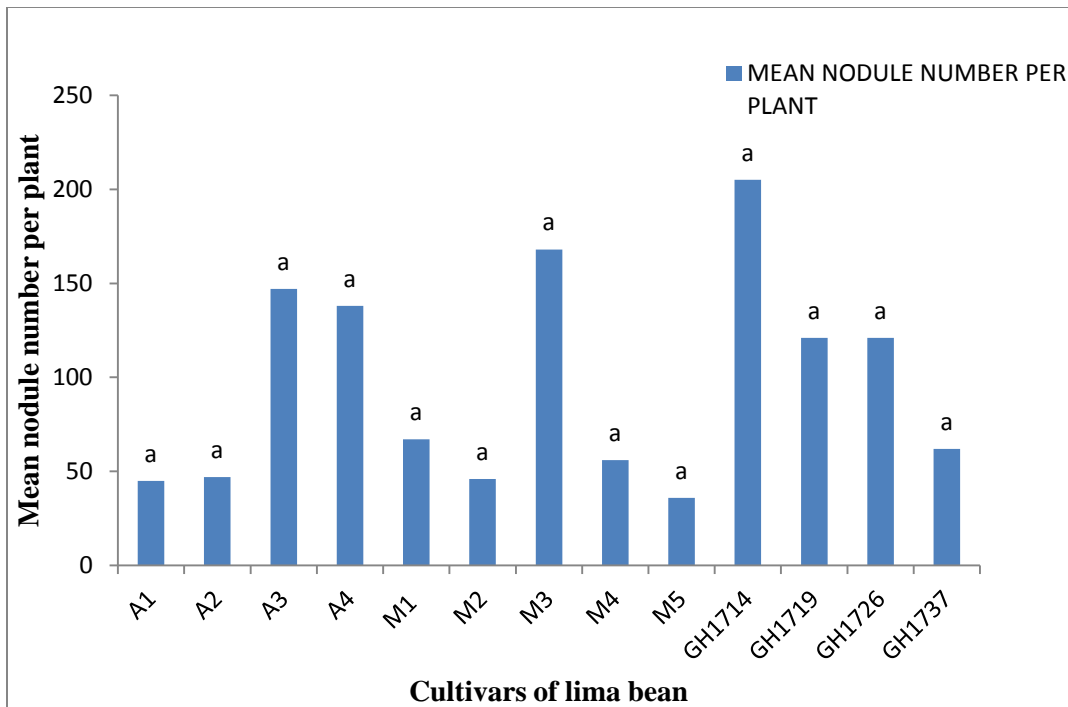


Figure 4.6 Mean nodule number per plant of thirteen cultivars of lima bean.

Bars with identical letters in the same bars are not significantly different at 5 % significance level by Duncan's Multiple Range Test.

#### 4.4.6 Mean number of Effective and Non-effective nodules

There were no significant differences ( $p \geq 0.05$ ) observed in the mean number of effective nodules in all thirteen cultivars of the lima bean. However, cultivars GH 1714 and GH 1719 had the highest mean effective nodule number of 91.7 and 55.2, respectively, with the lowest mean value observed in M2. The remaining lima bean cultivars, A1, A2, A3, A4, GH 1726, GH 1737, M1, M3, M4 and M5 had mean values which ranged from 10.7 to 54.1 (Figure 4.7).

For results obtained for non-effective nodules, however, there were no significant differences ( $p \geq 0.05$ ) among the thirteen lima bean cultivars. The highest mean non-

effective nodules were recorded in GH 1714 and M3 (Figure 4.7). The least mean numbers, however, were observed in M4 and M2 with 3.9 and 16.8 respectively. The other lima bean cultivars, A1, A2, A3, A4, GH 1719, GH 1726, GH 1737, M1 and M5 had mean numbers ranging from 24.1 and 93 (Figure 4.7).

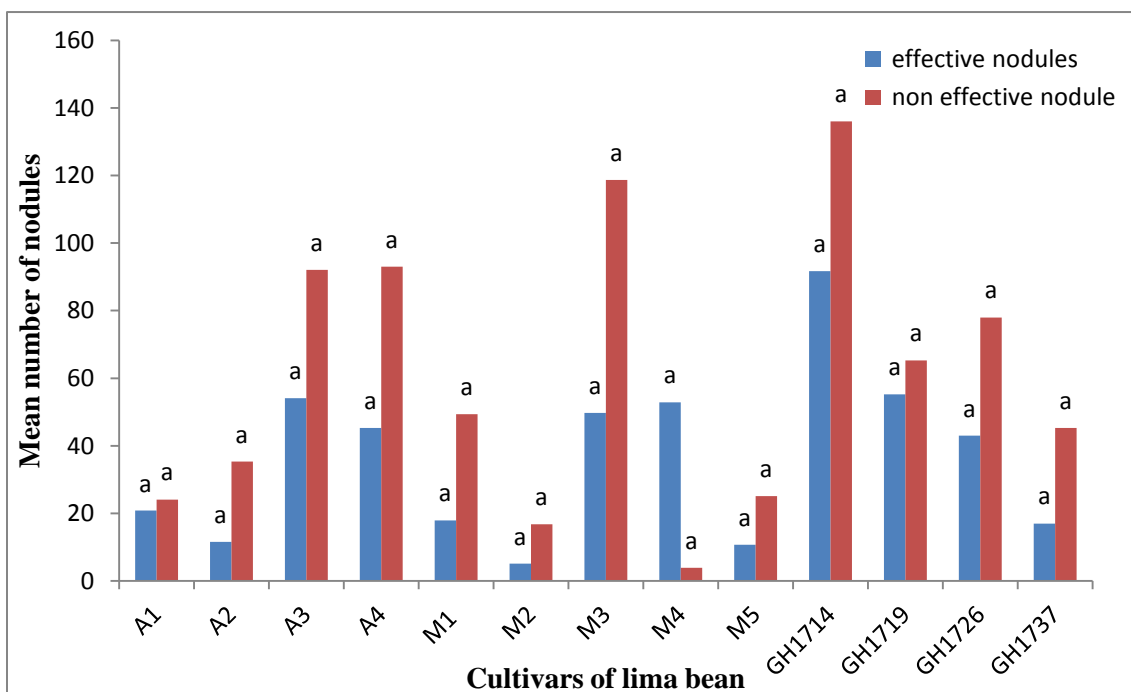


Figure 4.7 Mean effective and non effective nodules of thirteen cultivars of lima bean.

Bars with identical letters in the same bars are not significantly different at 5 % significance level by Duncan's Multiple Range Test.

#### **4.4.7 Mean Fresh and Dry Nodule Weight per Plant**

For fresh nodule weight, there were statistical similarities among twelve lima bean cultivars, with the exception of cultivar M4, which was significantly different ( $p \leq 0.05$ ), with fresh nodule weight of 8.061 g. The least mean values were recorded by M2, M5 and A2 with 0.022, 0.054 and 0.072 respectively. The other remaining cultivars ( A1, A3, A4, GH 1714, GH 1719, GH 1726, GH 1737, M1 and M3) had mean fresh nodule weight that ranged between 0.114 g and 0.447 g (Table 4.1).

The mean dry nodule weight of the lima bean cultivar M4 was significantly higher ( $p \leq 0.05$ ) compared to values for other remaining cultivars, which had statistically similar mean dry nodule weight. Additionally, the lima bean cultivar M4 had the highest mean dry nodule weight of 6.881 g while cultivars M2 and M5 had the least mean dry nodule weight of 0.012 g and 0.028 g, respectively. The other cultivars (A1, A2, A3, A4, GH 1714, GH 1719, GH 1726, GH 1737, M1 and M3) had mean dry nodule weight that ranged between 0.046 g to 0.267 g (Table 4.1).

**Table 4.1 Mean Fresh Nodule Weight (FNW) and Dry Nodule Weight (DNW) of thirteen cultivars of lima bean.**

<b>Cultivars</b>	<b>Mean Fresh nodule weight (g)</b>	<b>Mean Dry nodule weight (g)</b>
A1	0.114 a	0.048 a
A2	0.072 a	0.046 a
A3	0.493 a	0.227 a
A4	0.434 a	0.202 a
M1	0.138 a	0.079 a
M2	0.022 a	0.012 a
M3	0.257 a	0.173 a
M4	8.061 b	6.881 b
M5	0.054 a	0.028 a
GH 1714	0.447 a	0.267 a
GH 1719	0.445 a	0.188 a
GH 1726	0.368 a	0.192 a
GH 1737	0.127 a	0.057 a

Means with identical letters in the same column are not significantly different ( $p \leq 0.05$ ) according to Duncan's Multiple Range Test.

#### **4.4.8 Correlation between Nodulation and Growth Parameters in Lima Bean Cultivars**

The mean nodule number correlated highly and positively with mean effective nodule, mean non-effective nodule, mean fresh nodule weight and mean fresh shoot weight with values of 86.8 %, 96.4 %, 77.2 % and 52.5 %, respectively. It, however, correlated

positively but poorly with mean dry nodule weight, mean dry shoot weight, mean fresh root weight and mean dry root weight and negatively with average germination rate and total shoot dry matter. Similarly, the mean effective nodules correlated well and positively with mean non-effective nodules (73.5 %), mean dry nodule weight (68.1%), fresh shoot weight (63.3 %) and dry shoot weight (65.4 %). It was however negatively correlated with mean dry root weight and total shoot dry matter and weakly correlated with mean fresh nodule weight, average germination rate, fresh root weight and dry root weight at 40.1 %, 11.3 %, 10.2 % and 30.7 % respectively (Table 4.2).

The mean non-effective nodules only correlated highly and positively with mean fresh nodule weight (89.2 %), and positively but poorly with mean dry nodule weight, mean fresh shoot weight, mean dry shoot weight, mean fresh root weight and mean dry root weight with values at 16.7 %, 44.9 %, 24.2 %, 30.4 % and 40.9 % . With respect to mean fresh nodule weight, there was poor correlation with mean fresh shoot weight, mean fresh root weight and mean dry root weight. There was a positive correlation between the mean dry nodule weight with fresh shoot weight and dry shoot weight. However, it showed poor correlation with mean germination rate. The mean germination rate poorly correlated with mean fresh shoot weight, mean dry shoot weight and total shoot dry matter and correlated negatively with mean fresh root weight and mean dry root weight. The mean fresh shoot weight correlated positively with mean dry shoot weight with a value of 79.4 %. There was, however, a weak correlation between the mean fresh shoot weight with both mean fresh root weight and mean dry root weight. There was also poor correlation between mean dry shoot weight and mean fresh root weight, mean dry root weight and total shoot dry matter at 39 %, 30.9 % and 26 %, respectively. The mean

fresh root weight correlated highly and positively with mean dry root weight at 90.3 % but negatively with total shoot dry matter. Also a negative correlation existed between the mean dry root weight and total shoot dry matter. (Table 4.2)

**Table 4.2 Correlation analysis of nodulation and growth parameters for thirteen cultivars of lima bean.**

	MNN	MEN	MNEN	(MFNW)	(MDNW)	AVG	MFSW	MDSW	MFRW	MDRW	TSDM
<b>MNN</b>	1										
<b>MEN</b>	<b>0.868</b>	1									
<b>MNEN</b>	<b>0.964</b>	<b>0.735</b>	1								
<b>MFNW</b>	<b>0.772</b>	0.421	<b>0.892</b>	1							
<b>MDNW</b>	0.353	<b>0.681</b>	0.167	-0.196	1						
<b>AVG</b>	-0.079	0.113	-0.266	-0.497	0.373	1					
<b>MFSW</b>	<b>0.525</b>	<b>0.633</b>	0.449	0.325	<b>0.595</b>	0.034	1				
<b>MDSW</b>	0.397	<b>0.654</b>	0.242	-0.066	<b>0.884</b>	0.329	<b>0.794</b>	1			
<b>MFRW</b>	0.233	0.102	0.304	0.370	-0.338	-0.306	0.216	0.039	1		
<b>MDRW</b>	0.392	0.307	0.409	0.357	-0.050	-0.116	0.348	0.309	<b>0.903</b>	1	
<b>TSDM</b>	-0.660	-0.532	-0.626	-0.557	-0.076	0.303	-0.088	0.026	-0.076	-0.125	1

MNN- Mean nodule number, MEN- Mean effective nodule, MNEN- Mean non-effective nodules, MFNW- Mean fresh nodule weight, MDNW- Mean dry nodule weight, AVG- Average germination, MFSW- Mean fresh shoot weight, MDSW- Mean dry shoot weight, MFRW- Mean fresh root weight, MDRW-Mean dry root weight, TSDM- Total shoot dry matter

## 4.5 DISCUSSION

### 4.5.1 Germination Percentage

The germination percentage among the lima bean cultivars varied from 40.2 % to 90.3 %. Lima bean cultivars GH 1714, GH 1726, GH 1737 and A1 had germination percentages less than 50 % which could be attributed to conditions of the soil as at the time of sowing. Lima bean seeds are sensitive to prevailing soil conditions which can affect rate of germination. Thus germination rate can decrease when soil conditions are wet or compacted. Cultivars M4 and M2 recorded germination percentages of 90.3 % and 80.5 %, respectively, suggesting that their larger seeds in comparison with other cultivars could have played a role in their higher germination rates. Lush and Wien (1980) attested that large seeds generally produce seedlings that are vigorous than those derived from small seeds.

Sangakkara (1989) observed a positive relationship between seed size and emergence of *Phaseolus*. In further support, Bonfil (1998) working with two species of *Quercus* found a clear effect of seed size on seedling survival, with large seeds having the highest and small seeds the lowest survival. Hojjatt (2011) reported that large seeds germinated early and showed better germination than small seeds of lentil genotypes. In contrast to these reports, Stamp (1990) found a decreased rate of germination for large seeds in *Erodium brachycarpum*, indicating that seed size may not necessarily increase germination or emergence and that correlation between seed size and germination could probably be crop species related.

Another factor that could reduce germination percentage is the presence of nematodes in the soil. Gibson (1977) reported on the deleterious effect of nematodes on nodulation and nitrogen fixation of legumes.

#### **4.5.2 Fresh and Dry Shoot Weights per Plant**

The fresh shoot weights per plant showed significant differences among the thirteen cultivars of the lima bean and it ranged from 20.5 g to 47.8 g. The cultivar GH 1719 recorded the highest fresh shoot weight of 47.8 g. Differences in mean fresh shoot weights could be due to genetic attributes or environmental limitations.

For the mean dry shoot weight, significant differences existed with highest value of 25.09 g recorded in cultivar M4 and the least value of 7.32 g in M2. This differed from studies by Thamer *et al.* (2011) on lima bean, who observed mean dry shoot weights of less than 0.2 g after 5 WAP. It can be extrapolated that at 8WAP, dry shoot weights could not have reached values attained in this study. Also, observations made by Otieno *et al.* (2009) on lima bean had mean dry shoot weights per plant of 30.3 g and 21.0 g in both long and short rainy periods, respectively. This contrast values observed in this present study involving twelve of the lima bean cultivars having low dry shoot weights except M4, which had a higher weight. Thus, it is suggestive that *Rhizobium* strains of M4 were effective in contributing to shoot dry matter accumulation. Moawad *et al.* (1998) stated that evidence of certain *Rhizobium* strains in the soil can improve nitrogen fixation and yields in specific bean cultivars.

#### 4.5.3 Mean Fresh and Dry Root Weights

Dry root weights for the thirteen lima bean cultivars ranged from 0.92 g and 4.07 g. The lima bean cultivar M3 had the highest dry root weight of 4.07 g. Studies by Thamer *et al.* (2011) showed less than 0.3 g and 0.2 g for control and rhizobia colonised lima bean plants at 5 WAP, respectively. Although dry root weights was taken at 5 WAP in Thamer *et al.* (2011) work, it could be possible that at 8WAP, results could be similar to that obtained in this present study. In addition, observations made by Otieno *et al.* (2009) showed mean dry root weights of 287.7 mg and 248.3 mg, taken from un-inoculated lima bean cultivars during long and short rainy periods at 7WAP, respectively. This is in contrast to results obtained in present study, which had higher dry root weights. Thus it can be suggested that *Rhizobium* strains in the Coastal Savannah agro-ecological zone are quite effective and contribute significantly to root dry matter accumulation. This is supported by Zaman-Allah *et al.* (2007) who stressed that rhizobial strains of different origins vary in their symbiotic efficiency.

#### 4.5.4 Total Shoot Dry Matter

Significant differences existed in the total shoot dry matter of the thirteen cultivars of lima bean. Total shoot dry matter varied from 259 kg/ha in cultivar GH 1714 and 731 kg/ha in cultivar M5. The total shoot dry matter is an indicator of resources use strategy by plants (Granier *et al.*, 2001). Lima bean cultivars M5 and A2 had the highest total shoot dry matter in comparison with others. This can be attributed to differences in genetic make-up that enhance their ability to capture and utilise solar energy efficiently.

Dry matter accumulation is directly related to amount of solar radiation intercepted by plants (Chevula, 1991). Also, cultivar M5 and GH 1714 had similar germination percentages of 47.5 % and 47.2 %, respectively, but had contrasting total shoot dry matter yields.

#### **4.5.5 Mean Nodule Number per Plant**

The mean number of nodules per plant were not significantly different among the thirteen cultivars of the lima bean. Results obtained in present study had higher nodule numbers, which was in contrast to work done by Tayyar (1987) on lima bean, who had low nodule numbers of 4.67 per plant. This contrast suggest that native populations of *Rhizobium* are well adapted to the environmental conditions and cultural practises as observed by Danso *et al.* (1984) in soils of the Coastal Savannah Agro-ecological zone where this experiment was undertaken. Additionally, reports by Kellman *et al.* (2005) on common bean inoculated with *Rhizobium* strains recorded mean nodule number of 20.6 per plant at 54 DAI, lower in comparison to all thirteen lima bean cultivars used in this study. This suggests that *Rhizobium* inoculum competing with native populations may not be effective in nodulating lima bean. In support of this, Gonzalez *et al.* (2008) working on common bean, recorded mean nodule numbers per plant below 100 at 81 days after germination, as compared with lima bean cultivars A3, A4, M3, GH 1714, GH 1719 and GH 1726. This suggests that lima bean is capable of fixing nitrogen much more than the common bean, which is noted to exhibit unstable behaviour with respect to biological nitrogen fixation (Mostasso *et al.*, 2002; Hafeez *et al.*, 2005). This was further supported by Bliss (1993) and Isoi and Yoshida (1991) that many common bean cultivars are poor

nitrogen fixers in comparison to other legumes. Thus, it is important that the lima bean should be given much more attention due to this important attribute. The cultivar GH 1714 outperformed the other cultivars which could be attributable to effective strains of *Rhizobium* nodulating it or genetic attributes of the cultivar.

#### **4.5.6 Mean Effective and Non-effective Nodule Numbers**

Significant differences were not detected among the cultivars with regard to effective and non-effective nodules. The cultivar GH 1714 had higher values for both effective and non-effective nodules. However, non-effective nodules were more than effective nodules. This could be suggestive of continual utilisation of effective nodules and formation of new and non-effective nodules as the plant ages or reaches its final phase. It is also worth mentioning that reduction in effective nodules could be explained that as plants reach the flowering stage, effective nodules begin to deteriorate. Linderman and Glover (1990) observed that annual crops usually have their nodules being short lived and are replaced constantly during the growing season. Linderman and Glover (1990) also stressed that pink or red nodules which are supposed to be effective predominate on a legume in the middle of the growing season. Where white, grey or green nodules, which are supposed to be non-effective predominate, it may be an indication that little nitrogen is occurring. This could be attributed to inefficient *Rhizobium* strains, poor nutrition, pod filling or other plant stress. Cultivars M2 and M5 had the least effective nodules suggesting of low nitrogen fixation capability due to inefficient *Rhizobium* strains whereas GH 1714 has the highest effective nodule suggesting otherwise. However, this does not necessarily ensure that more effective nodules correspond to high nitrogen fixation. The reason is that plants

also have the ability to engage in associative nitrogen fixation (Ibeawuchi, 2008). The intensity of nodulation does not necessarily indicate the establishment of efficient nitrogen fixation. Thus, although fewer effective nodules may be developed, plants can fix appreciable amounts of nitrogen.

#### **4.5.7 Mean Fresh and Dry Nodule weight per Plant**

The mean fresh and dry nodule weights were statistically similar among the twelve lima bean cultivars, with exception of M4. This can be attributed to the size of nodules developed on cultivar M4 which were bigger as compared to the other nodules. The mean dry nodule weights ranged from 0.046 g to 6.88 g. This result was in contrast with mean dry nodule weights observed by Otieno *et al.* (2009) in non-inoculated lima bean during the long and short rainy seasons where they obtained 13.3 g and 18.3 g, respectively, after 7WAP. Variations existing in nodule weights can be suggestive of varied number of nodules for each cultivar in the present study.

#### **4.5.8 Correlation among Nodulation and Growth Parameters**

The relationship analysis indicated strong correlations among nodulation parameters than among growth parameter measured. The mean nodule number correlated with mean effective nodules, mean non-effective nodules, mean fresh nodule weight and mean fresh shoot weight. This indicated that large nodule numbers could not necessarily be a selection criterion for determination of effective nodulation potential. However, some cultivars, despite having low nodule numbers, are able to compensate with large nodule

size or weight thereby ensuring efficient nitrogen fixation for plant growth and development. Also, large nodule numbers may not be a guarantee that a higher shoot biomass would be attained. Thus, increase in nodulation cannot be a criterion for high shoot or root biomass accumulation.

The mean nodule dry weight which correlated with mean fresh shoot weight and mean dry shoot weight could be used as a selection criterion in identifying lima bean cultivars with greater shoot biomass. Hitherto, an increase in nodule mass or weight can result largely to high increase in plant growth. This can be an indication of efficiency in utilisation of the rhizobial symbiosis. Consequently, increase of nitrogen in plants stimulated high levels of biomass or plant growth which can be a function of symbiotic nitrogen fixation. A further advantage to cultivars with high shoot biomass is their high radiation use efficiency for dry matter production. The mean effective nodules correlated positively with fresh and dry shoot weight. This can function as a selection criterion for identifying cultivars with highly efficient *Rhizobium* strains that can contribute in increasing shoot biomass necessary for light interception, manufacture of food and transport of solutes to all parts of the plant.

## REFERENCES

- Bliss, F.A. (1993). Breeding common bean for improved biological nitrogen fixation. *Plant and Soil* 152. 71-79.
- Bonfil, C. (1998). The effect of seed size, cotyledon reserves, and herbivory on seedling survival and growth in *Quercus rugosa* and *Q. laurina* (Fagaceae). *American Journal of Botany* 85: 79-87.
- Bundy, G.L. and Andraski, W.T. (2005). Recovery of fertiliser nitrogen in crop residues and cover crops in an irrigated sandy soil. *Soil Science Society, American Journal* 69: 640-648.
- Chevula, K. M. (1991). Growth, light interception and yield of bambara groundnut (*Vigna subterranean* (L) Verdc.) in response to soil moisture. *Msc. Thesis, University of Nottingham*.
- Danso, S. K. A. and Eskew, D. L. (1984). Enhancing biological nitrogen fixation, IAEA Bulletin, volume, 26. Number 2. pp 29-35.
- Ennin, S. A., Dapaah, H. K. and Abaidoo, R. C. (2004). Nitrogen Credits from Cowpea, Soybean, Groundnut and *Mucuna* to Maize in Rotation. *West African Journal of Applied Ecology* 6:65-74.
- Garnier, E., Shipley, B., Roumet, C. and Laurent, G. (2001). A standardized protocol for the determination of specific leaf area and leaf dry matter content. *Functional Ecology* 15:688-695.
- Gentili, F., Wall, L.G. and Huss-Danell, K. (2006). Effects of phosphorus and nitrogen on nodulation is seen already at the stage of early cortical cell divisions in *Alnus incana*. *Annals of Botany*; 98:309-315.
- Gibson, A. H. (1977). The influence of the environment and managerial practices on the legume Rhizobium- symbiosis. In *A treatise on dinitrogen fixation. IV. Agronomy and ecology* (eds R. W. F. Hardy and A. H. Gibson). John Wiley, New York. pp. 393-450.
- Gonzalez, T.O., Campanharo, J.C. and de Macedo Lemess, E.G. (2008). Genetic characterisation and nitrogen fixation capacity of *Rhizobium* strains on common bean. *Pesq. agropec, Brasilia* V 43, n.9, pp 1177-1184.
- Hafeez, F.Y., Naeem, F.I., Naeem, R., Zaidi, A.H. and Malik, K.A. (2005). Symbiotic effectiveness and bacteriocin production by *Rhizobium leguminosarum* bv. *viciae* isolated from agriculture soils in Faisalabad. *Environmental and Experimental Botany*, v.54, p.142-147, 2005.

Hojjat, S.S. (2011). Effect of seed size on germination and seedling growth of some lentil genotypes. *International Journal of Agriculture and Crop Sciences*, 3: 1-5.

Ibeawuchi, I.L., Obiefuna, J.C., Nwifo, M.I. and Ofoh, M. C. (2008). Nodulation and nitrogen fixation in landrace legumes in Yam/ Cassava Based Cropping Systems of the tropical rainforest. *New York Science Journal*, Vol (1): pp 1-12.

Isoi, T. and Yoshida, S. (1991). Low nitrogen fixation of common bean ( *Phaseolus vulgaris*) *Soil Science and Plant and Nutrition* 37. 559-563.

Kellman, A.W., Hill, G.D. and Mckenzie, B.A. (2005). Variability in nodulation of *Phaseolus vulgaris* L. with different rhizobial strains. *Agriculture and Life Sciences Division; Agronomy*, NZ 35; pp 59-61.

Lee, J.Y. and Song, S.H. (2007). Evaluation of Ground water quality in coastal areas: implications for sustainable agriculture. *Environmental Geology*, 52:1231-1242.

Lindemann W. C and Glover C. R. (1990). Nitrogen fixation by legumes Cooperatives Extension Service Guide A-29. College of Agriculture and Home Economics New Mexico State University.

Lush, W.M. and Wien, H.C. (1980). The importance of seed in early growth of wild and domesticated cowpeas. *Journal of Agricultural Sciences Cambridge.*, 94: 177-182.

Martínez-Romero, E. (2003). Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspectives. *Plant and Soil* 252, 11-23.

Martínez-Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P. and Pardo, M.A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena sp.* trees. *International Journal of Systematic Evolutionary Microbiology* 41, 417-426.

Mkandawire, A.B.C., Mpahangwe, N.I.K., Mangwele, A.M. and Lupwayi, N.Z. (1998). Biological Nitrogen Fixation in African common bean (*Phaseolus vulgaris*) genotype. African Crop Science Conference Proceedings. 3: 315-322.

Moawad, H., El Din, S.M.S.B. and Abdel Aziz, R. A. (1998). Improvement of biological nitrogen fixation in Egypt winter legumes through better management of *Rhizobium*. *Plant and Soil*. 204: 95-106.

Mostasso, L., Mostasso, F., Dias, B.G., Vargas, M.A.T. and Hungria, M. (2002). Selection of bean (*Phaseolus vulgaris* L.) rhizobial strains for the Brazilian Cerrados. *Field Crops Research*, v.73, p.121-132.

- Otieno, P.E., Muthomi, J.W., Chemining'wa, G.N. and Nderitu, J.H. (2009). Effect of rhizobia inoculation, farmyard manure, nitrogen fertilizer on nodulation and yield of food grain legumes. *Journal of Biological Sciences.*; 9:326-332.
- Pearson, T., Grimland, S. and Brown, S. A. (2010). Spatial analysis of greenhouse gas emissions from agricultural fertilizer usage in the US. Report to Packard Foundation under 2008- 32689.
- Rigby, D. and Caceres, D. (2001). Organic farming and the sustainability of agricultural systems. *Agricultural Systems*, 68: 21-40.
- Sangakkara, U. R. (1989). Relationship between seed characters, plant growth and yield parameters of *Phaseolus vulgaris*. *Journal Agronomy Crop Science* 163, 105–108.
- Stamp, N.E. (1990). Production and effect of seed size in a grassland annual (*Erodium brachycarpum*, Graminaceae). *American Journal of Botany*, 77: 874-882.
- Souza, V., Eguiarte, L., Ávila, G., Capello, R., Gallardo, C., Montoya, J. and Pinero, D. (1994). Genetic structure of *Rhizobium etli* biovar *phaseoli* associated with wild and cultivated bean plants (*Phaseolus vulgaris* and *Phaseolus coccineus*) in Morelos, Mexico. *Applied Environmental Microbiology* 60, 1260-1268.
- Tadawan, B., Padua, D., Tianza, G. and Sagalla, E.J. (2004). Germplasm collection of indigenous agronomic crops in Benguet. Paper presented during the sectors review held at ATI, NTC on July 16, 2004.
- Tayyar, R. (1987). Dinitrogen fixation and growth of Faba bean, Lima bean and soya bean as affected by three insecticides. Msc. Thesis submitted to the American University of Beirut.
- Thamer, S., Schädler, M., Bonte, D. and Ballhorn, D.J. (2011). Dual benefit from below ground symbiosis, nitrogen fixing rhizobia promote growth and defense against a specialist herbivore in a cyanogenic plant. *Plant and Soil*. pp 209-219.
- Wilson, P.J., Thompson, K. and Hodgson, J.G. (1999). Specific leaf area and leaf dry matter content as alternative predictors of plant strategies. *New Phytologist*, 143:155–162.
- Woldeyohannes, W.H., Dasilva, M.C. and Gueye, M. (2007). Nodulation and nitrogen fixation of *Stylosanthes hamata* in response to induced drought stress. *Arid Land Research and Management*, 21:157-163.
- Zaman Allah, M., Sifi, B., L'Taief, B., El-Aouni, M.H. and Drevon, J.J. (2007). Rhizobial inoculation and P-fertilisation response in common bean (*Phaseolus vulgaris*) under glasshouse and field conditions. *Experimental Agriculture*, 43:67-77.

## CHAPTER FIVE

### MOLECULAR DIVERSITY STUDIES OF LIMA BEAN ROOT NODULE USING POLYMERASE CHAIN REACTION (PCR)

#### 5.1 INTRODUCTION

Among soil micro-organisms with significant positive effects on plants, the legume - nodulating bacteria, commonly referred to as rhizobia, are the most studied (McInnes *et al.*, 2004). *Rhizobium spp* are of particular interest due to their considerable agricultural and economic significance in converting atmospheric nitrogen to ammonia for the benefit of plants. The high abundance and diversity of rhizobia in the soils facilitates the cultivation of legumes. Thus, the selection of indigenous strains with high nitrogen tendencies is an important strategy in maximising legume production. Due to the diverse nature of rhizobia at species and strain levels, Sikora *et al.* (2002) alluded that classification methods are needed to identify genotypes displaying superior nitrogen-fixation capacity.

The successful management of the *Rhizobium*-legume symbiosis will require that specific strains of the bacteria can be identified reliably (Fening *et al.*, 2004; Mpeperekki *et al.*, 1997; Richardson *et al.*, 1995). In recent times, the use of molecular techniques based on Polymerase Chain Reaction (PCR) have become convenient for characterisation and analysis of microbial organisms, especially rhizobia, due to their rapid, simple and discriminative qualities. Polymerase Chain Reaction techniques used in conjunction with short arbitrary oligonucleotide primers of random sequence have been shown to be an effective means of differentiating complex genomes (Williams *et al.*, 1990; Welsh and

McClelland, 1990; Caetano-Anolles *et al.*, 1991). The use of GC-rich oligonucleotide primers create highly characteristics pattern when distinguished in agarose gel, providing well separation of strain level (Adiguzel, 2006). The assessment of diversity within rhizobial populations in various regions of the world has received increased attention (Chen *et al.*, 2000; Ando and Yokoyama, 1999). Thus, the study of rhizobia diversity in lima bean will aid in the selection of effective strains adapted to local environmental conditions which will enhance its nitrogen fixation tendencies, cultivation and utilisation in Ghana. The objective of this experiment is to investigate the relatedness between and within the *Rhizobium* strains observed in selected cultivars of the lima bean.

## **5.2 MATERIALS AND METHOD**

### **5.2.1 Experimental Site**

The field experiment was carried out at the research farm of the Biotechnology and Nuclear Agricultural Research Institute (BNARI) of the Ghana Atomic Energy Commission (GAEC) as previously described in chapter three. The laboratory experiment was conducted at the Microbiology and Compost Plant laboratory and the Molecular Biology Laboratory, all at BNARI.

### **5.2.2 Lima bean collection**

Thirteen cultivars of lima bean (*Phaseolus lunatus* L.) were used. Cultivars obtained from the CSIR-PGRRI include GH 1714, GH 1719, GH 1726 and GH 1737. Nine other

cultivars (A1, A2, A3, A4, M1, M2, M3, M4 and M5 were obtained from markets in various parts of Ghana. Seeds were planted on the 10th of August, 2013.

### **5.2.3 Nodule Harvesting, Sterilization and Preparation of Template DNA**

Lima bean plants were carefully removed from the soil contained in the polybags, and roots cut off from the shoots. The roots were washed under running water to remove soil particles and debris from the nodules. Intact nodules, especially the effective ones, were harvested and kept in cryovials which were labelled according to cultivars. The labelled cryovials were stored in the refrigerator at a temperature of 0 °C for future use. Selected nodules were later subjected to surface sterilisation using 70 % of ethanol and later with sodium hypochlorite. Nodules were immersed in 70 % ethanol for 5 seconds and transferred into diluted 3 % of sodium hypochlorite solution and soaked for 15 minutes. Nodules were removed from the solution and rinsed in six changes of distilled water. The forceps used were sterilised firstly by quickly dipping in 70 % ethanol and later flamed. Each nodule was then picked with the forceps into well labelled 1 ml eppendorf tubes and 50 µl of sterile distilled water added. The forceps then were used in crushing each nodule after which it was sterilised before using for the next nodule. The crushed nodule suspension was kept in the refrigerator at 4 °C. The sterilization process was carried out in the laminar flow hood.

#### **5.2.4 Preparation of PCR reaction mix and DNA amplification**

Polymerase Chain Reaction amplifications were performed on crude extracts from crushed nodules of the lima bean. Each crushed nodule from lima bean cultivars was diluted with distilled water in a ratio of 50:50. Two eppendorf tubes well labelled containing primers (RP01, RP04, ERIC 1 and 2) and master mix were placed on the ice maker. 30 micro litre of master mix was pipetted into an empty tube. Also added were 45 micro litre of the primer and 105 micro litre of MilliQ water making a total volume of 180 micro litres. A volume of 12 micro litre of the PCR mix was distributed into 15 eppendorf tubes for all cultivars. 1 micro litre of DNA template or extract from each crushed nodule suspension of the thirteen lima bean cultivars of the lima bean were added to its Polymerase Chain Reaction mix in each tube to make a total volume of 13 micro litre. All laboratory activities were done under microbiological axenic conditions to avoid contamination. Primers used were obtained from Inqaba Biotech, South Africa and reference sources of primers are indicated in Table 5.1

**Table 5.1 Primers used for the amplification and detection of *Rhizobium* DNA obtained from root nodules of lima bean (*Phaseolus lunatus* L.)**

Primer	Type	Reference
RPO1	Specific nif-directed	Richardson <i>et al.</i> , 1995
RPO4	Arbitrary oligonucleotide	Richardson <i>et al.</i> , 1995
ERIC1(1&2)	Arbitrary oligonucleotide	Ampomah <i>et al.</i> , 2008

The PCR was performed in a 96 well Eppendorf thermal cycler (Nexus, Wagtech, Hamburg, Germany). The PCR programme used for the three primers include; 35 cycles of denaturation at 92 °C for 7 minutes, annealing at 40 °C for 2 minutes, extension for 78 °C at 90 seconds and final extension at 72 °C for 3 minutes for RPO1. For RPO4, 35 cycles of denaturation at 95 °C for 1 minute, annealing at 50 °C for 1 minute, extension at 72 °C for 2 minutes and final extension at 72 °C for 3 minutes. For ERIC ( 1 and 2), it was initiated at a temperature of 95 °C for initial denaturation for 7 minutes. This was followed by 30 cycles of denaturation at a temperature of 94 °C for 1 minute and primer annealing at 52 °C for 1 minute, primer extension at 65 °C for 8 minutes. This procedure was a modified protocol of de Bruijn (1992).

**Table 5.2 PCR reaction mix for rhizobial work on thirteen (13) lima bean cultivars**

Reagent	× 1	× 15
Master Mix	2 µl	30 µl
Primer	3 µl	45 µl
MilliQ water	7 µl	105 µl
DNA template	1 µl	15 µl
Total reaction volume	13 µl	195 µl

### 5.2.5 Agarose Gel Electrophoresis

Gel electrophoresis was done using a modified protocol of Ampomah *et al.* (2008). Three percent (3 % W/V) of agarose gel was prepared by weighing 4.5g of gel-grade agarose (sigma) into 150 ml of 1 X TAE buffer. The mixture was then boiled in a microwave oven until a clear solution was obtained before allowing it to cool to about 55 °C. For every 50 ml of agarose solution, 8 µl of ethidium bromide solution was added. This was then placed on a shaker for thorough mixing. The solution was then poured into a horizontal gel electrophoresis tray mounted in a gel casting tray and filled with a 20-tooth comb and allowed to solidify. The tray was then removed from the gel caster and placed in an electrophoresis tank filled with 1 X TAE buffer to about 2 mm above the top of the gel. The combs were then removed gently from the mounted wells. 12 µl aliquots of the PCR reaction mix were then loaded into the wells and run at a constant 90 V for 50 minutes. The gel was subsequently visualized under a high performance ultraviolet transilluminator (UVP, Cambridge, UK) and images captured with the aid of a UVP Life

Science Software (Doc-It. Ls Image Acquisition). Based on the results that were obtained from the thirteen cultivar of the lima bean with the different primers, five (5) nodules from each cultivar were selected and used together with the RPO1 primer to determine the similarities or diversity among the different cultivars of lima bean. The procedures used were the same as previously described in sections 5.4.2 and 5.4.3.

#### **5.2.6 Scoring for bacteria DNA fingerprint polymorphism and statistical analysis**

The DNA bands that were obtained were converted into one (1) as being present and zero (0) as being absent for the different base pairs (bp) that were obtained through the amplification. Cluster analysis using the NTSYS-pc version 2.1 was used in constructing a UPGMA (unweighted pair group method with arithmetic averages) dendrogram indicating distance based interrelationships between isolates of rhizobia strains from the thirteen cultivars.

## 5.3 Results

### 5.3.1 Specific *nif*-directed RPO1

The PCR amplification profile generated from *Rhizobium* DNA using the specific *nif*-directed RPO1 in cultivars A3, A4 and M5 ranged from 100 to 3000 base pairs (Figure 5.1). The *Rhizobium* strains 1, 2, 3, 4, 5, 7, 8, 11, 13, 14 and 15 in cultivar A3 were strongly amplified. These were followed by strain 6 which was weakly amplified while no amplification profile was found in *Rhizobium* strains 8 and 10. (Figure 5.1.a)

The *Rhizobium* strains 1, 2, 4, 7, 8, 10, 11, 14 and 15 of cultivar A4 were amplified strongly. Weakly amplified bands were indicated in *Rhizobium* strains 5, 6, 9 and 13. However, no amplification were found in *Rhizobium* strain 3 (Figure 5.1.b). Polymerase Chain Reaction (PCR) amplification profile in cultivar M5 had strains 1, 2, 5, 6, 8, 10, 11, 12, 13, 14 and 15. *Rhizobium* strain 9 was weakly amplified while *Rhizobium* strains 3, 4 and 7 had no amplification profile (Figure 5.1c.).

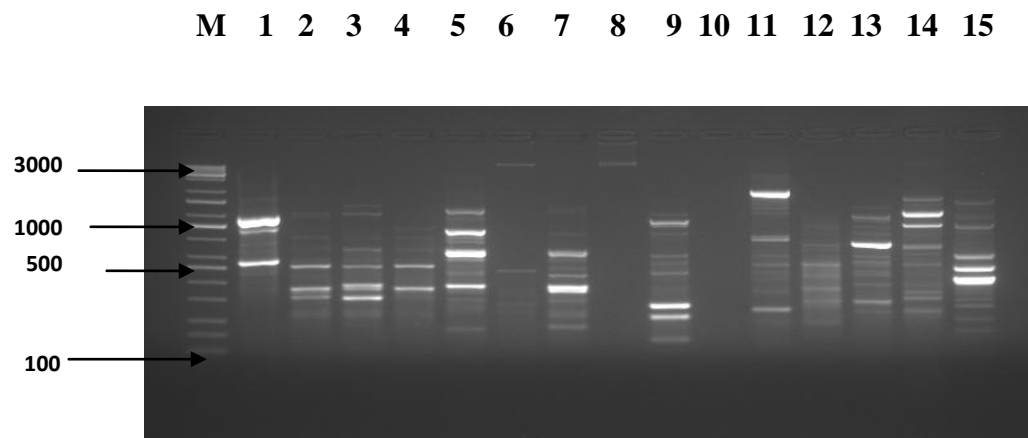


Figure 5.1( a) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the specific *nif*-directed RPO1. M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar (A3) following sterilisation.

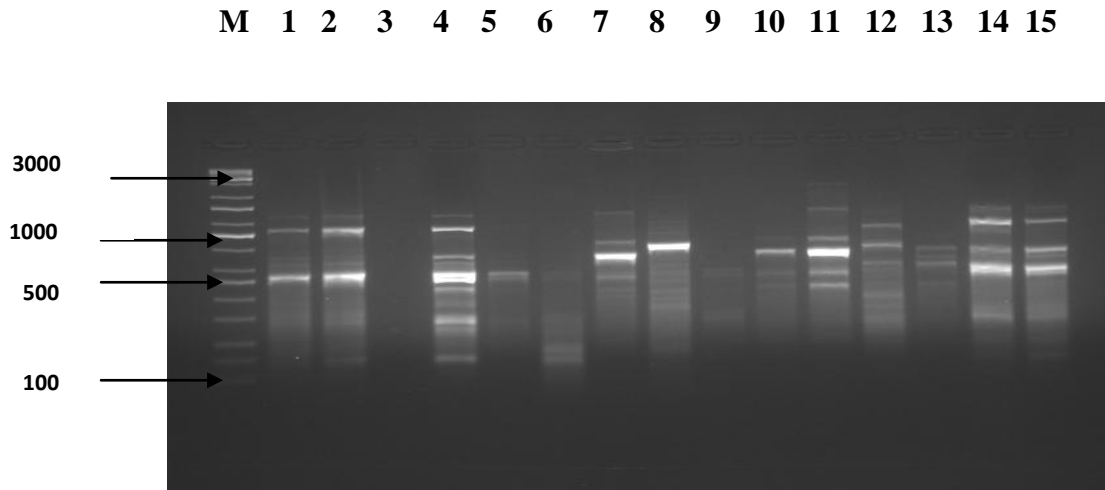


Figure 5.1(b) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the specific nif-directed RPO1. M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar (A4) following sterilisation.

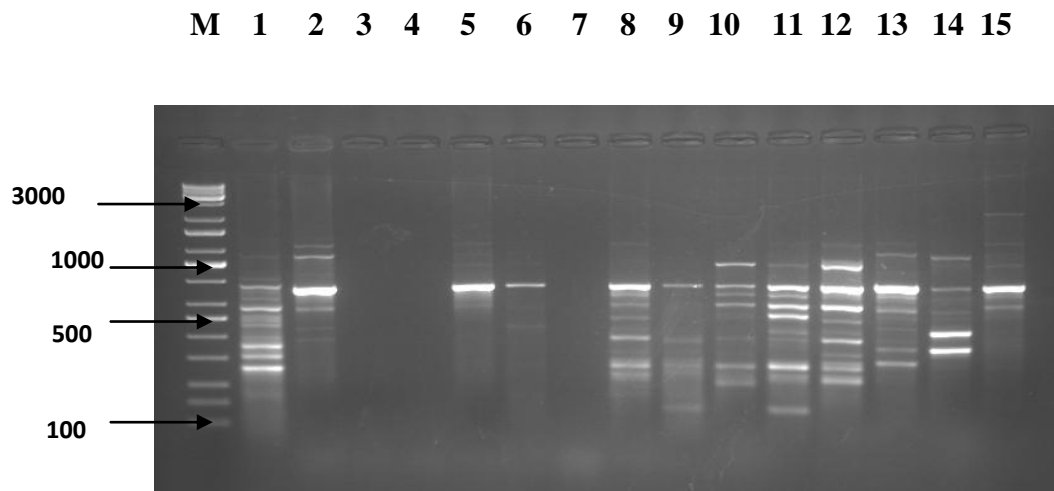


Figure 5.1(c) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the specific nif-directed RPO1. M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar (M5) following sterilisation.

### 5.3.2 The arbitrary primer RP04

DNA amplification profiles were generated from *Rhizobium* DNA using the arbitrary RPO4 primer in lima bean cultivars A3, A4, M2 and M5. (Figure 5.2). The *Rhizobium* strains that exhibited strong amplification bands or profiles in cultivar A3 were 1, 2, 3, 4, 5, 7, 9, 11, 12, 13, 14 and 15. Weakly amplified profiles were indicative in strain 6 and 8 and showed no amplification in strain 10 (Figure 5.4a). With respect to cultivar A4, *Rhizobium* strains 2, 4, 5, 7, 9, 10, 11, 12, 13, 14 and 15 were strongly amplified while weakly amplified profiles were observed in strains 1, 6 and 8. No amplification band was shown for *Rhizobium* strain 3 (figure 5.2b). *Rhizobium* of lima bean cultivar M5 were strongly amplified in strains 2, 3, 5, 6, 8, 9, 10, 12, 13, 14 and 15. *Rhizobium* strain 1 was weakly amplified with no amplified profile recorded in *Rhizobium* strains 4, 7 and 11 (Figure 5.2c)

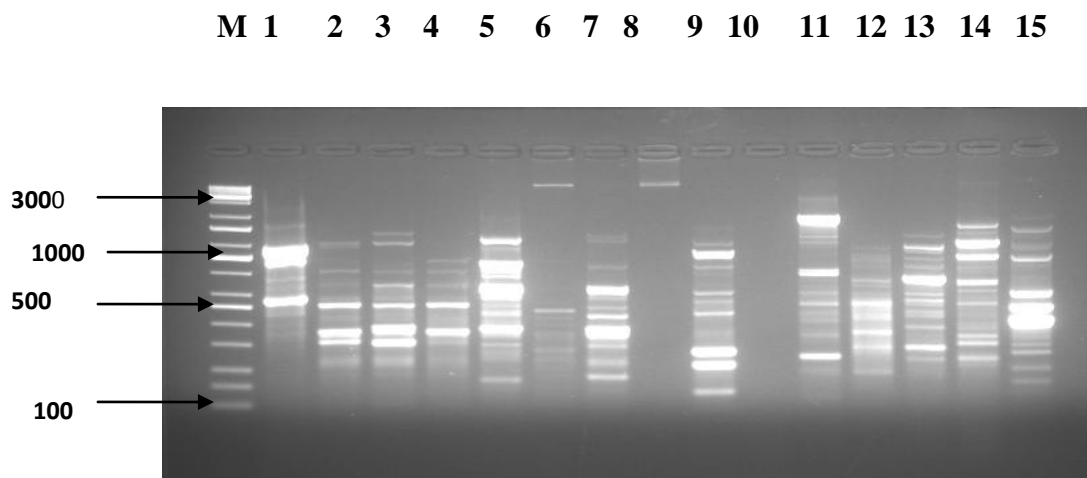


Figure 5.2 (a) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the arbitrary primer RPO4. M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar (A3) following sterilisation.

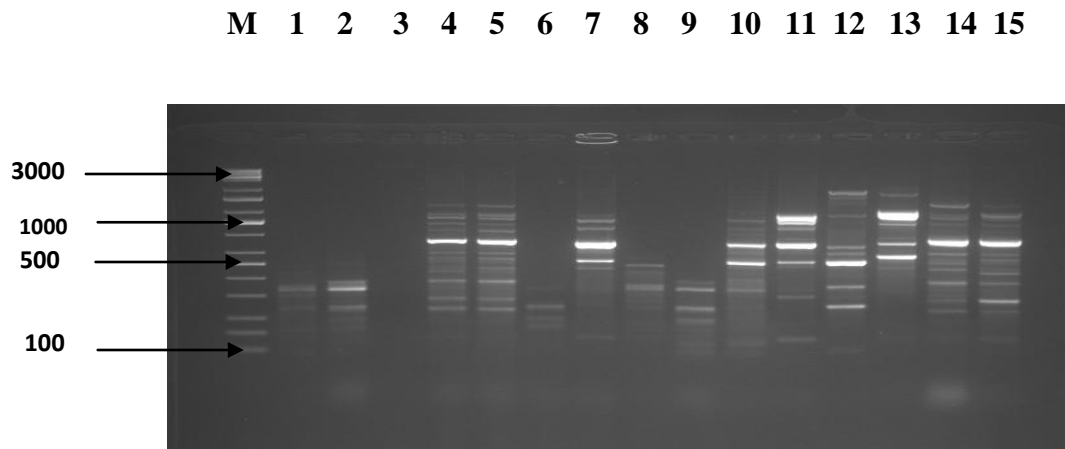


Figure 5.2 (b) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the arbitrary primer RPO4. M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar (A4) following sterilisation.

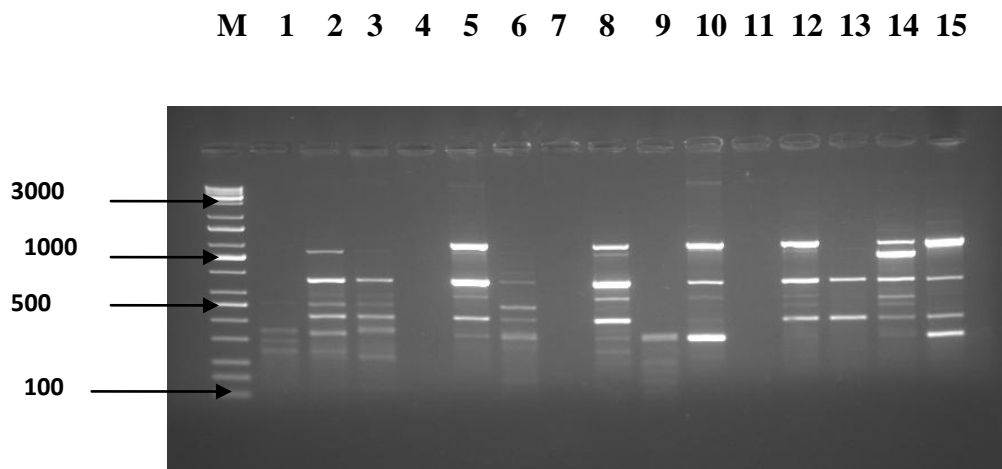


Figure 5.2 (c) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the arbitrary primer RPO4. M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar (M5) following sterilisation.

### 5.3.3 The arbitrary primer ERIC 1 and 2 primers

The ERIC 1 and 2 primers were able to generate DNA bands from some of the *Rhizobium* strains extracted from nodules of the lima bean cultivars A1 and A2. It however indicated very low amplification (Figure 5.3). With respect to cultivar A1, there were strong amplifications in *Rhizobium* strains 6, 7 and 11. *Rhizobium* strains 1, 5, 8, 10, 13, 14 and 15 were weakly amplified, with bands not shown for in *Rhizobium* strains 2, 3, 4, 9 and 12 (Figure 5.3a). For the lima bean cultivar A2, strong amplifications were shown for *Rhizobium* strains 2, 3, 11, 12 and 14. Weak amplified profiles were observed in *Rhizobium* strains 4, 9, 10 and 15 with low amplification in strains 5, 6, 7, 8 and 13 (Figure 5.3b).

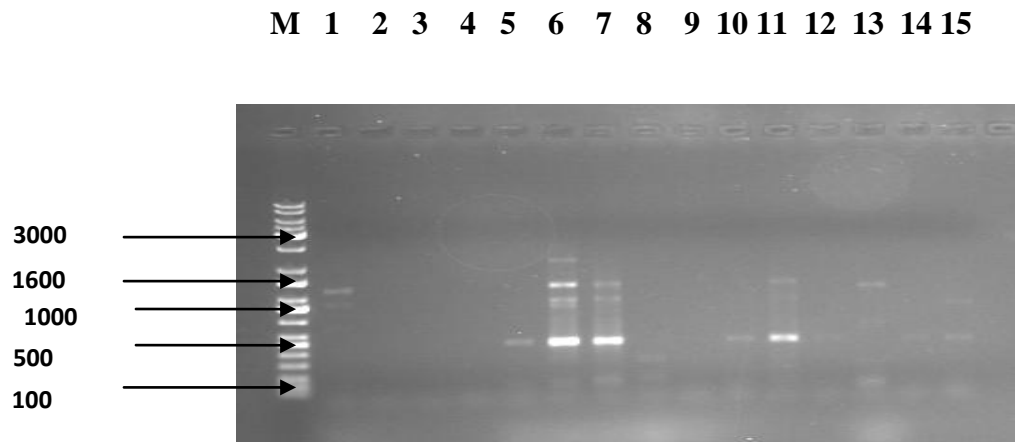


Figure 5.3(a) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the arbitrary primer ERIC . M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar A1 following sterilisation.

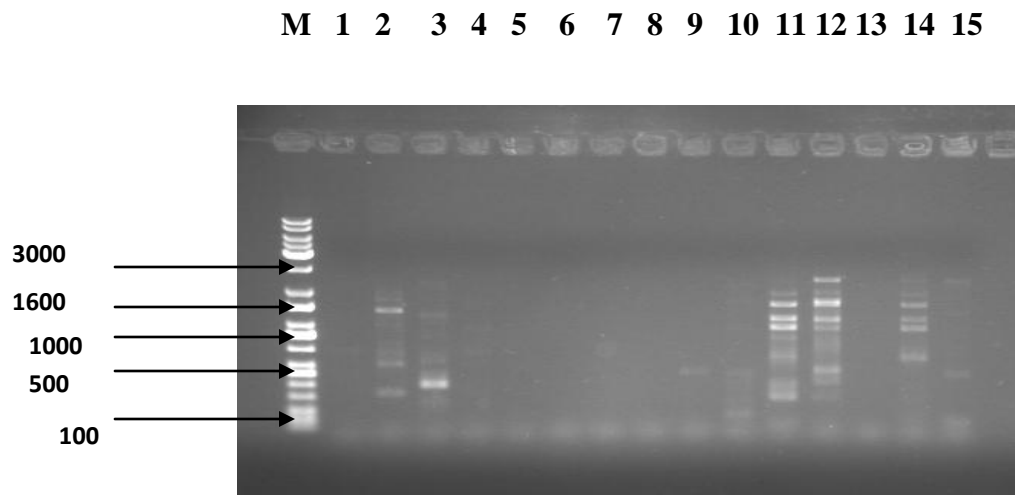


Figure 5.3(b) Gel electrophoresis of PCR amplification of *Rhizobium* DNA using the arbitrary primer ERIC . M is the ladder and 1-15 represents *Rhizobium* strains from squashed extracts of lima bean cultivar A2 following sterilisation.

#### 5.3.4 Inter-accessional DNA amplification profile using the *nif*-directed primer RPO1

DNA amplification profiles were generated from *Rhizobium* DNA between and among the thirteen cultivars of the lima bean using the *nif*-directed RPO1 primer (Figure 5.4). The *Rhizobium* strains that exhibited strong amplification bands or profiles in cultivar A1 were 1, 2, 4 and 5; in cultivar A2, *Rhizobium* strains 6, 7, 8, 9 and 10; cultivar A3, *Rhizobium* strains 11, 13, 14 and 15.

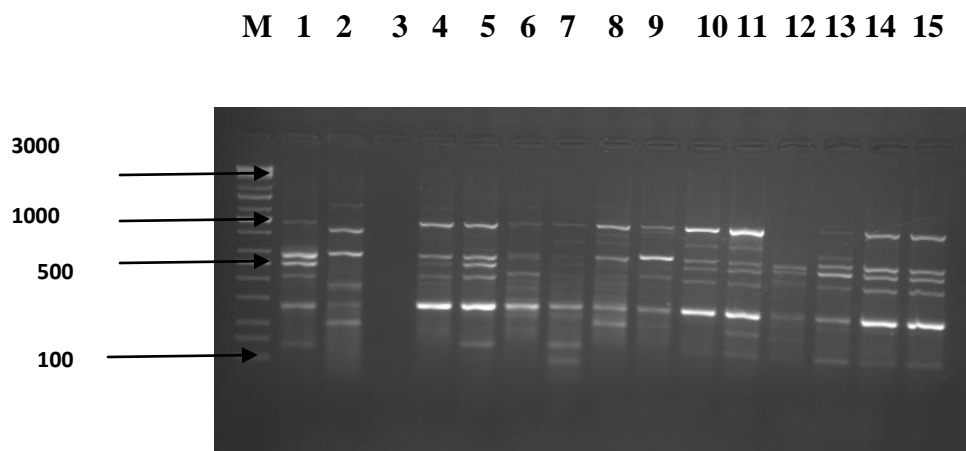


Figure 5.4 Representative DNA amplification profile using the *nif*-directed primer RPO1 from five nodules each of all thirteen cultivars of lima bean for dendrogram generation where A1(1-5), A2(6-10), A3(11-15).

### 5.3.5 Inter-accessional DNA amplification profile using the arbitrary primer RP04

DNA amplification profiles were generated from *Rhizobium* DNA between and among the thirteen cultivars of the lima bean using the arbitrary RPO4 primer (Figure 5.5). The *Rhizobium* strains that exhibited strong amplification bands or profiles in cultivar A1 were 1, 2, 3, 4 and 5; In cultivar A2, *Rhizobium* strains 6, 7, 9 and 10; cultivar A3, *Rhizobium* strains 11, 14 and 15.

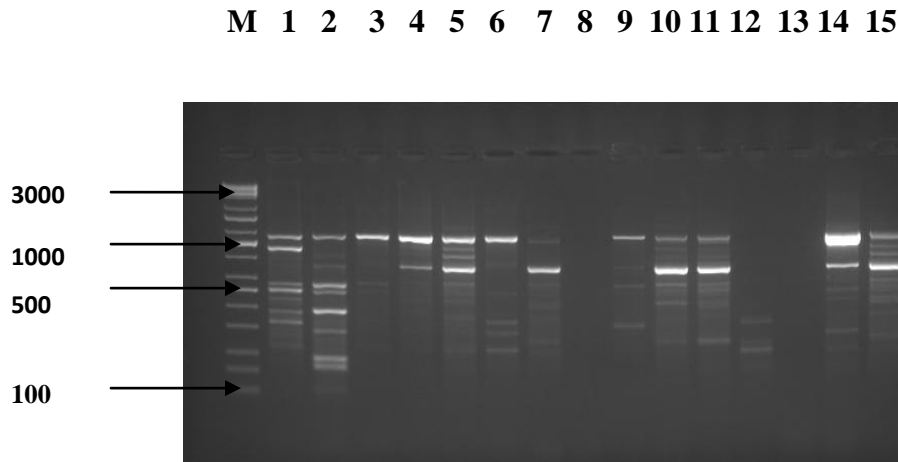


Figure 5.5 Representative DNA amplification profile using the arbitrary primer RPO4 from five nodules each of all thirteen cultivars of lima bean for dendrogram generation where A1(1-5) , A2(6-10), A3(11-15).

### 5.3.6 Genetic relationship within *Rhizobium* strains in lima bean cultivars

#### 5.3.6.1 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar

##### A1

The dendrogram generated clustered the *Rhizobium* strains into two main clusters at a level of 47 % genetic distance. Cluster A was divided into two subclusters at 50 % genetic distance. Subcluster AI consisted of *Rhizobium* strain 13 and AII of *Rhizobium* strains 4, 6 and 7. Cluster B was divided into two subclusters at 53.3% genetic distance. Subcluster BI was made up of *Rhizobium* strains 8 and 15 and subcluster BII, *Rhizobium* strains 1, 2, 3, 5, 9, 10, 11, 12 and 14. At 100 % genetic distance, *Rhizobium* strains 5 and 14 were similar. Also, at genetic distance of 80 % and 73.3 %, *Rhizobium* strains 1, 11 and 2, 3, 8 were similar, respectively. In addition, at 53.3 % genetic distance, *Rhizobium*

strains 12 and 13 showed similarities. The farthest genetic distance was between *Rhizobium* strain 1 and 13 (Figure 5.6).

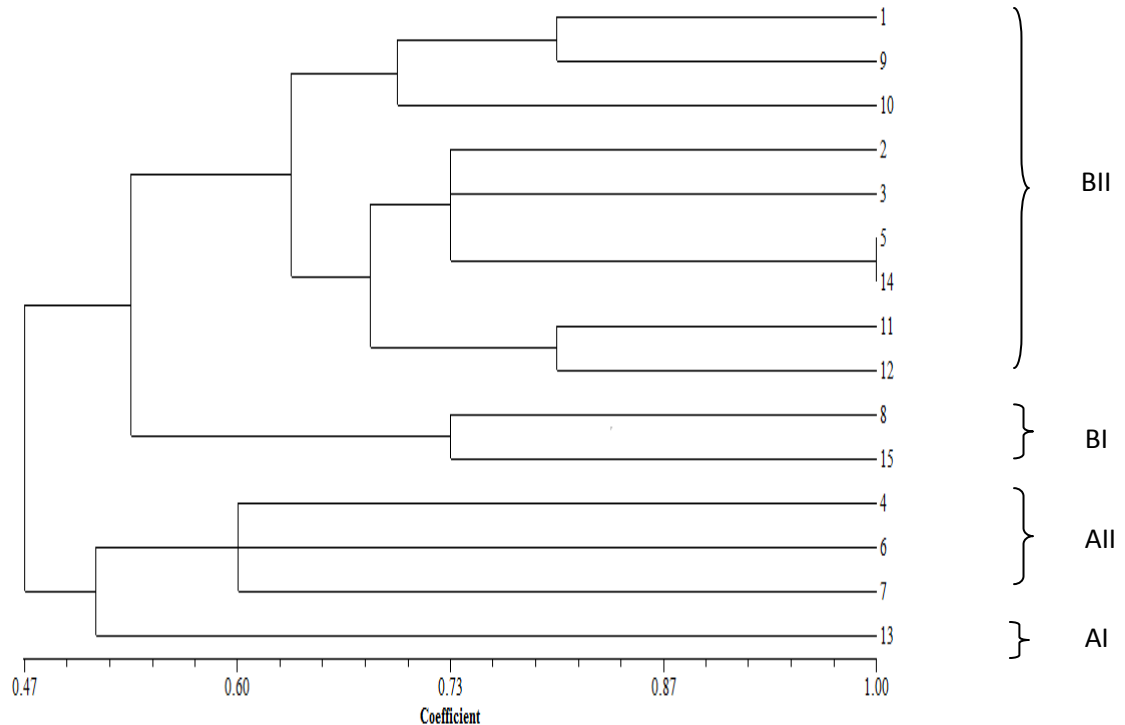


Figure 5.6 Dendrogram showing the genetic relationship among 15 nodules of the A1 cultivar.

### 5.3.6.2 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar A2

At a genetic distance of 49 %, *Rhizobium* strains were clustered into two main clusters, with cluster A being made up of *Rhizobium* strain 10. At a similarity distance of 49.2 %, cluster B was divided into two subclusters. Subcluster BI was made up of *Rhizobium* strain 14. Subcluster BII was further divided into three subgroups: BII (1) consisted of *Rhizobium* strains 4, 8, 9, 12, 13, BII (2) of *Rhizobium* strains 2, 5, 6, 15, BII (3) of

*Rhizobium* strains 1, 3, 7 and 11. At 100% level of genetic distance, similarities existed between *Rhizobium* strains 1 and 11. Also at 68.4%, *Rhizobium* strains 4 and 6 were similar. The farthest, however, in terms of genetic distance was between *Rhizobium* strain 1 and 10 (Figure 5.7).

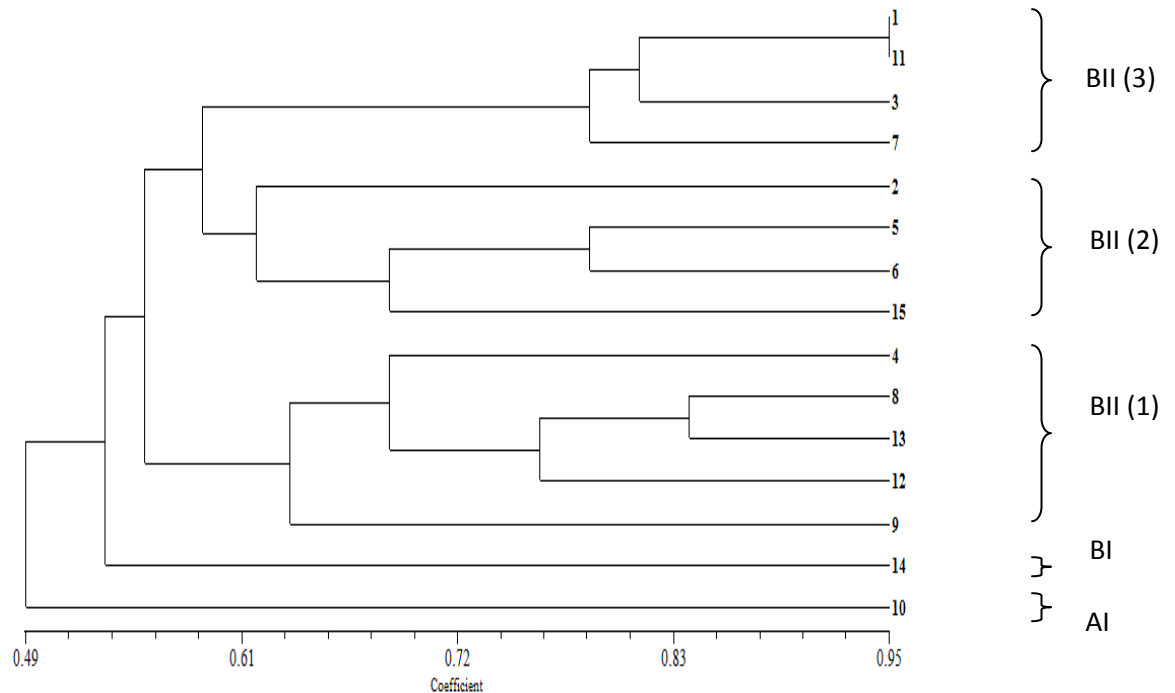


Figure 5.7 Dendrogram showing the genetic relationship among 15 nodules of A2 cultivar.

### 5.3.6.3 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar A3

Two major clusters A and B were formed at a genetic distance of 41 %. Cluster A was divided into two subclusters, AI comprised *Rhizobium* strain 6 and AII of *Rhizobium* strain 10. Cluster B was also divided into two subclusters at a genetic distance of 59.8 %. Subcluster BI contained *Rhizobium* strains 5, 9, 12 and 15. Subcluster BII was further

divided into two groups: BII (1) consisted *Rhizobium* strains 7, 14 and BII (2) of *Rhizobium* strains 1, 2, 3, 4, 8 and 13. At the level of 100 % genetic similarity, *Rhizobium* strains 2 and 3 were similar. Also, *Rhizobium* strains 9, 7 and 13 were similar to each other at 83.3 % genetic distance. The farthest genetic distance was however between *Rhizobium* strain 1 and 6 (Figure 5.8).

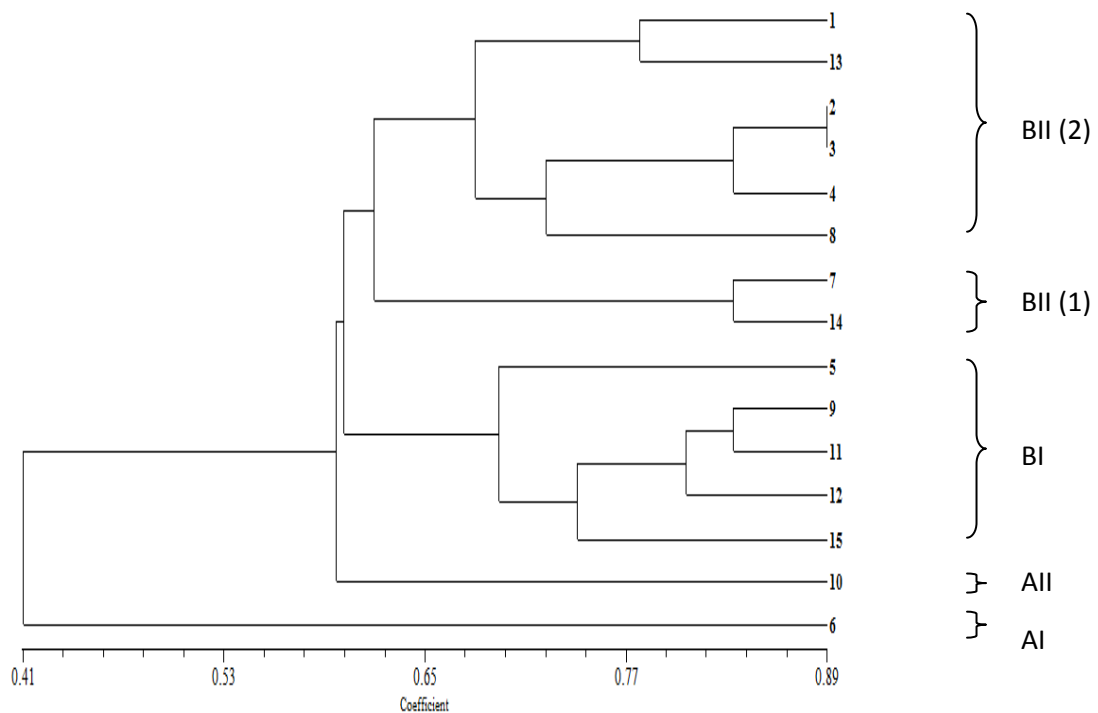


Figure 5.8 Dendrogram showing the genetic relationship among 15 nodules of the A3 cultivar.

#### 5.3.6.4 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar

##### A4

The dendrogram generated clustered *Rhizobium* strains into two main clusters at 57 % genetic distance. Cluster A and B was divided at a similarity distance of 62.5 %. Cluster

A was grouped into two clusters which had AI made up of *Rhizobium* strains 1, 7, 8, 10, 12, 13 and AII, *Rhizobium* strains 3, 5, 6 and 9. Cluster B, made up of subcluster BI, consisted of only *Rhizobium* strain 4 while subcluster BII contained *Rhizobium* strains 1, 2, 14 and 15. At genetic similarity levels of 78.9 % and 85.7 %, *Rhizobium* strains 5, 9, 10 and *Rhizobium* strains 5, 7 were similar to each other. At 100 % genetic distance, *Rhizobium* strains 14 and 15 showed similarity. The farthest distance was indicated between *Rhizobium* strain 1 and 11 (Figure 5.9).

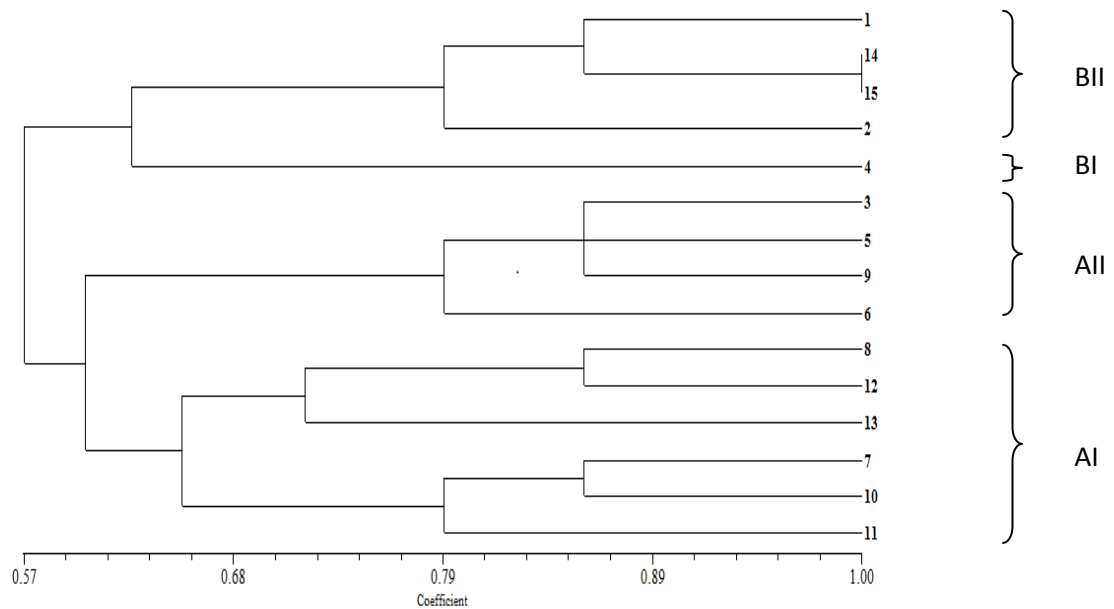


Figure 5.9 Dendrogram showing the genetic relationship among 15 nodules of the A4 cultivar.

### 5.3.6.5 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar M1

At a genetic distance of 53 %, two main clusters were generated. Cluster A was divided into two subclusters; subcluster AI consisted of *Rhizobium* strains 5 and 12. Subcluster

AII was further divided into two groups: AII (1) was made up of *Rhizobium* strains 6, 7 and 8 and AII (2), *Rhizobium* strains 3 and 13. At 59.2 % similarity distance, cluster B was divided into two subclusters. Subcluster BI comprised of *Rhizobium* strains 4 and 9 while subcluster BII was further divided into two groups at 68.8 % level of genetic distance. Subcluster BII (1) constituted *Rhizobium* strains 2, 10, 14, and 15 while BII (2) had *Rhizobium* strains 1 and 11. *Rhizobium* strains 1 and 3 were similar at 80 % genetic distance. At 100 % genetic similarity level, *Rhizobium* strains 6 and 8 were similar. However, the farthest distance was between *Rhizobium* strain 1 and 12 (Figure 5.10).

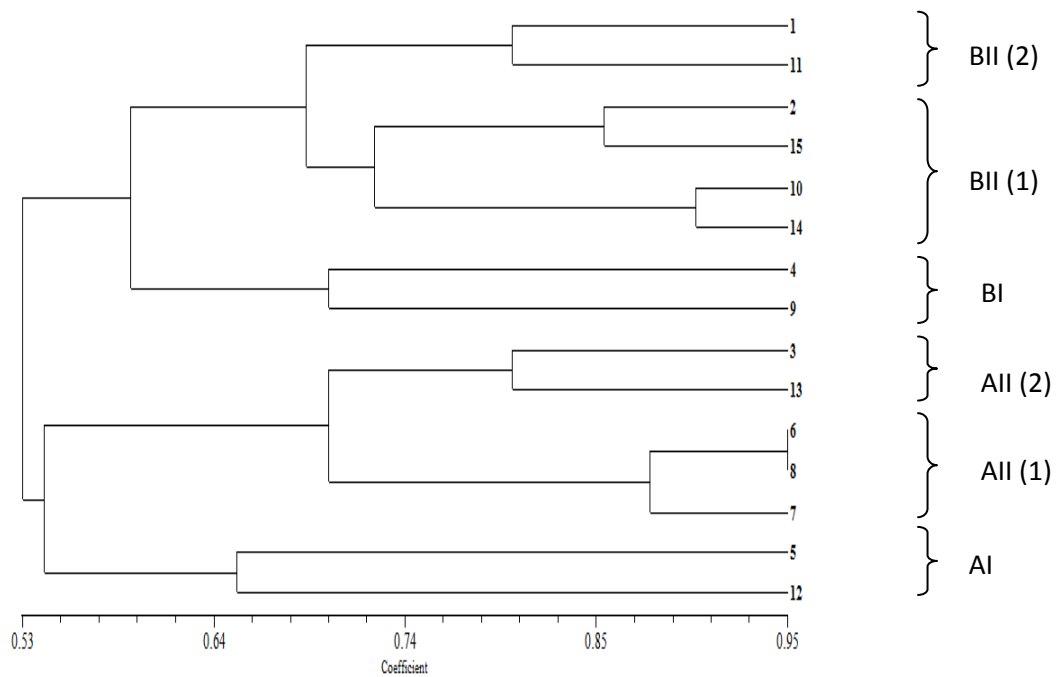


Figure 5.10 Dendrogram showing the genetic relationship among 15 nodules of the M1 cultivar.

### 5.3.6.6 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar M2

At 50 % genetic distance, two main clusters were formed. Cluster A comprised only of *Rhizobium* strain 3, while the remaining strains were found in the second cluster. The second cluster, cluster B, was divided into two subclusters I and II at 52.4 % genetic distance. Subcluster BI consisted of *Rhizobium* strains 8, 10, 11 and 13. Subcluster BII was divided into three groups: BII (1) comprised *Rhizobium* strains 14 and 15, BII (2) incorporated *Rhizobium* strains 4, 5, 6, and 12 and BII (3), *Rhizobium* strains 1, 2, 7 and 9. *Rhizobium* strains 5, 10 and 1, 6, 14 and 4, 6 were similar to each other at 71.4 %, 81 % and 100 % similarity level respectively. However *Rhizobium* strain 1 and 3 were farthest apart in genetic distance (Figure 5.11).

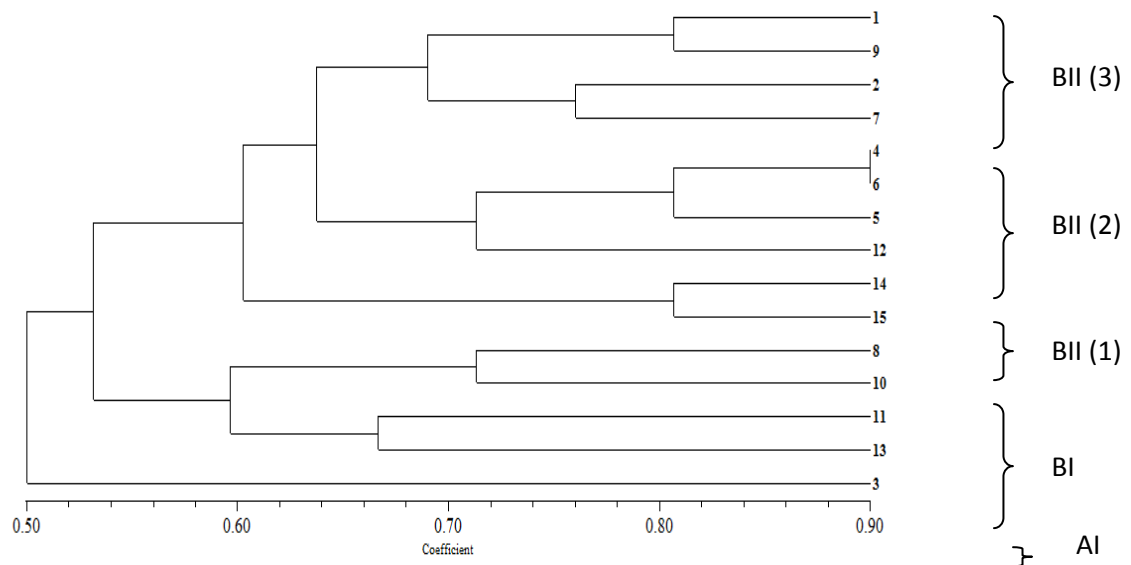


Figure 5.11 Dendrogram showing the genetic relationship among 15 nodules of the M2 cultivar.

### 5.3.6.7 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar M3

From the dendrogram below, all the *Rhizobium* strains were clustered into two main groups at 47 % genetic distance. Cluster A was further divided into two subclusters: subcluster AI consisted of *Rhizobium* strains 11, 12, 14 and 15 while subcluster AII was made up of *Rhizobium* strains 7, 8 and 13. Cluster B was also divided into two subclusters: BI made up of *Rhizobium* strains 3 and 10. Subcluster BII was however, divided into two subgroups: BII (1) composed of *Rhizobium* strains 2, 4, 6, 9 and BII (2), *Rhizobium* strains 1 and 5. *Rhizobium* strains 1 and 5 showed genetic similarity at level 100 %. The farthest in genetic distance was seen in *Rhizobium* strain 1 and 15 (Figure 5.12)

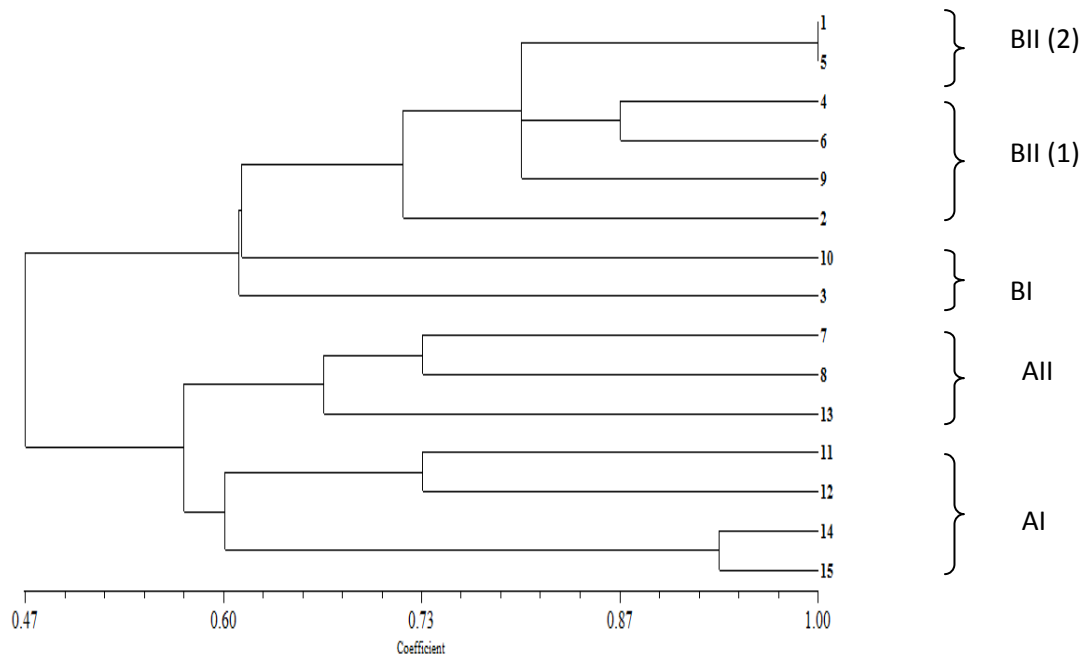


Figure 5.12 Dendrogram showing the genetic relationship among 15 nodules of the M3 cultivar.

### 5.3.6.8 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar M4

At 27 % genetic distance, two main clusters were formed. Cluster A was divided into two: AI comprised *Rhizobium* strains 4, 5, 6, 7, 9, 10, 12, 13, 14 and 15 while AII was constituted *Rhizobium* by strains 2 and 3. Cluster A and B were divided at a similarity of 55 %. Cluster B was divided into two groups. Subcluster BI consisted of *Rhizobium* strain 8 and subcluster BII made up of *Rhizobium* strains 1 and 11. *Rhizobium* strains 5, 6, 7, 9, 12, 13, 14 and 15 showed similarity at 100 % genetic distance. The farthest apart was shown between *Rhizobium* strain 1 and 15 (Figure 5.13).

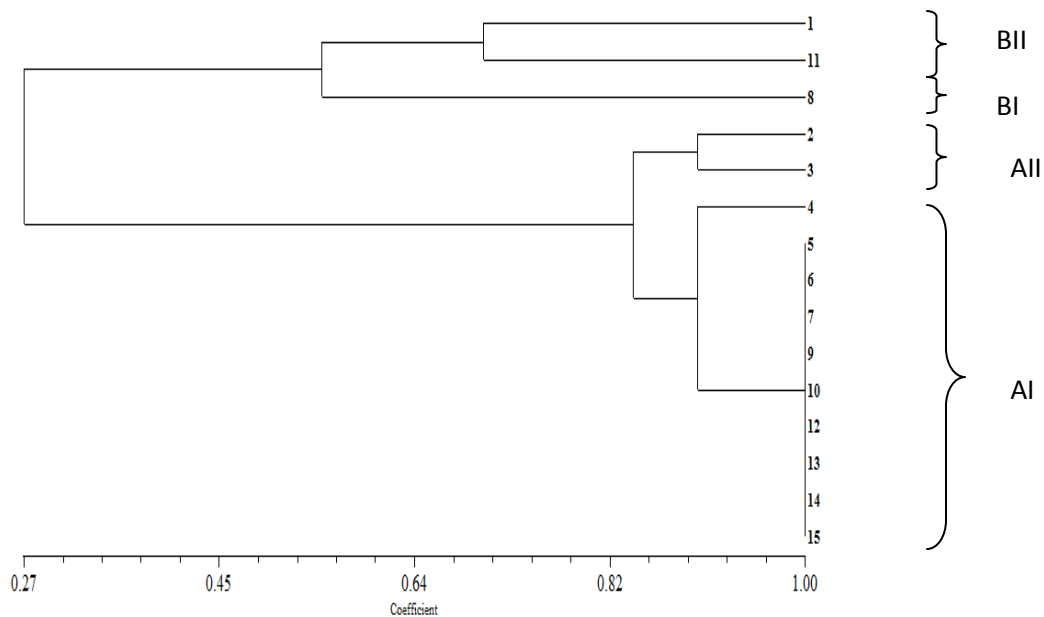


Figure 5.13 Dendrogram showing the genetic relationship among 15 nodules of the M4 cultivar.

### **5.3.6.9 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar M5**

Two major clusters A and B were formed at the genetic distance of 52 % level. Cluster A was divided into two subclusters AI and AII. Subcluster AI consisted of only *Rhizobium* strain 12. Subcluster AII was divided further into two subgroups: AII (1) made up of *Rhizobium* strains 3, 4, 7 and 9 with AII (2) having *Rhizobium* strains 2, 5, 6, 10, 13 and 15. Cluster B was divided into two subclusters, BI and BII at 57.2 % genetic distance. Subcluster BI had two subgroups: BI (1) comprised *Rhizobium* strains 11 and 14 with BI (2) having only *Rhizobium* strain 8. Subcluster BII only had *Rhizobium* strain 1 in its group. Similarities existed at the genetic distances of 100 %, 77.8 % and 83.3 % in *Rhizobium* strains 3, 4, strains 7, 11 and strains 5, 6, respectively. The farthest genetic distance however was indicated between strain *Rhizobium* 1 and 12 (Figure 5.14).

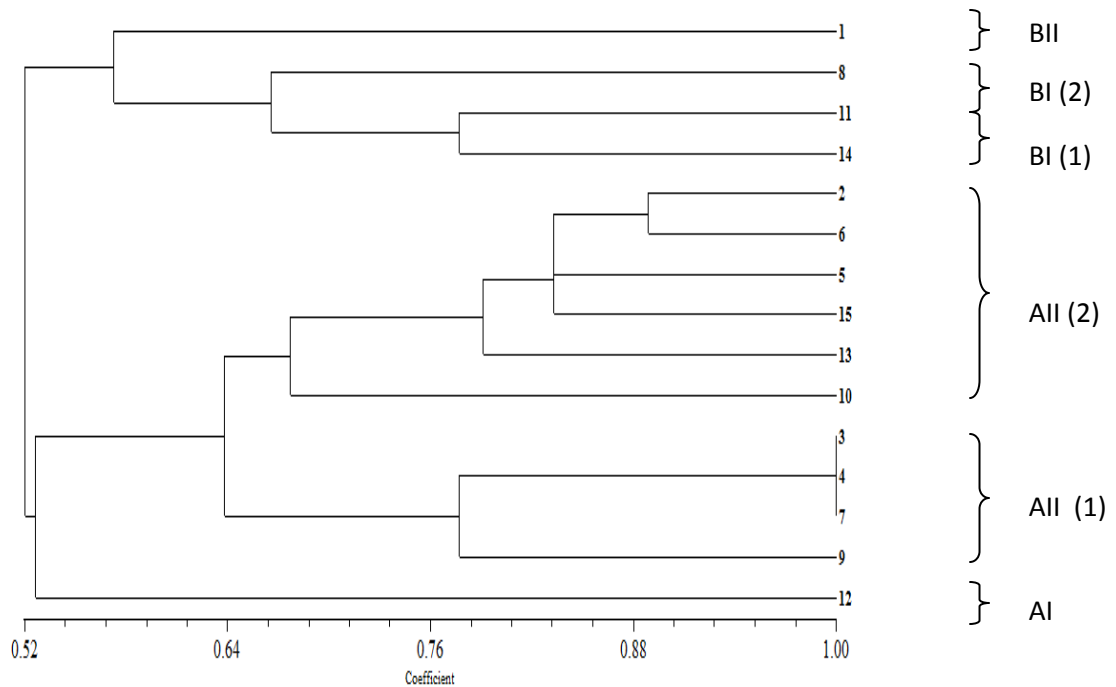


Figure 5.14 Dendrogram showing the genetic relationship among 15 nodules of the M5 cultivar.

### 5.3.6.10 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar GH1714

The dendrogram generated clustered the *Rhizobium* strain into two main clusters at 30 % genetic distance. Cluster A and B were divided at genetic distance of 46.6 %. Cluster A was subdivided into two: subcluster AI consisted of *Rhizobium* strain 7 with AII having *Rhizobium* strains 2 and 8. Cluster B was also divided into two groups with subcluster BI further been divided into two subgroups. BI (1) comprised *Rhizobium* strains 4, 5 and 6 with BI (2) having *Rhizobium* strains 3, 5, 9, 10, 11, 12, 14 and 15. Subcluster BII had only *Rhizobium* strain 1. At 100 % genetic distance, *Rhizobium* strains 3, 4, 5, 6, 9, 10,

11, 14 and 15 showed similarities. The farthest apart in genetic distance was between *Rhizobium* strain 1 and 7 (Figure 5.15).

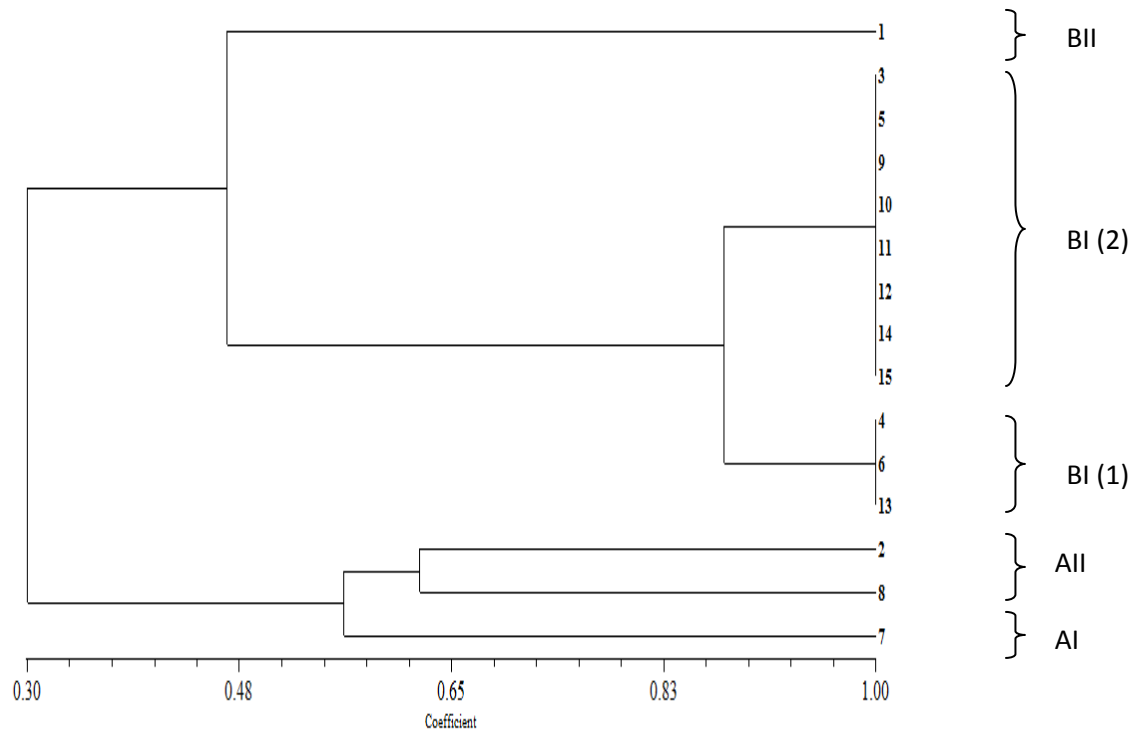


Figure 5.15 Dendrogram showing the genetic relationship among 15 nodules of the GH 1714 cultivar.

### 5.3.6.11 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar GH 1719

Two major clusters A and B were formed at a genetic distance of 42 %. Cluster A consisted of *Rhizobium* strains 11 and 12. Cluster B was divided into two. Subcluster BI comprised only *Rhizobium* strain 7 while BII was further subdivided into two groups: BII (1) was made up of the *Rhizobium* strain 3 and BII (2), with *Rhizobium* strains 1, 2, 4, 5, 6, 8, 9, 10, 13, 14 and 15. Similarities existed between strains 1, 2, 4, 5, 6, 9, 10, 13, 14

and 15 at genetic distance of 100 %. The farthest genetic distance was however between *Rhizobium* strain 1 and 12 (Figure 5.16).

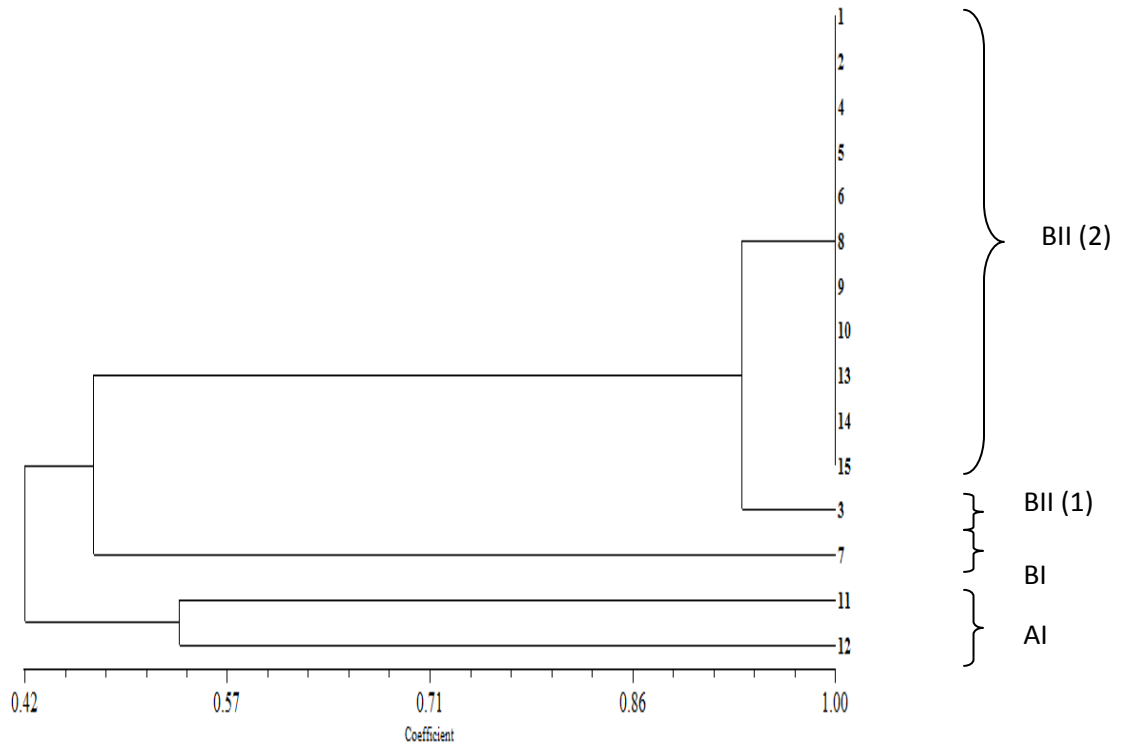


Figure 5.16 Dendrogram showing the genetic relationship among 15 nodules of the GH 1719 cultivar.

### 5.3.6.12 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar GH 1726

At a genetic distance of 42 %, two main clusters A and B were formed. The cluster A was divided into two subclusters, AI and AII. Subcluster AI was made up of *Rhizobium* strain 10 whereas subcluster AII was further subdivided into two groups. AII (1) comprised *Rhizobium* strain 13 with AII (2) constituted by *Rhizobium* strains 2, 3, 5, 6, 8, 9, 12, 14

and 15. Cluster B was also divided into two subclusters: BI showed *Rhizobium* strain 7 with BII consisting of *Rhizobium* strains 1,4 and 11. At a similarity level of 100 %, *Rhizobium* strains 3, 4, 5, 6, 9, 10, 11, 14 and 15 were similar to each other. The farthest genetic distance was between *Rhizobium* strain 1 and 10 (Figure 5.17).

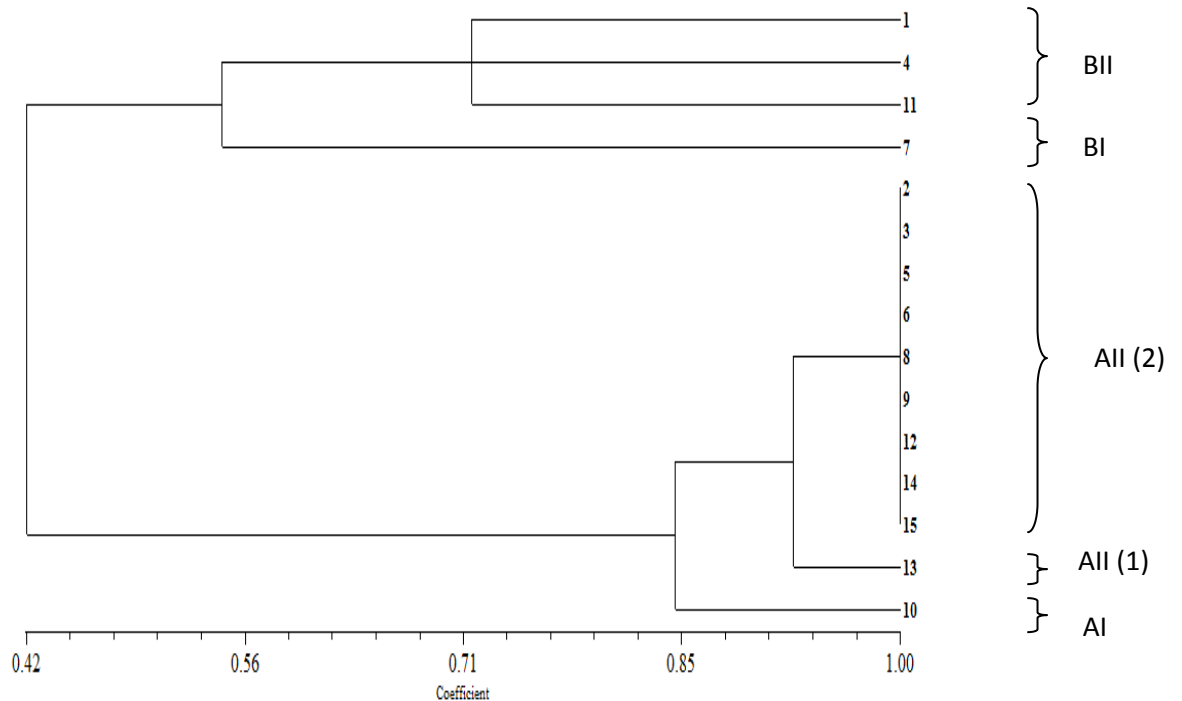


Figure 5.17 Dendrogram showing the genetic relationship among 15 nodules of the GH 1726 cultivar.

### 5.3.6.13 Genetic relationship among *Rhizobium* bacteria DNA in lima bean cultivar GH 1737.

At a genetic distance of 28 %, two major clusters were formed. Clusters A and B were divided at genetic similarity level of 46 %. Cluster A was made up of *Rhizobium* strain 15. However, cluster B was divided into two groups with subcluster BI having two subgroups. BI (1) contained *Rhizobium* strain 4 with BI (2) having *Rhizobium* strains 2, 3,

6, 7, 8, 9, 10, 11, 12, 13 and 14. Subcluster BII comprised *Rhizobium* strain 1 and 5. Similarities existed between *Rhizobium* strains 2, 3, 6, 7, 9, 10, 11, 12, 13 and 14 at 100 % genetic distance. The farthest genetic distance was between *Rhizobium* strain 1 and 15 (Figure 5.18).

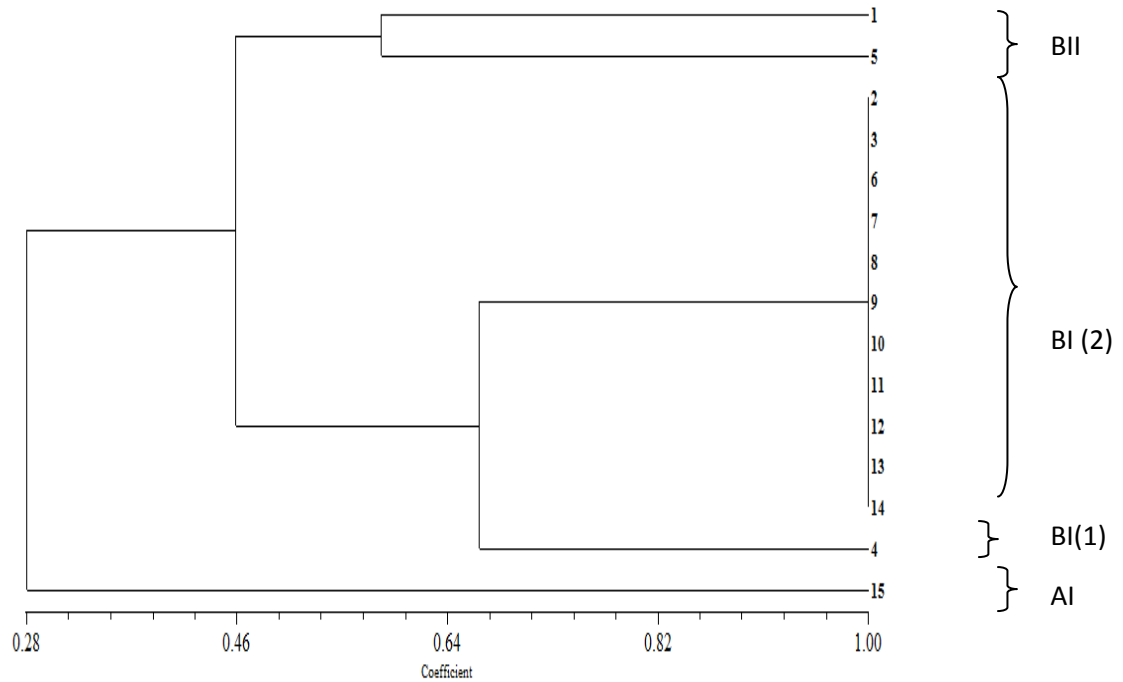


Figure 5.18 Dendrogram showing the genetic relationship among 15 nodules of the GH 1737 cultivar.

### **5.3.7 Inter-accessional genetic relationship between and among *Rhizobium* bacteria**

#### **5.3.7.1 Inter-accessional genetic relationship between and among *Rhizobium* bacteria DNA in lima bean using RPO1 primer.**

Two major clusters were formed at 49 % level of genetic distance. Cluster A consisted of *Rhizobium* strains D1, D2 and F5. Cluster B was divided into two subclusters BI and BII. Subcluster BI was made up of *Rhizobium* strains E1, E4 and G4. Subcluster BII was further subdivided into five groups. BII (1) comprised *Rhizobium* strains A3, H1, A4, M5, L4, B3, M2, E2 AND M4. BII (2) consisted of *Rhizobium* strains B4, H2, I3, K1, D3, M3, I4, E5, G1 and L3. BII (3) had *Rhizobium* strains A2, K2, A5, G3, B1, F1, F3, E3, F4 and G5. Also BII(4) was composed of *Rhizobium* strains B2, J2, C2, L2, M1, L1, C1, C3, F2, I2, C4, C5, J4 and J3. BII (5) had *Rhizobium* strains A1, J1, D5, D4, G2, I1, L5, H3 and K2. *Rhizobium* Strains B2, J2 ,L M1, C4, C5, F1 and F3 were all similar at 100 % level of similarity. The farthest apart existed between *Rhizobium* strains A1 and F5 (Figure 5.19).

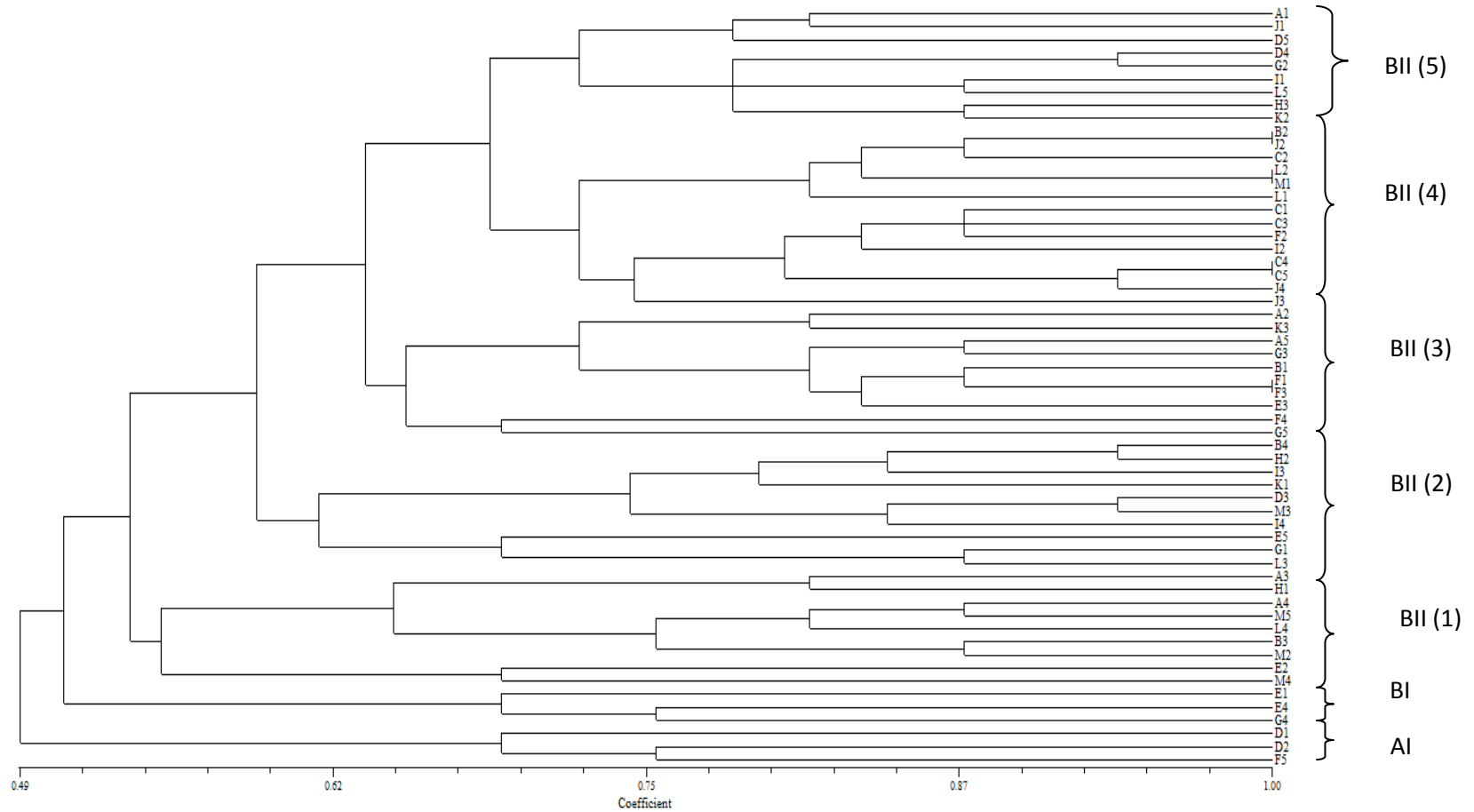


Figure 5.19 Dendrogram showing the genetic relationship among 65 nodules of the thirteen lima bean cultivars using RPO1 primer, A1(A1-A5), A2(B1-B4), A3(C1-C5), A4(D1-D5), M1(E1-E5), M2(F1-F5), M3(G1-G3), M4(H1-H5), M5(I1-I5), GH1714(J1-J3), GH1719(K1-K5), GH1726(L1-L4), GH1737(M1-M4).

### **5.3.7.2 Inter-accessional genetic relationship between and among *Rhizobium* bacteria DNA in lima bean using RPO4 primer.**

Two major clusters were formed at 49 % level of genetic distance. Cluster A was constituted by *Rhizobium* strains D1 and D3. Cluster B was divided into two subclusters BI and BII. Subcluster BI comprised *Rhizobium* strains D5 and D4. Subcluster BII was further subdivided into six groups. BII (1) was composed of *Rhizobium* strains A5, H4, B5, G4, M2, H3, K1, H5, L3, L1, E4, E1 and E2. BII (2) consisted of *Rhizobium* strains D2, M4, C1, C3, C2, C4, E5, M1 and M3. BII (3) had *Rhizobium* strains G1 and G3. BII (4) comprised *Rhizobium* strains A3, E3, J3, G5, H2, C5 and K4. BII (5) contained *Rhizobium* strains A4, G2, B1, L4 F4, I2, I4, K2, J2, J1, L2, L5 and H1. BII (6) consisted of *Rhizobium* strains A1, F5, B3, L3, B4, A2, F1, F3, I1, B2, F2 and K3. At 100 % level of genetic similarity, *Rhizobium* strains F4 and I2 were similar whereas the farthest genetic distance existed between *Rhizobium* strains A1 and D3. (Figure 5.20).

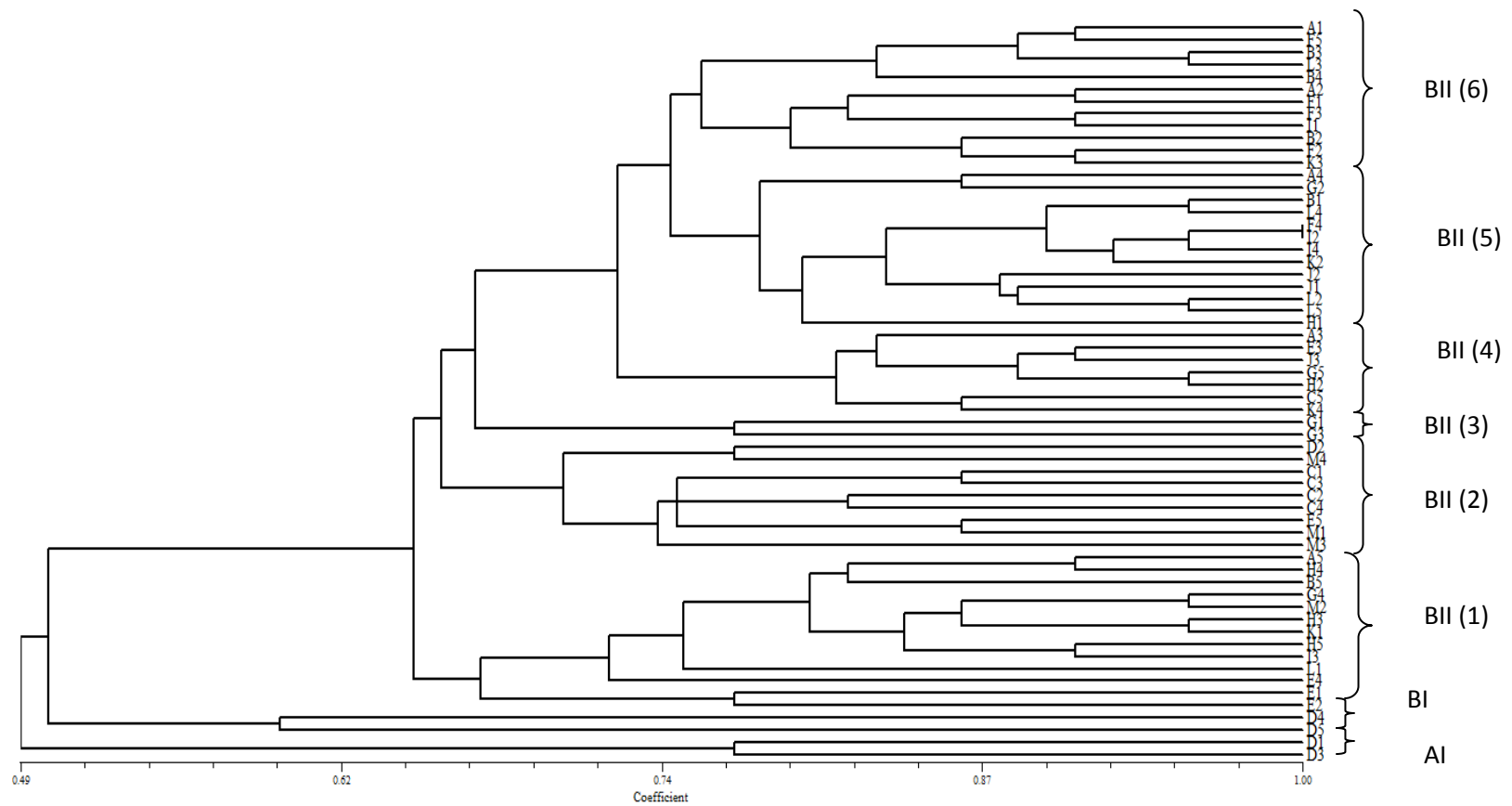


Figure 5.20 Dendrogram showing the genetic relationship among 65 nodules of the thirteen lima bean cultivars using RPO4 primer, A1(A1-A5), A2(B1-B5), A3 (C1-C5), A4(D1-D5), M1(E1-E5), M2(F1-F5), M3( G1-G5), M4(H1-H5), M5( I1-I4), GH1714(J1-J3), GH1719 (K1-K4), GH1726(L1-L5),GH1737( M1-M4).

## 5.4 DISCUSSION

### 5.4.1 Differentiation of *Rhizobium* strains using three primers

Genetic diversity studies on *Rhizobium* strains of lima bean were done using two arbitrary primers (RPO4 and ERIC) and nif directed primer (RPO1) for PCR amplification. The profiles generated indicated very distinct profiles for most of the DNA of the bacteria for two of the primers specifically RPO1 and RPO4. The primers RPO1 and RPO4 were very discriminatory by virtue of the generation of high polymorphic DNA bands. This result correlated with observation made by Harrison *et al.* (1992) who reported that primers RPO1 and RPO4 were highly discriminate in nature and generated a greater degree of polymorphism on *Rhizobium* DNA. Also Richardson *et al.* (1995) stated that primers RPO1 and RPO4 are suitable or useful in differentiation of *Rhizobium* species to the strain level. The unique amplification profiles by both RPO1 and RPO4 is an indication of their wide applicability to differentiate strains of *Rhizobium* residing in the nodules of lima bean.

Richardson *et al.* (1995) reported that RPO1 primer was effective in amplifying and generating fragment length polymorphism for various strains of *R. meliloti* and *R. leguminosarum* bv. *viciae* and a wider range of rhizobia including *B. japonicum*. Thus, it can be said that the nif-directed RPO1 was able to amplify the diverse lima bean rhizobia nodulating the lima bean. Schofield and Watson (1985) also reported that the sequence of the RPO1 primer corresponded to a conserved region located within the *Rhizobium leguminosarum* bv. *trifoli* nif HDK promoter, which includes the *Rhizobium* nif promoter consensus element. Based on this, it is highly possible that *Rhizobium* strains amplified

by the RPO1 could mainly be the strains of *Rhizobium leguminosarum*. However, lima bean can be nodulated by species of *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* using the classification of Wang *et al.* (2006) in Brazilian soils (Santos *et al.*, 2008). The arbitrary primer ERIC, however, was not highly discriminatory due to the lower degree of polymorphism on the *Rhizobium* DNA for all cultivars of the lima bean investigated. The low degree of polymorphisms generated by the ERIC primer could be due to the high annealing temperature of 52°C used for the experiment. Kundu and Dudeja (2008) observed high polymorphisms using ERIC primer with an annealing temperature of 45°C on rhizobial populations infecting mung bean.

Thus based on results attained, the primers RPO1 and RPO4 were selected for use in inter-cultivar *Rhizobium* amplification which generated varied and distinct amplification in the differentiation of *Rhizobium* DNA in the lima bean cultivars studied.

#### **5.4.2 Genetic relatedness and diversity within *Rhizobium* strains in thirteen lima bean cultivars**

Genetic diversity was present in the lima bean cultivar A1as shown by the dendrogram generated (Figure 5.6) which linked isolates of bacteria at a similarity distance of 47 % where two major clusters were joined. Possible duplicates were shown in *Rhizobium* strains 5 and 14 which were linked at the 100 % genetic similarity level.

At the genetic distance of 49 % two major clusters were identified showing dissimilarity among isolates or strains of bacteria in lima bean cultivar A2. Two *Rhizobium* strains namely 1 and 11, were shown as possible duplicates at the 100 % level of similarity.

For lima bean cultivar A3, two major clusters were identified for all the isolates of bacteria and were joined at a similarity distance of 41 %, indicating diversity among the *Rhizobium* strains. Diversity of subclusters were also observed at a genetic distance of 60.2 %. Possible duplicate of *Rhizobium* strains identified at 100 % similarity were observed in *Rhizobium* strains 2 and 3.

At 57 % similarity level, two major clusters were identified in the dendrogram generated for isolates of bacteria for the lima bean cultivar A4. There were diversity among the *Rhizobium* strains less than one quarter of the strains were possible duplicates at genetic distance of 100 %. *Rhizobium* strains 14 and 15 showed similarity between each other.

Some amount of genetic diversity was observed in bacteria isolates or *Rhizobium* strains of lima bean cultivar M1, as shown by dendrogram generated (Figure 5.10) in which major clusters were formed at similarity distance of 53 %. Diversity between the subclusters A and B were formed at the 53.3 % similarity level. In cultivar M1, possible duplicates were not identified, which is indicative of the varied isolates generated by the dendrogram.

For the lima bean cultivar M2, two major clusters were identified in the dendrogram generated which linked bacteria isolates at the genetic distance of 50 %. Only two *Rhizobium* strains were identified as possible duplicates at 100 % level of similarity showing the distinct nature of the bacteria strains for the lima bean cultivar.

Diversity of isolates of bacteria in lima bean cultivar M3 were identified by dendrogram generated at a similarity level of 47 % where two major clusters were joined. This

suggests high dissimilarity among isolates of bacteria for this cultivar. Possible duplicates formed at the level of 100 % similarity level were observed in *Rhizobium* strain 1 and 5.

At a genetic distance of 27 %, the major clusters in the lima bean cultivar M4 were identified by dendrogram generated which suggests a high dissimilarity among strains of *Rhizobium*. Although, there were diversity among isolates, a high number of possible duplicates in one major cluster was observed at 100 % genetic distance. The *Rhizobium* strains 5, 6, 7, 9, 12, 13, 14 and 15 were similar to each other.

For the lima bean cultivar M5, the major clusters were joined at 52 % similarity level through the dendrogram generation. Possible duplicates were identified in *Rhizobium* strains 3 and 4 at 100 % similarity.

The dendrogram generated linked two major clusters at a genetic similarity of 30 % which indicated great diversity among strains of *Rhizobium* for the lima bean cultivar GH 1714. The diversity in *Rhizobium* strain was expressed in subclusters at 46.6 % similarity levels. However, almost half of the isolates could be considered as possible duplicates as they were linked at a genetic distance of 100 %.

A high degree of diversity among *Rhizobium* isolates existed for the lima bean cultivar GH 1719 as two major clusters were linked at a genetic distance of 42 %. However, at 100 % similarity level, more than half of the strains of bacteria were identical. At genetic distance of 42 %, two major clusters were formed. This is indicative of great diversity among isolates of bacteria in the cultivar GH 1719. Possible duplicates from isolates of bacteria were exhibited in *Rhizobium* strains 1, 2, 4, 5, 6, 9, 10, 11, 12, 14 and 15 .

Also for the lima bean cultivar GH 1726, two major clusters at similarity distance of 42 % were observed, quite indicative of genetic diversity. However, most of the isolates or *Rhizobium* strains were similar to each other at 100 % similarity level.

At 33 % level of similarity, the dendrogram generated linked two major clusters together for the lima bean cultivar GH 1737. A great amount of diversity existed among *Rhizobium* isolates as indicative of the genetic distance. More than half of the *Rhizobium* isolates were possible duplicates at 100 % similarity.

#### **5.4.3 Inter-accessional diversity among *Rhizobium* strains**

The dendrogram generated from use of the two primers, RPO1 and RPO4, which showed unique amplifications in the differentiation of *Rhizobium* strains, were suggestive of high diversity among isolates of the bacteria. Both primers had similarity levels of 49 % as indicated in the dendrogram where two major clusters were joined. The dendrograms derived from both the *nif*-directed and arbitrary primer were similar and this consistency provides basis that strain groupings does reflect true relationship among *Rhizobium* strains investigated. There were however some differences exhibited by the two primers. With the *nif*-directed RPO1 primer, a total number of five groups from subcluster BII were generated. Additionally, four groups of possible duplicates of isolates were found.

With respect to the arbitrary RPO4 primer, a total number of six groups of subcluster BII were formed. Also, contrary to that of RPO1, only one set of possible isolates was indicated. Most interestingly, some *Rhizobium* isolates nodules used for the inter-cultivar studies for all the thirteen lima bean cultivars were identified in similar

subclusters for both primers (RPO1 and RPO4). This can be suggestive of the wider ability or applicability to identify *Rhizobium* strains by both primers. In addition, two distinct *Rhizobium* isolates D1 and D3 formed in main cluster A and D4 and D5 in main cluster B using the RPO4 can be suggestive of unique or elite *Rhizobium* strains obtained from lima bean cultivar A4. From the same cultivar A4, D1, D2 and F5 were identified in main cluster A in RPO1. These isolates for cultivar A4 could be vital in improving nitrogen fixing tendencies in the lima bean plant.

## REFERENCES

- Adiguzel, A. (2006). Molecular Characterization of Thermophilic Bacteria Isolated From Water Samples Taken From Various Thermal Plants. PhD Thesis, Ataturk University, Graduate School at Natural and Applied Sciences, Erzurum, Turkey.
- Ampomah, O. Y., Ofori-Ayeh, E., Solheim, B. and Svenning, M. M. (2008). Host range symbiotic effectiveness and nodulation competitiveness of some indigenous cowpea bradyrhizobia isolates from the transitional savanna zone of Ghana. *African Journal of Biotechnology* 7(8):988-996.
- Ando, S. and Yokoyama, T. (1999). Phylogenetic analyses of *Bradyrhizobium* strains nodulating soybean (*Glycine max*) in Thailand with reference to the USDA strains of *Bradyrhizobium*. *Canadian Journal Microbiology*, 45 pp 639-645.
- Caetano-Anolles, G., Bassam, B.J. and Gresshoff, P.M. (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology*, 553-557.
- Chen, L.S., Figueredo, A., Pedrosa, F.O. and Hungria, M. (2000). Genetic characterisation of soyabean rhizobia in Paraguay. *Applied Environmental Microbiology*. 66 p 5099-5103.
- de Bruijn, F. J. (1992). Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergeneric Consensus) sequence and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied Environmental Microbiology* 58:2180-2187.
- Fening, J. O., Sessitsch, A., Offei, S. K. And Danso, S. K. A. (2004). Genomic heterogeneity within cowpea bradyrhizobia isolated from Ghanaian soils. *West African Journal of Applied Ecology* 6:20-29.
- Harrison, S.P., Mytton, L.R., Skot, L., Dye, M. and Cresswell, A. (1992). Characterisation of *Rhizobium* isolates by amplification of DNA polymorphism using random primers. *Canadian Journal of Microbiology* 38, 1009-1015.
- Kundu, S. and Dudeja, S. (2008). Molecular diversity, effectiveness and competitiveness of indigenous rhizobial population infecting mung bean *Vigna radiata* ( L. Wilczek) under semi-arid conditions. *Indian Journal Microbiology* V 48(4): 445-452.
- McInnes, A., Thies, J.E., Abbott, L.K. and Howieson, J.G. (2004). Structure and diversity among rhizobial strains, populations and communities. A review. *Soil Biology Biochemistry* 36:1295-1308.

- Mpepereki, S., Makonese, F. And Wollum, A. G. (1997). Physiological characterisation of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. *Symbiosis* 22:275-292.
- Richardson, A.E., Viccars, L.A., Watson, J.M. and Gibson, A.H. (1995). Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biology and Biochemistry*. 27, 515-524.
- Santos, J.O., Araújo, A.S.F., Gomes, R.L., Lopes, A.C. and Figueiredo, M.V. (2008). Rhizobia- *Phaseolus lunatus* Symbiosis: Importance and diversity in Tropical soils. A review. *Dynamic Soil, Dynamic Plant* 2 (2) pp 56-60.
- Schofield, P.R. and Watson, J.M. (1985). Conservation of nif- and species specific domains within repeated promoter sequences from fast growing *Rhizobium* species. *Nucleic Acid Research* 18, 7213-7218.
- Sikora, S., Dedzepovic, S. and Bradic, M. (2002). Genomic fingerprinting of *Bradyrhizobium japonicum* isolates by RAPD and rep-PCR. *Microbiological Research*, 157: 213-219.
- Wang, F.Q., Wang, E.T., Zhang, Y.F. and Chen, W.X. (2006). Characterization of rhizobia isolated from *Albizia spp.* in comparison with microsymbionts of *Acacia spp.* and *Leucaena leucocephala* grown in China. *Systematics and Applied Microbiology* 29, 502-517.
- Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* 18, 7213-7218.
- Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*. 18, 6531-6535.

## CHAPTER SIX

### GENERAL CONCLUSIONS AND RECOMMENDATIONS

#### 6.1 Conclusions

At the end of the research, the following conclusions were drawn from the three major experiments undertaken on the thirteen lima bean cultivars.

1. Majority of the thirteen lima bean cultivars grown showed relatively high germination percentages in the Coastal Savannah Agro-ecological zone where the experiment was undertaken.
2. Significant differences were observed in all morphological quantitative traits (leaflet length, leaflet width, pod length, pod width, seed length, seed width, seed weight/10 seeds and days to 50% emergence) studied and these contributed highly to divergence among the lima bean cultivars.
3. Morphological qualitative traits differed greatly in terms of pod beak shape, leaf shape and seed coat colour and seed colour pattern.
4. Majority of the lima bean cultivars were of the same morphotype in the major clusters identified with the exception of lima bean cultivars A2 and M4.
5. Significant differences in mean nodule numbers, effective and non-effective nodules did not exist. However, GH 1714 showed to be a superior cultivar with respect to nodule numbers and effective nodules.
6. Similarities and differences were observed within and between the lima bean cultivars with respect to nodule bacteria isolates. Possible duplicates of *Rhizobium* isolates existed both within and between lima bean cultivars.

## 6.2 Recommendations

Further studies should be conducted on agronomic traits and improvement in cultivars of lima bean.

1. The number of primers used to differentiate *Rhizobium* strains should be increased.
2. Reduction in annealing temperature used for ERIC primer may enhance its amplification of *Rhizobium* strains.
3. Cross inoculation studies on *Rhizobium* strains for cultivars with superior performances should be conducted to improve nitrogen fixing potentials.
4. Phylogenetic studies should be performed on *Rhizobium* strains to determine the groupings with other well known *Rhizobium* strains which can lead to a novel *Rhizobium* strain being identified.

**APPENDICES****APPENDIX 1.** Different parameters assessed for qualitative traits

<b>TRAITS</b>	<b>DIFFERENT PATHS OF SCORING</b>
Growth habit	Determinate (1) , indeterminate (2)
Main stem Pigmentation	No pigmentation(0), localised to nodes (3), extensive (5) , almost solid (7)
Leaflet shape	Round (1), ovate (3) , ovate lanceolate (5) , lanceolate (7) , linear lanceolate (9)
Clear markings along fully developed leaf (colour)	Absent (0) , narrow (3) , wide (7)
Flower wings colour	White (1) , light pink (3) , deep pink (5) , violet (7)
Flower standard colour	White (1), light pink (3), deep pink (violet) (5) , purple (7)
Flower keel colour	Greenish (1) , tinged (2)
Wing opening (freshly opened flowers)	Parallel wings, closed (0), intermediate opening (3), wings widely diverging (7)
Standard hairiness	Absent (0), sparsely hairy on tip (3) moderately hairy (5), densely hairy all over (7)
Seed background (lightest colour)	Green (1) , white (2) , grey (3) , yellow (4) , buff (5) , light brown(6) , maroon(7) , pink(8) , red (9) , dark red(10) , purple red (11) , black (12)
Pattern colour	No pattern (0) , green (1) , light brown (2) , dark brown(3),

	red (4 ), purple red (5), black (6)
Pod colour (mature Pod)	Brown (1 ), brown with red spotting or mottling (2 ), red, purple or black (3)
Pod beak shape	Short beak (1), medium length beak (2), long beak (3 ), thick beak (4)
Pod curvature	Straight (0 ), slightly curved (3 ), curved (5 )
Seed Second pattern colour (darkest pattern of colour)	Monocoloured pattern (0), dark red (1), purple red (2), black (3 )
Seed coat pattern	No pattern (0), pattern around eye only (1), eye distinct with few specks on body (2), eye distinct with many specks on body (3), eye distinct with blotches on <50% of body (4), eye distinct with blotches on >50% of body (5), eye linked to other parts of pattern, blotches in hilar region, some specks maybe present (6), Eye linked to other parts of pattern, blotch covering hilar region and front side, some specks maybe resent (7), eye linked to other parts of pattern, blotch covering hilar region, back, front and keel, specks present on rest of body (8), eye linked to other parts of patern, blotch in hilar region, body ha bands radiating from hilar region (9), eye linked to other parts, blotch in hilar region, body has bands oriented radially and transversely (10), body sparsely mottled (11), body

	moderately mottled, some jointed to form a blotch (12), body intensely mottled, seed background is almost hidden (13)
--	---

**APPENDIX 2. Different Parameters assessed for morphological quantitative traits**

<b>TRAITS</b>	<b>DIFFERENT PATHS OF SCORING</b>
Leaflet length	Measured on the terminal leaflet of third trifoliolate leaf from pulvinus to leaf tip.
Leaflet width	Measured on the terminal leaflet of third trifoliolate leaf according to width.
Pod length	Average of 20 randomly chosen mature pods.
Pod width	Of the largest width from 20, randomly chosen, mature pods.
Seed length	Average of 10 ripe seeds chosen at random
Seed width	Average of 10 ripe seeds chosen at random.
Days of 50% emergence	Average number of seedlings to germinate in each replicate.
Seed weight	Average of 10 ripe seeds weighed

**APPENDIX 3. Similarity matrix of thirteen lima bean accessions based on sixteen (16) qualitative morphological traits**

<b>ACC</b>	<b>A1</b>	<b>A2</b>	<b>A3</b>	<b>A4</b>	<b>M1</b>	<b>M2</b>	<b>M3</b>	<b>M4</b>	<b>M5</b>	<b>GH</b> <b>1714</b>	<b>GH</b> <b>1719</b>	<b>GH</b> <b>1726</b>	<b>GH</b> <b>1737</b>
<b>A1</b>													
<b>A2</b>	.92												
<b>A3</b>	0.77	0.83											
<b>A4</b>	0.77	0.83	1.00										
<b>M1</b>	1.00	0.92	0.77	0.77									
<b>M2</b>	1.00	0.92	0.77	0.77	1								
<b>M3</b>	0.77	0.83	1	1	0.77	0.77							
<b>M4</b>	0.79	0.71	0.69	0.69	0.79	0.79	0.69						
<b>M5</b>	1.00	0.92	0.77	0.77	1.00	1.00	0.77	0.79					
<b>GH</b> <b>1714</b>	1.00	0.92	0.77	0.77	1.00	1.00	0.77	0.79	1.00				
<b>GH</b> <b>1719</b>	0.77	0.83	1.00	1.00	0.77	0.77	1.00	0.69	0.77	0.77			
<b>GH</b> <b>1726</b>	0.77	0.83	1.00	1.00	0.77	0.77	1.00	0.69	0.77	0.77	1.00		
<b>GH</b> <b>1737</b>	0.77	0.83	1.00	1.00	0.77	0.77	1.00	0.69	0.77	0.77	1.00	1.00	

**APPENDIX 4.** ANOVA for Days to 50% emergence (average germination)

Source of variation	Df	s.s	m.s	v.r	f pr.
Block	2	0.41121	0.2056	3.02	
Accessions	12	1.20037	0.10003	1.47	0.204
Residual	24	1.63621	0.06818		
Total	38	3.24778			

**APPENDIX 5.** Leaflet length

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	1.9163	0.9581	2.63	
Accessions	12	99.0623	8.2552	22.69	< 0.01
Residual	24	8.7304	0.3638		
Total	38	109.709			

**APPENDIX 6.** ANOVA for Leaflet width

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	0.499	0.2495	1.16	
Accessions	12	40.6467	3.3872	15.81	< 0.01
Residual	24	5.141	0.2142		
Total	38	46.2867			

**APPENDIX 7. ANOVA for Pod length**

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	9.574	4.787	2.62	
Accessions	12	500.37	41.697	22.85	< 0.01
Residual	24	43.799	1.825		
Total	38	553.743			

**APPENDIX 8. ANOVA for Pod width**

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	0.002051	0.001026	0.22	
Accessions	12	1.764103	0.147009	31.71	
Residual	24	0.111282	0.004637	< 0.01	
Total	38	1.877436			

**APPENDIX 9. ANOVA for Seed length**

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	0.38513	0.19256	5.63	
Accessions	12	0.57692	0.04808	1.4	0.231
Residual	24	0.82154	0.03423		
Total	38	1.78359			

**APPENDIX 10.** ANOVA for Seed width

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	0.08359	0.04179	4.02	
Accessions	12	0.22256	0.01855	1.78	0.11
Residual	24	0.24974	0.01041		
Total	38	0.5559			

**APPENDIX 11.** ANOVA for Seed weight/ 10 seeds

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	0.026296	0.013103	3.76	
Accessions	12	1.122725	0.09356	26.84	<0.001
Residual	24	0.083668	0.003486		
Total	38	1.232599			

**APPENDIX 12.** ANOVA for Germination Percentage

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	3913.4	1956.7	2.83	
Accessions	12	11406.4	950.5	1.38	0.243
Residual	24	16567.4	690.3		
Total	38	31887.2			

**APPENDIX 13.** ANOVA for Mean fresh shoot weight

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	694.21	347.11	4.16	
Accessions	12	2173.01	181.08	2.17	0.051
Residual	24	2002.27	83.43		
Total	38	4869.5			

**APPENDIX 14.** ANOVA for Mean dry shoot weight

Source of variation	Df	s.s	m.s	v.r	f pr.
Block	2	10.03	5.02	0.17	
Accessions	12	790.94	65.91	2.26	0.043
Residual	24	700.83	29.2		
Total	38	1501.8			

**APPENDIX 15.** ANOVA for Mean fresh root weight

Source of variation	Df	s.s	m.s	v.r	f pr.
Block	2	50.826	35.413	3.27	
Accessions	12	171.483	14.29	1.84	0.099
Residual	24	186.79	7.783		
Total	38	409.099			

**APPENDIX 16.** ANOVA for Mean dry root weight

Source of variation	Df	s.s	m.s	v.r	f pr.
Block	2	3.068	1.534	0.85	
Accessions	12	29.249	2.437	1.35	0.256
Residual	24	43.322	1.805		
Total	38	75.638			

**APPENDIX 17.** ANOVA for Total shoot dry matter

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	112441	56220	2.07	
Accessions	12	736907	61409	2.26	0.043
Residual	24	652136	27172		
Total	38	1501484			

**APPENDIX 18.** ANOVA for Mean nodule number per plant

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	15700	7850	1.22	
Accessions	12	112328	9361	1.45	0.21
Residual	23	154573	6441		
Total	38	282601			

**APPENDIX 19.** ANOVA for Mean effective nodules

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	231	115	0.08	
Accessions	12	225339	1878	1.36	0.253
Residual	24	33265	1386		
Total	38	56035			

**APPENDIX 21.** ANOVA for Mean non-effective nodule

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	7342	3671	1.18	
Accessions	12	60439	5037	1.62	0.151
Residual	24	74496	3104		
Total	38	142277			

**APPENDIX 22.** ANOVA for Mean fresh nodule weight

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	1.0507	0.5253	1.9	
Accessions	12	170.104	14.1753	51.38	<0.01
Residual	24	6.6216	0.2759		
Total	38	177.7762			

**APPENDIX 23.** ANOVA for Mean dry nodule weight

Source of variation	df	s.s	m.s	v.r	f pr.
Block	2	0.8183	0.4092	1.67	
Accessions	12	126.6009	10.5501	43.02	< 0.01
Residual	24	5.8859	0.2452		
Total	38	133.3052			

**APPENDIX 24.** Nucleotide lengths of primers used

Primer	Sequence	Length
RPO1	5-AATTTTCAAGCGTCGTGCCA-3	20 nucleotides
RPO4	5-GGAAGTCGCC-3	10 nucleotides
ERIC 1	3-CACTTAGGGGTCCTCGAATGTA-5	22 nucleotides
ERIC 2	5-AATGAAGTGACTGGGGTGAGCG-3	22 nucleotides