Variation in Root Nodulation Traits among Parental Genotypes and Segregating F_2 Pigeonpea Plant Populations

By

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A dissertation submitted in fulfilment of the requirements for the degree of Master of Science in Agriculture (Crop Science) (MScAgric)

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November 2022

Abstract

Pigeonpea (*Cajanus cajan L. Millsp.*) is an important grain legume that originated in the Indian sub-continent. In South Africa, it is grown either as single plants or as a hedge, mainly in Kwazulu-Natal, Limpopo, and Mpumalanga Provinces. The crop provides highly nutritious food for human consumption and fixes considerable amounts of atmospheric nitrogen (N) thus contributing to the improvement of soil fertility. Root nodulation in pigeonpea is an integral part of the symbiotic process that results in N fixation thus contributing to the productivity of the crop. Currently, there are no reports that determined the genetics of root nodulation in pigeonpea. Therefore, this study was designed to determine the mode of inheritance for selected root nodulation traits.

The experiment was conducted in a greenhouse at the Agricultural Research Council-Plant Health and Protection (ARC-PHP). The average day and night temperatures in the greenhouse were 28°C and 15°C- respectively, with 14 hours of daylight. A randomized complete block design with two replications was used to set up the experiment. Six pigeonpea genotypes were used in the study together with thirty-six rhizobia strains originating from soil that was collected from diverse locations across South Africa. The nodulation variables which were measured included leaf chlorophyll content (LCC), shoot dry weight (SDW), root dry weight (RDW) and nodule dry weight (NDW). The data sets for each of these quantitative variables were subjected to the analysis of variance followed by mean separation using the least significant difference at the 5% probability level using statistical software (Statistix 10.0), and subsequently to analysis of goodness of fit test using standard Chi-square procedures for various Mendelian ratios.

The results revealed that the method which was employed to phenotype both the parental genotypes and the F_2 progenies was effective and enabled a distinction between the phenotypic classes among the treatments hence a rapid, simple technique to identify contrasting parental genotypes for specific nodulation traits for use in the subsequent genetic study. The GGE biplot analysis revealed that the rhizobial strains ‘R24’, ‘R28’, ‘R31’ and ‘R34’ were clustered around the origin. In contrast, the rhizobial strains ‘R7’, ‘R8’, ‘R10’, ‘R27’ and ‘R29’were positioned far away from the origin. The biplot also indicated that the pigeonpea parental genotypes (coded as environment scores), ‘Gen-1’ (E1), ‘Gen-2’ (E2), ‘Gen-3’ (E3) and ‘Gen-5’ (E5) were separated by acute angles between them and grouped in the same quadrant. The ‘which-won where’ biplot explained 56.05% total variation of which PC1 and PC2 accounted for 29.40% and 26.65% of the total variation, respectively. The results also revealed that the rhizobial strains (depicted as

genotypes) on the vertices of the polygon 'R10', 'R11', 'R27' and 'R35' performed best with the pigeonpea parental genotypes (depicted as environments) 'Gen-2' (E2), 'Gen-5' (E5), 'Gen-4' (E4) and 'Gen-6' (E6), respectively. The genotype 'Gen-5' (E5) showed the longest vector line, suggesting a high discriminating ability.

The frequency distribution curve for the F_2 plant population that was derived from the cross P_6 x P_1 showed approximately a normal distribution curve but with a slight skew to the right suggesting the presence of epistatic gene action for the LCC trait. The segregation ratio of 9 high:7 low chlorophyll content in the cross P_4 x P_2 (P-04-SST x P-02-DC) suggested duplicate recessive epistasis in which there is complete dominance at both gene pairs; but, when either gene is homozygous recessive, it masks the effect of the other gene. For SDW, the results also confirmed that the 9:7 ratio was the best fit. The segregation pattern, based on the LCC, of the F_2 population in the cross P-04-SST x P-02-DC, best fitted the 9:7 ratio. The results showed that the 9:7 ratio was generally predominant for the traits that were studied thus indicating a high probability that more than one gene, with epistasis are involved in their genetic control. The LCC showed a weak negative correlation with each of NDW and SDW in the F_2 progenies that were derived from P-04-SST x P-02-DC. However, there was a positive but weak correlation between NDW and SDW in this set of progenies. In contrast, there was a highly significant ($P < 0.01$) positive correlation between NDW and SDW in 'cross 2'. The LCC was positively correlated to both NDW and SDW in the F_2 progenies that were derived from the cross involving P_6 x P_1. It is recommended that future studies should include the determination of heritability values that can be used in breeding programs aimed at the genetic improvement of N fixation in pigeonpea. It may also be necessary to combine classical Mendelian genetics with modern genomics tools to gain a better understanding of the complex nature of N fixation in pigeonpea as well as its genetic manipulation.

Key words: pigeonpea, root nodulation trait, Mendelian ratio, rhizobia strain, inheritance.

Manuscripts in preparation from this dissertation:

1. Mthombeni T, Gerrano AS and Gwata ET (2022). A genetic study of leaf chlorophyll score in F_2 pigeonpea (*Cajanus cajan*) germplasm. ***Journal of Heredity***.
2. Mthombeni T, Gerrano AS and Gwata ET (2022). Inheritance of nodule dry weight in pigeonpea. ***Euphytica***.

Dedication

This dissertation is dedicated to the memory of my beloved mother Girly Tintswalo Mthombeni who to me was the pillar of my strength.

Declaration

I, Mthombeni Tinyiko, hereby declare that this dissertation, for the Master of Science in Agriculture (Crop Science) in the Department of Plant and Soil Sciences at the University of Venda, hereby submitted by me, has not previously been submitted for a degree at this or any other University. It is my own work, design and execution. All reference material contained therein has been duly acknowledged.

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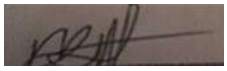
Signature: …  …… Date: …12/11/2022 …...

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Acknowledgements

I would like to acknowledge and thank my supervisors, Prof. E.T. Gwata who has been the best supervisor for his advice, insightful criticisms, patience and spending hours going through the dissertation. I would like also to thank my co-supervisor Dr. A.S. Gerrano for giving me opportunity to be part of research team and also his insightful suggestions on how to conduct a research trial. I would like to acknowledge and give big thanks to Ms. F Bopape for her contribution throughout the trial, by helping me to set up my laboratory and the greenhouse trial to a successful one. I would also like to acknowledge the Agricultural Research Council for funding this research, whose financial support for this project was seriously needed and deeply appreciated.

Table of Contents

List of Abbreviations

ANOVA = analysis of variance

ARC - PHP = Agricultural Research Council – Plant Health and Protection

BNF = biological nitrogen fixation

FAO = Food and Agriculture Organization

GGE = genotype plus the genotype by environment

ICRISAT = International Crops Research Institute for the Semi-Arid Tropics

LCC = leaf chlorophyll content

LCS = leaf color score

LSD = least significant difference

N = atmospheric nitrogen

NDW = nodule dry weight

RDW = root dry weight

SARC = South African Rhizobium Collection

SDW = shoot dry weight

YM-CR = yeast mannitol congo red

List of Figures

List of Tables

List of Appendices

1.0 CHAPTER ONE: INTRODUCTION

1.1 Introduction

Pigeonpea (*Cajanus cajan L. Millsp.*) is an important grain legume that originated in the Indian Sub-continent (Zavinoni and Sagbadja, 2019). Currently, the crop is produced in many countries (Table 1.1). The crop provides highly nutritious food for human consumption (Amarteifio *et al*., 2002) and fixes considerable amounts of atmospheric nitrogen (N) (Mapfumo *et al*., 1999), thus contributing to the improvement of soil fertility. Lack of improved pigeonpea varieties, the declining soil fertility and high fertilizer costs are some of the major limiting factors in pigeopnpea production by smallholder farmers (Zavinoni and Sagbadja, 2019).

Requirements for N exceed any other major nutrients and rarely do soils have enough of this nutrient to produce high grain yield (Mkandawire *et al*., 1998; Woldeyohannes *et al*., 2007). However, inorganic N fertilizers are not readily affordable for most smallholder farmers particularly in sub-Saharan Africa, indicating that alternative sources of N are necessary in smallholder cropping systems. Biological nitrogen fixation (BNF), which is a microbiological process that converts atmospheric N into organic plant-usable forms of bio-fertilizers, is a viable alternative for smallholder farmers.

Leguminous crops such as cowpea (*Vigna unguiculata*), soybean (*Glycine max*) and pigeonpea symbiotically interact with soil rhizobia during the BNF process. In the field, pieonpea can fix up to 235 kg/ha of N and produces relatively more N per unit area from plant biomass than most legumes (Egbe and Anyam, 2012). The pigeonpea plant also nodulates promiscuously (i.e. with naturally occurring rhizobia in the soil) (Bopape et al., 2021). Furthermore, even though pigeonpea exhibits high symbiotic capacity with rhizobia, it is necessary to identify the efficient rhizobial strains that can assist to attain high yield (Saxena *et al.,* 2002). Moreover, the identification of efficient inoculants that can be commercialized will be a useful contribution to efforts that are aimed at increasing the productivity of the crop in many parts of the world particularly in Southern Africa. However, the symbiotic interaction between the host plant and the micro-symbiont is a complex process involving multiple genes from both sides and is poorly understood (*Wang et al*., 2018). This is partly because studies that are aimed at elucidating the host genotype x rhizobial strain often involve mixed rhizobial stains (in the soil) against a promiscuous host plant.

Table 1.1 Major pigeonpea producing countries (FAO, 2019).

Country	Production (M t)	Average yield (kg/ha)	Proportion in world production (%)
India	3.0	652.0	67.4
Myanmar	0.6	921.0	12.8
Malawi	0.3	1268.0	6.2
Tanzania	0.2	855.0	5.3
Kenya	0.2	749.0	4.6
Haiti	0.1	803.0	2.0
Dominican Republic	0.0	1097.0	0.6
Nepal	0.0	905.0	0.4
World	4.4	718.0	100.0

1.2 Problem statement

Root nodulation in pigeonpea is an integral part of the symbiotic process that results in N fixation thus contributing to the productivity of the crop by optimizing available N. However, the variation and genetic control of root nodulation in pigeonpea has not been investigated adequately. Studies aimed at the investigation of this complex interaction are often confounded by using mixed rhizobial strains (in field soil) on a promiscuous host genotype. A dearth of knowledge in terms of the mode of inheritance of root nodulation traits in pigeonpea makes it difficult to design pigeonpea genetic improvement programs aimed at enhancing the N fixation process. Consequently, the productivity of the crop tends to attain a plateau due to limited genetic improvement and cultivar genetic gains.

1.3 Justification of the study

The determination of the variation and genetic control of root nodulation traits will facilitate the genetic improvement of pigeonpea aimed at enhancing N fixation, hence grain yield (or productivity) of the crop, providing opportunity for plant breeders to develop new and improved cultivars with desirable characteristics. Probably, this will increase the adoption of pigeonpea by growers, thus also increasing the production of the crop. In addition, the resultant improved pigeonpea genotypes will benefit end-users.

1.4 Aim and objectives of the study

The aim of this study was to evaulate the productivity of pigeonpea. The specific objectives of the study were to determine:

(i) genetic variation in selected root nodulation traits among pigeonpea parental lines

(ii) genetic variation in selected root nodulation traits among segregating F_2 pigeonpea plant populations

(iii) mode of inheritance for selected root nodulation traits among segregating F_2 pigeonpea plant populations.

1.5 Hypothesis

The study tested the null (H_O) hypotheses that:

(i) genetic variation in selected root nodulation traits among pigeonpea parental lines was not significant

(ii) genetic variation in selected root nodulation traits among segregating F2 pigeonpea plant populations was not significant.

(iii) the mode of inheritance for selected root nodulation traits among segregating F_2 pigeonpea plant populations was not controlled by a few genes.

1.6 Dissertation outline

This dissertation is arranged into six chapters. The first chapter introduces the problem of root nodulation in pigeonpea and outlines the objectives of the study as well as the hypotheses that were tested in the study. Chapter two focuses on the literature review of relevant aspects of root nodulation in pigeonpea and is followed by Chapter three covering the materials and methods that were used in the study. Chapter four and chapter five focus on the results and discussion of the results, respectively. The last chapter (six) summarizes the conclusions on the findings of the study and makes some tentative recommendations. The references that were cited throughout the document are listed at the end of the document. Partly because of the closeness of the various aspects of root nodulation in pigeonpea and similar legumes, inevitable overlaps in some of the concepts or citations occurred in this dissertation.

2.0 CHAPTER TWO: LITERATURE REVIEW

2.1 Origin, biology, and distribution of pigeonpea

Pigeonpea (*Cajanus cajan*) is a diploid species (2n = 2x = 22) which originated in the Indian sub-continent (Pazhamala *et al*., 2017; Zavinoni and Sagbadja, 2019). It is a perennial legume crop that is adaptable to a range of soil types, temperature, and rainfall. It can grow up to four meters in height, and it has a deep taproot system extending up to two meters (Kumar and Naik, 2017). It has zygomorphic flowers that are yellow in color with some variation (Kumar and Naik, 2017).

Currently, it is cultivated in several countries including Malawi and South Africa in southern Africa. In South Africa, it is grown either as single plants or as a hedge. Long-duration unimproved pigeonpea landraces are grown as shade plants in home gardens, especially in KwaZulu-Natal, Limpopo, and Mpumalanga Provinces (Mathews and Saxena, 2005). Currently, there are no commercial cultivars of pigeonpea that are cultivated in South Africa. Nonetheless, the small-scale production and field trials in the country have indicated that there is high potential for the crop (Gwata and Shimelis, 2010).

2.2 Uses of pigeonpea

Pigeonpea is a versatile crop which is grown primarily for human consumption. The grain of pigeonpea contains about 20-21 % protein, 1.2 % fat, 65 % carbohydrate, and 3.8 % ash (Sharma *et al.,* 2011). Therefore, pigeonpea is often considered as an inexpensive source of protein particularly for poor rural communities in Africa and Asia. Pigeonpea can also be used as a livestock feed (Rao *et al*., 2002). Pigeonpea is also used as a traditional medicine mostly in India and China where the flowers are used for treating illness such as bronchitis, coughs, and pneumonia (Saxena *et al*., 2010). The pigeonpea leaves can be used for curing sores, wounds, abdominal tumours, and diabetes (Li *et al*., 2001). The grain of pigeonpea is often traded in international markets thus also generating household income (Gwata and Shimelis, 2010)

2.3 Agronomic characteristics of pigeonpea

Pigeonpea landraces are characterized by late maturity, and low grain yield (Gwata and Shimelis, 2010). The landraces are tolerant to droughts that occur frequently in some of the production areas in Africa and Asia (Gwata and Siambi, 2009). However, pigeonpea improved cultivars fall into distinct categories based on their duration to maturity (Table 2.1). Short-duration and medium-duration types require 90 days and 91-150 days to mature respectively (Gwata and Shimelis, 2010). The long-duration types require >150 days to attain maturity. However, the classification is dependent on the specific agro-ecological conditions in the production area which in turn are defined by the altitude, temperature, latitude, and photoperiod.

The average grain yield obtained by pigeonpea farmers in most African countries is generally low. In Tanzania, growers obtained 0.4 t/ha (Mligo and Myaka, 1994) but 0.8 t/ha in 2013 (Gangarao et al., 2016). In Malawi about 1.33 t/ha of pigeonpea grain yield was reported (Kaoneka *et al*., 2016). In terms of N fixation into the soil, pigeonpea can contribute up to 234 kg/ha (Egbe and Anyama., 2012). Similar observations were also reported in other studies (Mapfumo *et al*., 1999). However, this productivity depends on the genotype and environmental factors (Myaka *et al*., 2006; Egbe *et al*., 2007).

Among the environmental factors, the soil conditions such as pH (Ferguson *et al*, 2013), presence of compatible rhizobia (Wang *et al*., 2018), moisture (Sprent, 2014), and temperature (Montanez *et al*., 1995) are critical for the formation of effective nodules and hence N fixation. Low pH conditions affect the rhizobia attachment to the root hair and colonization (Vargas and Graham, 1988), hence, reduces nodule formation (Ferguson *et al*., 2013). N fixation at low pH (pH 4.7 and 5.4) was significantly lower than at the higher pH levels (pH 6.2 and 7.0) (Schubert *et al*., 1990). Compatible rhizobia is important in the establishment of a successful process of symbiotic development (Wang *et al*., 2018). Water stress affects the formation and longevity of leguminous root nodules (Sprent, 2014), particularly where nodules occur around soil surface (Gan *et al*., 2008). High temperatures (≥35°C) decrease N fixation (Montanez et al., 1995), since, under such conditions, symbiotic processes including nodule formation, development, and activity, are inhibited (Ofosu-Budu et al., 1992).

Table 2.1 Examples of improved pigeonpea cultivars from distinct maturity categories.

Duration type	Example of Genotype	Origin	Notes	Reference (s)
Short (approx. 90 d)	ICPL 87091	India (ICRISAT)	Released for commercial production in 2003; cultivated mainly in India	Gwata *et al*., 2007
Medium (approx. 150 d)	ICEAP 00068	Kenya (ICRISAT)	Released for commercial production in 2003; cultivated mainly in Malawi / Tanzania	Silim *et al*., 2005
	ICEAP 00020	Kenya (ICRISAT)	Released fro commercial production 2003; cultivated mainly in Malawi / Tanzania	Gwata *et al*., 2007
Long (> 150 d)	ICEAP 00040	Kenya (ICRISAT)	Released for commercial production in 2003; cultivated mainly in Malawi / Tanzania / Mozambique; resistant to Fusarium wilt.	Silim *et a*l., 2005

ICRISAT= International Crops Research Institute for the Semi-Arid Tropics; ICPL 87091= Short duration type; ICEAP 00068= Medium duration type; ICEAP 00020= Medium duration type; ICEAP 00040= Long duration type

2.4 Constraints to pigeonpea production

The production of pigeonpea is constrained by a range of abiotic and biotic factors, which significantly reduce the grain yield (Brink *et al*., 2006). For instance, *Fusarium* wilt is a serious disease in many countries in the Eastern and Southern African region (Silim *et al*., 1995). Furthermore, pigeonpea is susceptible to a wide range of insect pests such as *Helicoverpa armigera* that attack the crop at both the vegetative and reproductive stages (Shanowe *et al*., 1999). Various abiotic stresses such as moisture stress, temperature, photoperiod, and mineral stress also negatively affect pigeonpea production (Choudhary *et al*., 2011). Yield loss (up to ≥50%) was previously reported (Gwata, 2010; Dialoke *et al*., 2014).

2.5 Genetics of root nodulation

To date, there are no documented studies that determined the genetics of root nodulation in pigeonpea. However, in soybean (*Glycine max L*), the segregation pattern of the nonpromiscuous root nodulation trait which was studied using nodule dry weight as an indicator of N fixation effectiveness and was reported to be partially dominant (h/d = 0.37) and controlled by four loci (Gwata *et al.,* 2004). The study utilized six plant populations among which four (i.e., the two first backcrosses to each parent and the F_2 generations) were segregating for the promiscuous trait and were each inoculated with a single rhizobial strain that would distinguish between promiscuous and nonpromiscuous types of nodulation. However, in the same study, the leaf colour score (as an additional indicator of N fixation effectiveness), the researchers reported that nonpromiscuity was almost completely dominant (h/d = 0.74) and controlled by two loci. The study concluded that promiscuous root nodulation was controlled by a few genes and nonpromiscuity was almost completely dominant. In another study involving nonnodulating and nodulating parental genotypes of peanut (*Arachis hypogaea L*), three genes were found to control nodulation (Gallo-Meagher *et al*., 2001). Similarly, in chickpea (*Cicer arientinum*), recessive alleles at three different loci governed root nodule formation (Davis *et al*., 1986).

Apart from the utilization of single rhizobial strains for simultaneous inoculation of the segregating plant populations, other approaches interested in determining the genetic control of traits of agronomic interest such as pod shattering in soybean, successfully used F_2 segregating generations in combination with X^2 tests for the goodness-of-fit of observed F_2 segregation data to the expected Mendelian ratios (Nevhudzholi *et al*., 2020; Mohammed *et al*., 2014; Singh *et al*., 2011; Bhor *et al*., 2004). Therefore, there is merit in using segregating F_2 plant populations (i.e., derived from a cross between contrasting parents for the given root nodulation trait, (see Gwata *et al*., 2003) to investigate the genetics of root nodulation traits in pigeonpea. Moreover, the root nodulation trait of interest in such segregating plant populations should be induced by single rhizobial strains because of the various reasons including promiscuity in root nodulation (Gwata *et al*., 2005), and competition among different strains (Mendoza-Suárez *et al*., 2021; Ji *et al*., 2017). Inoculation with a single strain in studies aimed at the genetics of root nodulation were reported recently (Agoyi *et al*., 2016).

Table 2.2 Examples of genetic studies of traits using segregating F_2 plant populations in legume species.

Legume species	Trait	Reference
Common bean (*Phaselous vulgaris*)	seed shiness; flower color	Koening and Gepts, 1989
Chickpea (*Cicer arietinum*)	pale green foliage; bronze foliage	Kazan et al., 1993
Birds foot trefoil (*Lotus japonicus*)	number of seeds per plant; leaf color; flowering period	Jiang and Gresshoff, 1997
Lentil (*Lens culinaris*)	penduncle pubescence; stem pigmentation; cotyledon color; seed coat pattern	Sarker et al., 1999
Cowpea (*Vigna anguiculata*)	pod pigmentation	Mustapha and Singh, 2008
Pigeonpea (*Cajanus cajan*)	*Fusarium wilt*	Odeney et al., 2009
Pigeonpea (*Cajanus cajan*)	number of pods per plant	Parekh et al., 2016
Faba bean (*Vicia faba L.*)	flower color	Khazaei et al., 2018
Cowpea (*Vigna anguiculata*)	nodule color; nodule fresh weight; nodule number; shoot dry weight	Ohlson et al., 2018
Chickpea (*Cicer arietinum*)	number of seeds per pod	Deokar et al., 2019
Cowpea (*Vigna anguiculata*)	seed color; pod shape; seed weight	Amusa et al., 2019
Chickpea (*Cicer arietinum*)	duration to flowering; seed size	Sundaram et al., 2019
Soybean (*Glycine max*)	pod shattering	Nevhudzholi et al., 2020
Pigeonpea (*Cajanus cajan*)	seed dimensions	Bohra et al., 2020
Hyacinth bean (*Lablab purpureus*)	photoperiod sensitivity	Basanaouda et al., 2022

Table 2.3 Examples of nitrogen fixation attributes of legume species that were used in previous studies.

Legume species	Nitrogen fixation attribute	Reference
Tepary bean (*Phaseolus acutifolius*)	nodule number; nodule dry weight; pod number; pod dry weight	Shisanya, 2003
Soybean (*Glycine max*)	nodule dry weight; leaf color score	Gwata *et al*., 2004
Pigeonpea (*Cajanus cajan*)	pods per plant; 100-seed weight per plant; seed yield per plant	Bhakaran *et al*., 2007
Cowpea (*Vigna anguiculata*)	numbet of pods; seed per pod; branches per plant	Mohammed *et al*., 2009
Pigeonpea (*Cajanus cajan*)	number of pods; plant height; seed per plant	Bhadru, 2011
Pigeonpea (*Cajanus cajan*)	seed yield per plant; pods per plant; plant height	Vanisree *et al*., 2013
Pigeonpea (*Cajanus cajan*)	plant height; pods per plant; seed per pod	Pandey *et al*., 2015
Soybean (*Glycine max*)	nodule number; nodule fresh weight; nodule dry weight	Agoyi *et al*., 2016
Tepary bean (*Phaseolus acutifolius*)	nodule size score; nodule number score	Mapp *et al*., 2016
Cowpea (*Vigna anguiculata*)	number of nodules; pods per plant; pod length; number of penduncles per plant	Emiri, 2016
Chickpea (*Cicer arietinum*)	number of nodules; nodule dry weight	Girma et al., 2019
Cowpea (*Vigna anguiculata*)	Shoot dry matter; number of nodules; nodule fresh matter	Seido *et al*., 2019
Chickpea (*Cicer arietinum*)	number of nodule; nodule fresh weight; nodule dry weight; seed yield per plant	Roy *et al*., 2019
Cowpea (*Vigna anguiculata*)	plant height; pod length; pods per plant; seed per pod	Owusu *et al*., 2020

2.6 Heritability

Heritability in plant breeding measures the resemblance between parents and offsprings. Two types of heritability, namely the broad-sense (H^2) and narrow-sense (h^2) heritability can be estimated. The broad sense heritability is defined as the ratio of total genetic variance to total phenotypic variance whereas the narrow-sense heritability estimates the ratio of additive genetic variance to the total phenotypic variance (Falconer and Mackay, 1996). Heritability is useful for determining the expected gain from selection (You *et al*., 2016; Holland *et al*., 2003). The variation in a plant population for a specific trait can be due to genetic differences or environmental influences. Therefore, heritability measures the proportion of variation in a plant population that is due to genetic differences (Holland *et al*., 2003).

Currently, there are no reports of heritability values of N fixation in pigeonpea which limit the genetic improvement efforts aimed at enhancing the productivity of the crop. However, in a study involving N fixation in the F_1 generation in chickpea, a moderate (53.0%) h^2 was reported for nodule dry weight (Girma *et al*, 2019). Another study which used F_2 and F_3 soybean plant populations found a moderate H^2 for shoot dry weight (Pazdernik *et al*., 1996). In contrast, a higher estimate (H^2 = 90.0%) for shoot dry weight was observed in cowpea (Seido *et al*., 2019). The limited studies on the genetics of N fixation in these legumes is partly attributed to the complexity of the fixation process which involves many genes and is influenced by several factors (Divito and Sadras, 2014; Kavamura *et al*., 2013). Nonetheless, both nodule dry weight and leaf color score were used as reliable indicators of N fixation in soybean (Gwata *et al*., 2004). Moreover, significant positive associations between nodule dry weight and shoot dry weight have been reported for several leguminous crops (Roy *et al*., 2019; Mohammed *et al*., 2018; Emiri, 2016). Therefore, determination of the heritability of these N fixation indicators is merited in terms of understanding the genetics of root nodulation in pigeonpea and other legumes.

2.7 Summary of the literature review

The literature review showed that:

(i) pigeonpea is a versatile crop which can be used as a livestock feed, traditional medicine, and gown for human consumption

(ii) there are no commercial cultivars of pigeonpea that are cultivated in South Africa, however field trials in the country shows good potential of the crop

(iii) the production of pigeonpea is constrained by multiple range of abiotic and biotic factors, hence the average grain yield obtained by famers in most producing African countries is markedly very low

(iv) the pigeonpea crop nodulates promiscuously and can fix considerable amounts of atmospheric N

(v) there is merit in using single rhizobial strains (strains) in studies aimed at understanding the host x symbiont relationships in the N fixation process

(vi) to date, the genetics of root nodulation traits in pigeonpea is poorly understood and there is no sufficient documentary evidence regarding this aspect of pigeonpea

(vii) the genetic improvement of pigeonpea productivity requires sufficient information about the genetic control of the various root nodulation traits

(viii) there is no documentary evidence regarding heritability values of N fixation attributes in pigepnpea

(ix) there is merit in determining the heritabilities of N fixation indicators in order to elucidate the genetics of root nodulation in pigeonpea.

3.0 CHAPTER THREE: MATERIALS AND METHODS

This study consisted of two experiments. The focus of the first experiment (Experiment 1), was to evaluate nitrogen fixation in pre-existing parental pigeonpea genotypes that were used to derive the F2 segregating plant populations. In the second experiment (Experiment 2), the nitrogen (N) fixation attributes of selected F_2 plant populations were studied as described below.

3.1 Experiment 1: *Determination of genetic variation in selected root nodulation traits among parental pigeonpea genotypes that were used to derive F_2 plant populations*

3.1.1 Study site

The experiment was conducted in a greenhouse at the Agricultural Research Council – Plant Health and Protection (ARC-PHP) Unit, (-25.6° S; 28.4° E), in Pretoria, South Africa. The greenhouse was set at a 14 h day temperature of 28°C and 10 h night temperature of 15°C.

3.1.2 Pigeonpea genotypes

A sample of six pigeonpea genotypes (that could potentially generate 15 possible crossing combinations) was used in the study (Table 3.1). The genotypes were previously used as parental lines to derive the F_1 plants that were subsequently selfed to generate the F_2 populations that were utilized in the second component of this study. The genotypes varied markedly in morphological and agronomic characters such as the duration to flowering and maturity (Table 3.1).

3.1.3 Rhizobial strains and inoculum

In order to optimize the chances of host plant x microsymbiont compatibility, thirty-six rhizobial strains that were previously straind from pigeonpea nodules were selected randomly and used in the study (Table 3.2). The strains originated from soil that was collected from diverse locations across several locations in South Africa and used for inoculating pigeonpea in a nitrogen-free medium in a controlled environment and subsequently purified (Bopape et al., 2021). Most of the rhizobial strains (about 80.0%) belonged to the genus *Rhizobium* and only one strain

of 13 each of *Paraburkholderia* and *Phyllobacterium* were available (Table 3.2). The remainder of the strains belonged to the genus *Bradyrhizobium.*

To prepare the inoculum, each of the strains was recovered from storage (at -70°C) at the South African *Rhizobium* Collection (SARC), which Is housed at the ARC-PHP Unit, Pretoria, South Africa and revived by streaking on yeast mannitol – congo red (YM-CR) and incubated at 28°C for 3-4 days for growth.The inoculum was prepared by suspending two loopfulls of a fresh single colony culture from an YM-CR agar plate in 4ml sterile distilled water in a McCartney bottle (28ml each; Nurrin Pharmalab, South Africa). The suspensions in the were mixed using a vortex mixer (Vortex-Genie 2T with integrated timer) to produce a homogeneous mixture.

Table 3.1 The pigeonpea genotypes that were used in the study.

Genotype		**Seed size** (100-seed weight (g)) (small ≤ 11.0; medium = 11.1-16.0; large > 16.0 g /100 seed)	**Seed color**	**Notes**
Designation	**Code**			
Gen-1	PP-01-01514	Medium	White / cream	Yellow flowers; medium duration; white pods
Gen-2	PP-02-DC	Medium	White / cream	Red flowers; early - medium duration; white pods
Gen-3	PP-03-0557	Large	White / cream	Yellow flowers; medium duration; broad, white pods
Gen-4	PP-04-SST	Small	Brown	Yellow flowers; early -medium duration; small, brown pods
Gen-5	PP-05-MJL	Large	Red	Red flowers; medium duration; long, brown pods
Gen-6	PP-06-MJ-HBR	Large	Red	Red flowers; medium duration; long, brown pods

Table 3.2 The rhizobial strains that were used in the study.

Rhizobial isolate		Genus	Species	Province of origin in South Africa
Code	Designation			
R1	5b2-PP1	*Rhizobium*	*Rhizobium leucaenae*	KwaZulu Natal
R2	16a2-PP1	*Rhizobium*	*Rhizobium sp*	Mpumalanga
R3	29a-PP1	*Rhizobium*	*Rhizobium* sp	North West
R4	17a-PP1	*Rhizobium*	*Rhizobium multihospilium*	Mpumalanga
R5	8b2-PP1	*Rhizobium*	*Rhizobium alamii*	KwaZulu Natal
R6	32a1-PP2	*Rhizobium*	*Rhizobium lupini*	Limpopo
R7	29a2-PP2	*Rhizobium*	*Rhizobium tropici*	North West
R8	29a1-PP2	*Rhizobium*	*Rhizobium* sp	North West
R9	30b-PP3	*Rhizobium*	*Rhizobium multihospilium*	Limpopo
R10	31b1-PP3	*Rhizobium*	*Rhizobium* sp	Limpopo
R11	31b2-PP3	*Rhizobium*	*Rhizobium gallicum*	Limpopo
R12	36a-PP3	*Rhizobium*	*Rhizobium* sp	Limpopo
R13	35a-PP3	*Rhizobium*	*Rhizobium* sp	KwaZulu Natal
R14	32b2-PP5	*Rhizobium*	*Rhizobium* sp	Limpopo
R15	30a2-PP3	*Paraburkholderia*	*Paraburkholderia phenolyruptix*	North West
R16	39a3-PP3	*Rhizobium*	*Rhizobium leucaenae*	Limpopo
R17	15a-PP3	*Rhizobium*	*Rhizobium* sp	Mpumalanga
R18	10a-PP3	*Rhizobium*	*Rhizobium tropici*	Gauteng
R19	19a1-PP3	*Bradyrhizobium*	*Bradyrhizobium elkanii*	Free State
R20	26b-PP3	*Rhizobium*	*Rhizobium* sp	North West
R21	18a-PP3	*Rhizobium*	*Rhizobium mayense*	Mpumalanga
R22	17a1-PP3	*Rhizobium*	*Rhizobium tropici*	Mpumalanga
R23	31b-PP4	*Phyllobacterium*	*Phyllobacterium leguminum*	Limpopo
R24	13b1-PP4	*Rhizobium*	*Rhizobium galegae*	Gauteng
R25	18a-PP4	*Bradyrhizobium*	*Bradyrhizobium* sp	Mpumalanga
R26	33a-PP4	Rhizobium	Rhizobium *elkanii*	Limpopo
R27	37a-PP4	*Rhizobium*	*Rhizobium multihospilium*	Limpopo
R28	19b-PP5	*Bradyrhizobium*	*Bradyrhizobium elkanii*	Free State
R29	23a-PP5	*Rhizobium*	*Rhizobium* sp	Northern Cape
R30	38a1-PP5	*Rhizobium*	*Rhizobium phaseoli*	Limpopo
R31	31b1-PP5	*Rhizobium*	*Rhizobium celliloscum*	Limpopo
R32	26a2-PP5	*Rhizobium*	*Rhizobium* sp	North West
R33	34a2-PP5	*Rhizobium*	*Rhizobium* sp	Limpopo
R34	27b2-PP5	*Bradyrhizobium*	*Bradyrhizobium japonicum*	North West
R35	11b2-PP5	*Bradyrhizobium*	*Bradyrhizobium elkanii*	Gauteng
R36	14a1-PP5	*Rhizobium*	*Rhizobium alamii*	Gauteng

3.1.4 Trial establishment and management

A total of 432 plastic pots (2.0L each; Calibre Plastic (Pty) Ltd, South Africa) (base diameter =12.0 cm; top diameter = 17.0c m; height = 13.0 cm) (Fig 3.1a) were washed in a bucket of water containing 3.5% sodium hypochlorite and rinsed several times in clean water to remove the residual effect of the bleach. Each pot was filled with 1.65 kg sterile river sand and saturated with 380.0 ml Hoagland solution (Appendix 2.1). In each pot, two holes (2.0 cm deep) for planting the seed were made with a sterile spatula (0.65 cm wide).

At planting, a single healthy seed of pigeonpea was placed in each of the holes per pot and immediately inoculated with approximately 2.0ml of the bacterial inoculum using a micro-pipette (Fig 3.1b) and covered with the sand. During the trial, a mixture of 150.0ml of sterile water and the Hoagland solution was applied around the plants in the pots after 2 or 3 days. The plants were raised in the greenhouse under 14-hr daylength, at day/night temperature of 28/15ºC. These conditions were previously found suitable for pigeonpea growth in a greenhouse (Bopape et al., 2021).

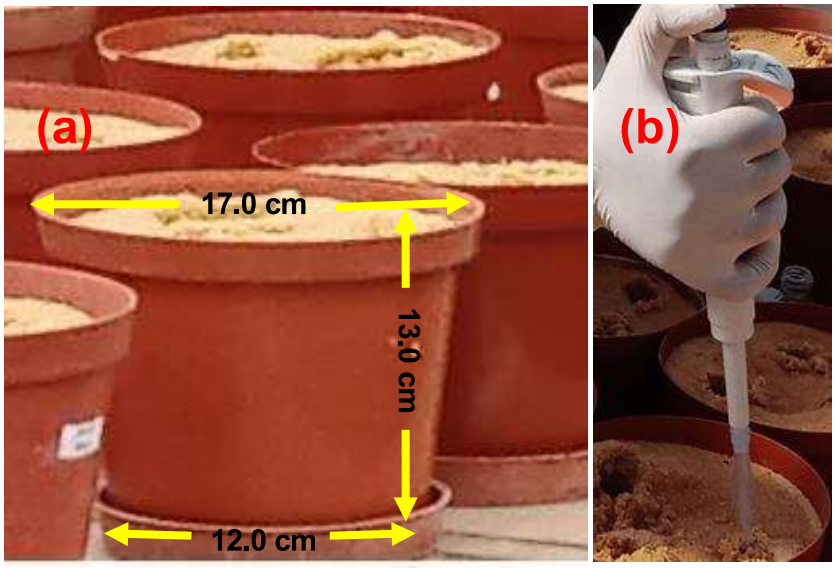

Fig 3.1 (a) An example of the plastic pots that were used in the study and (b) inoculation of the seed with the bacterial inoculum.

3.1.5 Measurements of nitrogen fixation traits

Prior to harvesting, a non-destructive, rapid, visual screening method for N-fixation involving the classification of the plants into leaf color score (LCS) was used (Gwata et al., 2003). In this method, the LCS was based on a modified scale (with 1 = chlorotic leaves, indicating ineffective nodulation and no nitrogen fixation; 3 = dark green leaves, indicating effective nodulation and active nitrogen fixation) that could accommodate partial N-fixation instead of a two-category scale that was used previously in a similar study (Gwata and Wofford, 2012; Gwata et al., 2003). Partially effective nodules of a legume, cowpea (*Vigna unguiculata*) were reported previously (Yu et al., 2014). Upon harvesting the plants at six weeks after planting, they were transferred to the laboratory for processing. Each plant was separated into the shoot and the root (including nodules) and placed into labelled brown paper bags after which they were oven-dried at temperature 75°C for 72 h and weighed thereafter to determine the shoot dry weight (SDW) and root dry weight (RDW). Previous studies showed that SDW consistently produced a positive relationship with nitrogen fixation ability in soybean (Neuhausen et al., 1988; Gwata et al., 2003).

3.1.6 Experimental design and data analysis

The experiment was conducted in a completely randomized design, with two replications. The data sets for all nodulation variables for each genotype x rhizobial strain were subjected to the analysis of variance (ANOVA) followed by mean separation using the least significant difference (LSD) at the 5% probability level. To understand the relationship between the rhizobial strains and the host plant genotypes, a further analysis using the genotype plus the genotype by environment interaction (GGE) biplot technique was performed (Yan et al., 2002; Yan and Tinker 2005; Yan and Tinker, 2005) using Genstat Software version 13 (Genstat 2010). The GGE biplot enables the graphic visualization of the information regarding the best performing genotypes and their winning environments, the interrelationship among the test environments as well as the ranking of genotypes based on both mean performance and stability (Yan, 2002; Yan and Kang 2002). Therefore, for this analytical approach, the rhizobial strains and the pigeonpea genotypes were coded as genotypes and environments, respectively.

3.1.7 Selection of contrasting parental genotypes for nodulation traits

The second component of this study required the identification of the contrasting parental genotypes (in terms of the measured nodulation attributes) that would segregate in the F_2 filial generation for those attributes. Consequently, the selection of such parental genotypes utilized the results of at least one or both the ANOVA and the GGE biplot analytical approaches to select the genotypes.

3.2 Experiment 2: *Determination of the genetic variation and mode of inheritance for selected root nodulation traits in segregating pigeonpea F_2 plant populations*

3.2.1 Study site

The experiment was conducted in the greenhouse at the same location under similar conditions as described above (section 3.1.1).

3.2.2 Crosses for deriving F_2 pigeonpea plant populations

The F_2 pigeonpea segregating plant populations which were derived from two distinct crosses each involving a pair of contrasting parental genotypes (Table 3.1) (which were determined from the results of the preceding experiment (Experiment 1), were used in the study. The identities of two crosses (see explanation of selection in section 4.1.3 below) were as follows:

(i) cross 1 = P_6 x P_1

(ii) cross 2 = P_4 x P_2

where:

P_1 = parental genotype ‘Gen-1’ = PP-01-01514

P_2 = parental genotype ‘Gen-2’ = PP-02-DC

P_4 = parental genotype ‘Gen-4’ = PP-04-SST

P_6 = parental genotype ‘Gen-6’ = PP-06-MJ-HBR.

3.2.3 Specific rhizobial strains for each cross

Two rhizobial strains (Table 3.2) that were selected (see explanation of selection in section 4.1.3 below) from the results of the preceding experiment (Experiment 1), and were used in the study. The identities of two strains were as follows:

(i) rhizobial strain for 'cross 1'

(a) 'strain 1' = rhizobial strain R14 = 32b2-PP5 = *Rhizobium sp*

and

(ii) rhizobial strain for 'cross 2'

(b) 'strain 2' = rhizobial strain R5 = 8b2-PP1 = *Rhizobium alamii*

The inoculum was prepared using the same method as described above (section 3.1.2.2).

3.2.4 Trial establishment and management

The F_2 seed of each cross was partitioned into two equal portions (consisting of 70 seeds each). A single seed from each portion was planted per 2.0L plastic pot (see section 3.1.2.3) as described above to constitute a total of 140 plants per cross. After filling each pot with 1.65kg sterile river sand and saturating it with 380.0ml Hoagland solution as described above, a single hole (2.0 cm deep) for planting the seed was made with a sterile spatula in the center of the pot. At planting, a single healthy seed of pigeonpea was placed in the hole per pot and immediately inoculated with approximately 2.0 ml of the bacterial inoculum using a micro-pipette (see section 3.1.2.3) and covered with the sand. Each of the plants in the F_2 population that was derived from the cross 'P_4 x P_2' (cross 1 = P_6 x P_1 (PI-06-MJ-HBR x PI-01-01514) was inoculated with rhizobial strain R14 (32b2-PP5) = *Rhizobium* sp). Similarly, the F_2 plants from the second cross, P_4 x P_2 (PI-04-SST x PI-02-DC) were inoculated with the rhizobial strain R5 (8b2-PP1) = *Rhizobium alamii*). During the trial, the plants were maintained as described above for Experiment 1 and the greenhouse conditions were set at 14-hr daylength and day/night temperature of 28/18ºC.

3.2.5 Measurements of nitrogen fixation traits

In this study, the segregation pattern of each of three traits namely leaf color content (LCC), shoot dry weight (SDW) and nodule dry weight (NDW) were used indicators of effective N fixation in the root nodules. Prior to harvesting, the LCC was measured with the aid of a chlorophyll meter (Minolta Chlorophyll Meter Spad-502, Minolta Co., Ltd., Tokyo, Japan) which quantitatively records numerical leaf color units ranging from high (green) to low (yellow) and automatically calculates a numerical value, which is linearly related to the leaf chlorophyll content (Markwell et al. 1995; Gwata et al., 2004). For each plant, both the shoots and nodules were first separated from root prior to oven-drying (see section 3.1.4) and weighing thereafter.

3.2.6 Experimental design and data analysis

For each cross, a completely randomized design with two replications (each replication consisting of 70 seeds) was used. The mode of inheritance for each of the three root nodulation (or N fixation) traits was determined using the Chi-square (X^2) tests (Nevhudzholi et al., 2020; Mohammed *et al.*, 2010; Gwata et al., 2005; Jackson *et al.*, 2005) for the goodness-of-fit of the observed F_2 segregation data to the expected ratios in each of the three root nodulation traits. Five Mendelian ratios (3:1; 9:7; 9:3:3:1; 13:3 and 15:1) were tested using the equation:

$$X^2 = \sum \frac{(\text{observed - expected})^2}{\text{expected}}$$

For each Mendelian ratio, the null hypothesis which was tested was that there was no difference between the observed values and the expected values. The significance of the observed deviations from the expected values was determined at the 5.0% probability level and where the chi-square calculated value was greater than the chi-square critical value, the null hypothesis was rejected (Steel et al., 1997).

4.0 CHAPTER FOUR: RESULTS

This Chapter presents the findings of the study covering both specific objectives. The first part of the Chapter presents the evidence for the variation in N fixation using three indicators of the fixation. The second part of the Chapter, presents the mode of inheritance the selected N fixation traits.

4.1 Experiment 1: *Determination of the genetic variation in selected root nodulation traits among parental pigeonpea genotypes that were used to derive F_2 plant populations.*

4.1.1 Variation in root nodulation traits among parental genotypes

The results showed that at six weeks after germination, there was significant variation in the root nodulation traits that were assumed to indicate symbiotic N fixation among the pigeonpea genotypes. The pattern of the variation depended on the source of N (or rhizobial strain). Some of the plants were chlorotic indicating that they were unable to fix adequate nitrogen for their growth and development (Fig. 4.1). The control plants that were inoculated with distilled water (R-36) were chlorotic at 6 weeks after germination. There were highly significant ($P < 0.01$) differences among the pigeonpea genotypes for leaf color score (LCS) and shoot dry weight (SDW) (Table 4.1). In contrast, there were no significant ($P < 0.05$) differences among the pigeonpea genotypes for root dry weight (RDW). The LCS ranged from 1.39 to 2.83 with an average of 2.24. In addition, the RDW of genotype 'Gen-6' (0.67 g) was 66.7% less than the trial mean NDW (0.21 g). The highest SDW (0.23 g) which occurred in genotype 'Gen-5' was more than two-fold heavier than that for the control genotype ('Gen-6) (Table 4.1).

Fig. 4.1 Variation in ability to fix nitrogen among (a) chlorotic (unable to fix nitrogen) and (b) green (able to fix nitrogen) pigeonpea plants that were inoculated separately with each of thirty-six rhizobial strains.

Table 4.1 Mean squares for leaf color score, root dry weight and shoot dry weight among six parental pigeonpea genotypes that were each inoculated separately with each of thirty-six rhizobial strains.

Source	df	Mean square		
		Leaf color score	Root dry weight	Shoot dry weight
Replication	1	0.67	1.70	0.22
Genotype (G)	5	17.39 **	0.98	0.15 **
Rhizobia Isolates (RI)	35	1.66 **	0.89	0.01 *
G x RI	175	1.62 **	0.97	0.01
Mean separation				
Gen-5		2.22 bc	0.35 a	0.23 a
Gen-3		2.42 bc	0.19 a	0.14 ab
Gen-4		2.08 c	0.36 a	0.19 ab
Gen-2		2.51 ab	0.15 a	0.18 b
Gen-1		2.83 a	0.17 a	0.18 b
Gen-6		1.39 d	0.07 a	0.10 c
Grand mean		2.24	0.21‡	0.18‡
Coefficient of variation (%)		39.88	1.37	25.27

**; * = significant at the 1.0 and 5.0% probability levels, respectively. In each column, means followed by a different small letter are significant at the 5.0% probability level.

‡measured in grams (g).

4.1.2 Genotype x rhizobial strain performance

The analysis of variance showed that there were highly significant ($P < 0.01$) differences in LCS between several pairs of potential parental genotypes including 'Gen-4' versus 'Gen-2' when inoculated separately with each of at least three rhizobial strains, namely 'R-1', 'R-5', 'R13 and 'R-25' (Table 4.2). In addition, five rhizobial strains ('R-5', 'R-10' 'R-11' 'R-15', 'R-25') could discriminate between 'Gen-1'and 'Gen-6'. In some cases, a single rhizobial strain, for instance, 'R-1', could discriminate between 'Gen-6' versus 'Gen-2' but not between 'Gen-1' versus 'Gen-3' nor between 'Gen-1' versus 'Gen-6' indicating the importance of identifying the appropriate genotype x rhizobial strain combinations that reflect contrasting responses for plant genetic studies involving segregating plant populations. Similarly, the results revealed highly significant ($P < 0.01$) differences in SDW between several pairs of candidate parental genotypes including 'Gen-4' versus 'Gen-2' when inoculated with the rhizobial strain 'R-14' (Table 4.3). The rhizobial strains 'R-1', 'R-7' and 'R-8' could induce the differential responses in SDW between 'Gen-2' versus 'Gen-6' while the strain 'R14' showed a contrasting pattern in SDW between 'Gen-1' on one hand and each of the remaining pigeonpea genotypes on the other hand (Table 4.3). Overall, only 12 rhizobial strains (33.3%) could discriminate the six parental genotypes for either LCS or SDW or both. Nonetheless, there was a marked variation in the effects of the individual strains on SDW among the parental pigeonpea genotypes (Fig. 4.2). The results also showed that the rhizobial strains 'R16' (*Rhizobium leucaenae*, 39a3-PP3) and 'R8' (*Rhizobium* sp., 29a1-PP2) induced the heaviest and lightest SDW, respectively (Fig. 4.2).

Table 4.2 Mean separation for leaf color score among pegeonpea parental genotypes that were each inoculated separately with specific rhizobial strains.

Genotype	Code	Rhizobial isolate						
		R-1	R-5	R-10	R-11	R-13	R-15	R-25
Gen-1	PP-01-01514	3.00 a	3.00 b	4.00 a	4.00 a	3.00 a	3.00 a	4.00 ab
Gen-2	PP-02-DC	0.00 c	**5.00 a**	3.00 ab	1.50 bc	3.00 a	3.00 a	5.00 a
Gen-3	PP-03-0557	3.00 a	2.00 bc	2.00 abc	3.00 ab	3.00 a	2.00 ab	2.50 bc
Gen-4	PP-04-SST	2.00 b	**1.00 c**	1.00 bc	2.00 abc	2.00 b	1.00 bc	2.50 bc
Gen-5	PP-05-MJL	2.50 ab	3.00 b	2.00 abc	2.00 abc	2.00 b	2.00 ab	1.00 cd
Gen-6	PP-06-MJ-HBR	3.00 a	1.00 c	0.00 c	0.00 c	2.50 ab	0.00 c	0.00 d
Grand mean		2.25	2.50	2.00	2.08	2.58	1.83	2.50
C.V.		12.83	23.09	46.1	41.57	11.17	31.5	29.21
Significance		**	**	**	*	*	**	**

**; * = significant at the 1.0% and 5.0% probability levels, respectively. In each column, means followed by a different small letter are significantly different ($LSD_{0.05}$).

Table 4.3 Mean separation for shoot dry weight (g) among pegeonpea parental genotypes that were each inoculated separately with specific rhizobial strains.

Genotype	Code	Rhizobial isolate				
		R-1	R-7	R-8	R-14	R-18
Gen-1	PP-01-01514	0.19 bc	0.14 bc	0.08 abc	**0.16 d**	0.17 b
Gen-2	PP-02-DC	0.00 d	0.21 b	0.15 ab	0.27 ab	0.00 c
Gen-3	PP-03-0557	0.24 ab	0.19 b	0.19 a	0.21 c	0.14 b
Gen-4	PP-04-SST	0.22 bc	0.37 a	0.08 bc	0.27 a	0.27 a
Gen-5	PP-05-MJL	0.32 a	0.08 c	0.03 c	0.28 a	0.18 b
Gen-6	PP-06-MJ-HBR	0.16 c	0.00 d	0.00 c	**0.23 bc**	0.10 b
Grand mean		0.19	0.16	0.09	0.23	0.14
C.V.		15.64	17.88	48.15	7.09	24.89
Significance		**	**	*	**	**

**; * = significant at the 1.0% and 5.0% probability levels, respectively. In each column, means followed by a different small letter are significantly different ($LSD_{0.05}$).

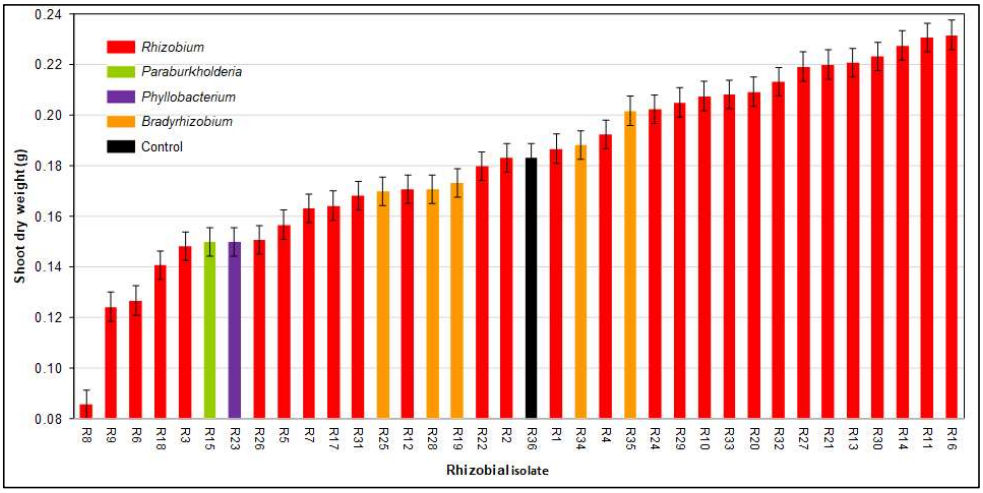

Fig. 4.2 Variation in the effects of individual rhizobial strains on shoot dry weight among the pigeonpea parental genotypes. The identities of the rhizobial strains is stated in Table 3.2.

4.1.3 Selection of parental genotypes for deriving F_2 populations and specific rhizobial strains

Based on the genotypic variation in response to rhizobial inoculations, the contrasting parental genotypes for 'cross 1' (Gen-1 x Gen-6) were selected based on significant differences in SDW that were induced by the rhizobial strain R14 (*Rhizobium* sp., 32b2-PP5) (Table 4.3). Similarly, the contrasting parental genotypes for 'cross-2' (Gen-2 x Gen-4) were selected based on the significant differences in LCS that were induced by the rhizobial strain R5 (*Rhizobium alamii*, 8b2-PP1) (Table 4.4).

4.1.4 GGE biplot analysis

4.1.4.1 Shoot dry weight

The GGE biplot analysis revealed that the rhizobial strains (coded as genotype scores) were scattered almost evenly across the four quadrants (Fig. 4.3). However, the rhizobial strains 'R24', 'R28', 'R31' and 'R34' were clustered around the origin. In contrast, the rhizobial strains 'R7', 'R8', 'R10', 'R27' and 'R29'were positioned far away from the origin suggesting that they

were unique in their influence on the genotypes. Nonetheless, a considerable number of the rhizobial strains (including 'R5', 'R7', 'R15', 'R18', 'R22' and 'R25') were positioned in the two bottom quadrants away from the genotypes. The biplot also showed that the pigeonpea parental genotypes (coded as environment scores), 'Gen-1' (E1), 'Gen-2' (E2), 'Gen-3' (E3) and 'Gen-5' (E5) were separated by acute angles between them and grouped in the same quadrant (Fig. 4.3). However, although both 'Gen-4' (E4) and 'Gen-6' (E6) were separated by an acute angle between them and grouped together in the top left quadrant, they formed obtuse angles with each of the remaining genotypes indicating that they were negatively related to the rest of the genotypes in terms of the SDW trait. Genotype 'Gen-3' (E3) which had the shortest absolute projection was the most stable (followed by 'Gen-1') across the rhizobial strains.

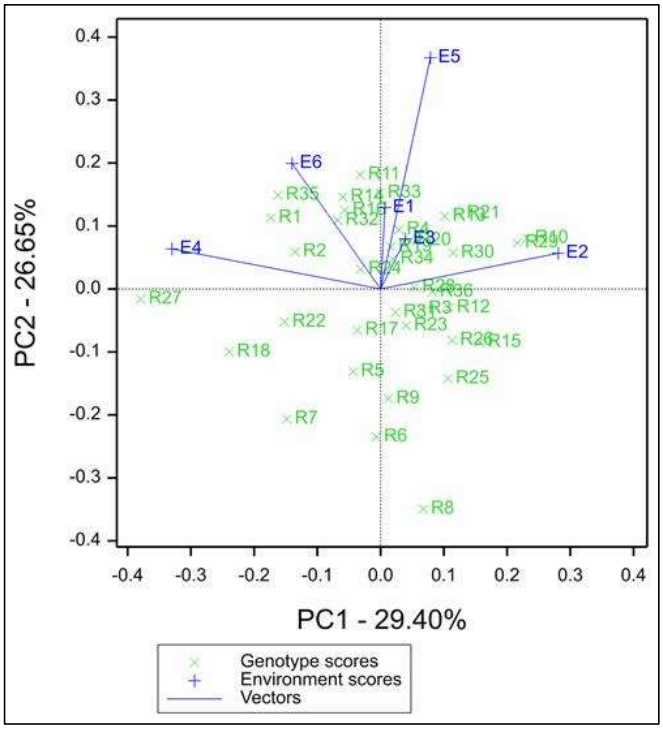

Fig. 4.3 GGE biplot analysis showing the relationship between the rhizobial strains (coded as genotype scores) and the parental pigeonpea genotypes (coded as environment scores). E = Gen-1 (PP-01-01514); E2 = Gen-2 (PP-02-DC); E3 = Gen-3 (PP-03-0557); E4 = Gen-4 (PP-04-SST); E5 = Gen-5 (PP-05-MJL); E6 = Gen-6 (PP-06-MJ-HBR).

4.1.4.2 Superior rhizobial strains with specific pigeonpea genotypes

The biplot consists of an irregular polygon with a set of lines drawn from the origin to dissect perpendicularly each side of the polygon and dividing the biplot into sectors as well as determining the winning rhizobial strains (coded as genotype scores) for each sector (Yan et al. 2007). The 'which-won where' biplot explained 56.05% total variation of which PC1 and PC2 accounted for 29.40% and 26.65% of the total variation, respectively (Fig. 4.4). The biplot produced six sectors and the rhizobial strains were clustered into all the sectors. The results also revealed that the rhizobial strains (depicted as genotypes) on the vertices of the polygon 'R10', 'R11', 'R27' and 'R35' performed best with the pigeonpea parental genotypes (depicted as environments) 'Gen-2' (E2), 'Gen-5' (E5), 'Gen-4' (E4) and 'Gen-6' (E6), respectively (Fig. 4.4). The GGE biplot analysis showing the discriminating power of the test environments (parental genotypes) and representativeness of the target environment (genotype) consists of vector lines that are drawn from the origin of the biplot to each test environment marker, measuring the discriminative power of the environment. Since in this method, the long vectors indicate test environments (i.e. genotypes) with relatively more discriminating power, the genotype 'Gen-5' (E5) showed the longest vector line, suggesting a high discriminating ability.

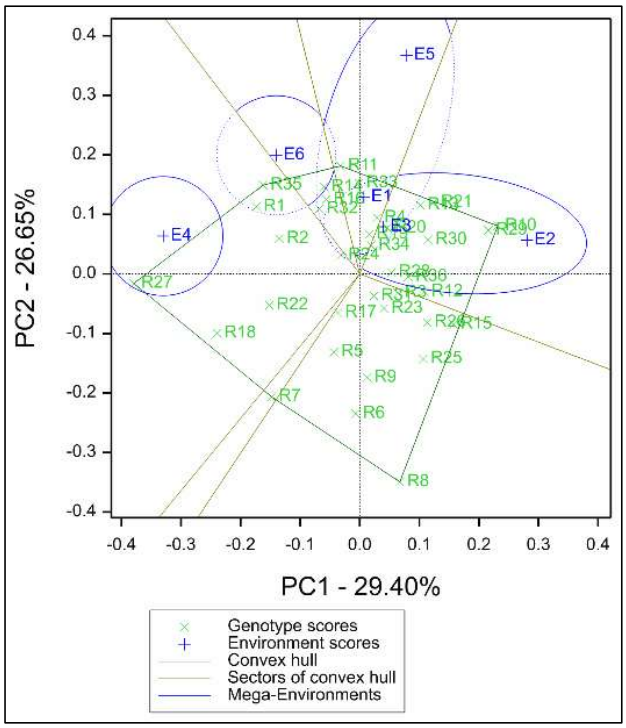

Fig. 4.4 A “which-won-were” pattern of GGE biplot polygon view showing the rhizobial strain main effect plus G x E interaction effect of 36 strains on the shoot dry weight in parental genotypes of pigeonpea. (Rhizobial strains and pigeonpea parental genotypes are coded as genotype scores and environmental scores, respectively). E = Gen-1 (PP-01-01514); E2 = Gen-2 (PP-02-DC); E3 = Gen-3 (PP-03-0557); E4 = Gen-4 (PP-04-SST); E5 = Gen-5 (PP-05-MJL); E6 = Gen-6 (PP-06-MJ-HBR).

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4.1.4.3 Comparison view of GGE biplot analysis of ideal rhizobial strain

According to Yan and Tinker (2006), the ideal rhizobial strain (coded as a genotype score in this case), is located in the innermost concentric circle, indicated by an arrowhead in the biplot. The rhizobial strain 'R11 was identified as ideal for SDW (Fig. 4.5). In contrast, rhizobial strains 'R6', 'R7'and 'R8' were furthest from the ideal genotype, indicating low stability.

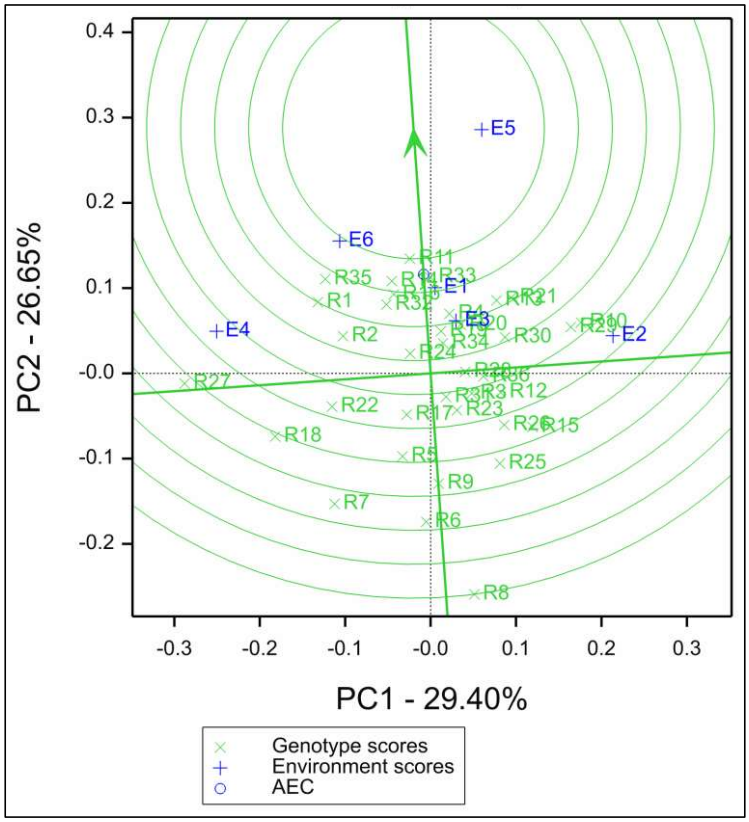

Fig. 4.5 A comparison view of GGE biplot showing an ideal rhizobial strain for shoot dry weight in parental genotypes of pigeonpea. (Rhizobial strains and pigeonpea parental genotypes are coded as genotype scores and environmental scores, respectively). E = Gen-1 (PP-01-01514); E2 = Gen-2 (PP-02-DC); E3 = Gen-3 (PP-03-0557); E4 = Gen-4 (PP-04-SST); E5 = Gen-5 (PP-05-MJL); E6 = Gen-6 (PP-06-MJ-HBR).

4.1.4.4 Comparison view of GGE biplot analysis of ideal parental pigeonpea genotype

To visualize the GGE biplot comparison of parental genotypes (coded as environments), relative to the ideal genotype, the 'Gen-5'(E5), followed by 'Gen-6' was positioned closest to the epicenter of the concentric circles, providing the most ideal production conditions for shoot dry weight (Fig 4.6). In contrast, genotypes 'Gen-2' (E2), 'Gen-4' (E4) and 'Gen-3'(E3) (in that order) were positioned farthest from the epicenter, hence considered the least discriminatory genotypes.

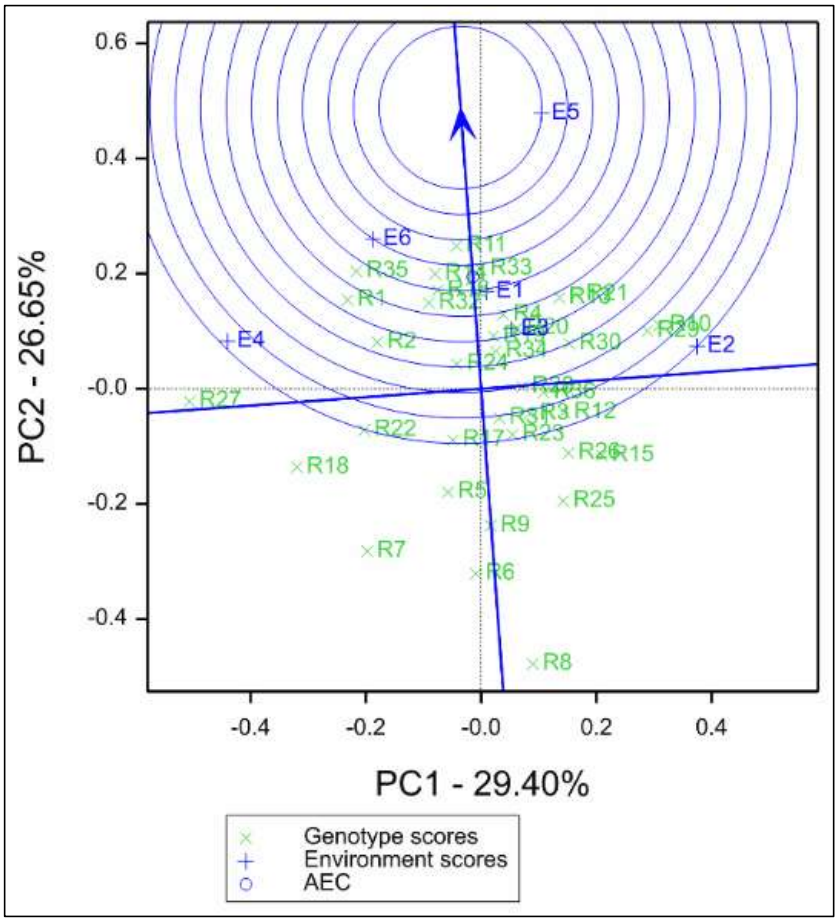

Fig. 4.6 The comparison view of GGE biplot showing the ideal environment for shoot dry weight in parental genotypes of pigeonpea. (Rhizobial strains and pigeonpea parental genotypes are coded as genotype scores and environmental scores, respectively). E = Gen-1 (PP-01-01514); E2 = Gen-2 (PP-02-DC); E3 = Gen-3 (PP-03-0557); E4 = Gen-4 (PP-04-SST); E5 = Gen-5 (PP-05-MJL); E6 = Gen-6 (PP-06-MJ-HBR).

4.2 Experiment 2: *Determination of the genetic variation and mode of inheritance for selected root nodulation traits in segregating pigeonpea F_2 plant populations*

4.2.1 Frequency distribution and segregation pattern for leaf chlorophyll content

The frequency distribution curve for the leaf chlorophyll content (LCC) among the F_2 progenies of the 'cross 1' (P_6 x P_1) showed an approximately normal distribution curve but with a slight skew to the left (Fig. 4.7). On average, the genotypes in this plant population attained 29.41 units of LCC. On the other hand, the frequency distribution curve for the F_2 plant population that was derived from 'cross 2' (P_4 x P_2) followed a normal distribution but with a slight skew to the right (Fig. 4.7).

The segregation pattern of F_2 populations indicated a segregation ratio of 9 high:7 low chlorophyll content in 'cross 1' involving P_6 x P_1 but there was a limited number of genotypes in this population partly due to poor germination (Table 4.4). However, the segregation pattern for LCC in 'cross 2' (P_4 x P_2) consistently fitted a segregation ratio of 3 high:1 low chlorophyll content (Table 4.5). The Mendelian ratio of 13 high:3 low also fitted the observed segregation pattern for LCC in this cross suggesting that at least two gene pairs were involved in controlling this trait in pigeonpea although this segregation ratio was less convincing than the 3:1 ratio.

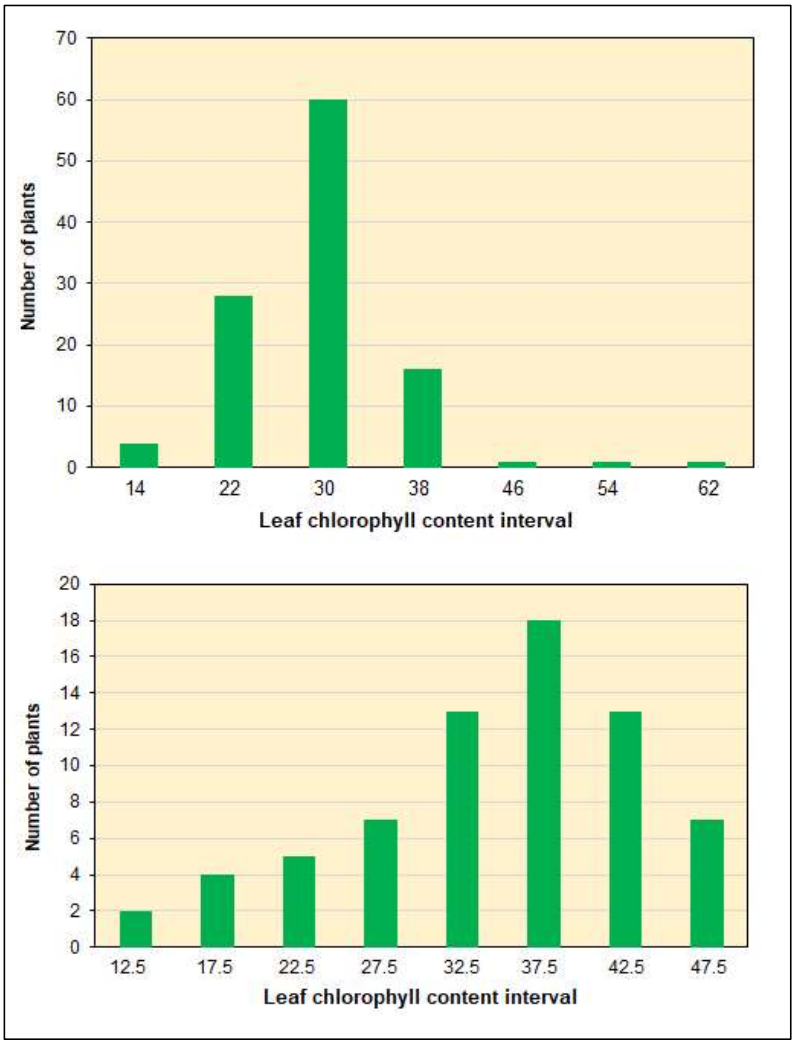

Fig. 4.7 Frequency distribution curve for leaf color score in pigeonpea F_2 populations derived from 'cross 1' (P_6 x P_1) (top) and 'cross 2' (P_4 x P_2) (bottom).

Table 4.4 Segregation pattern for root nodulation trait in pigeonpea F_2 populations ('cross 1') based on differences in leaf chlorophyll content of plants grown in a N-depleted medium and inoculated with the rhizobial strain R14 (*Rhizobium* sp., 32b2-PP5).

Cross	Sub-set	Number of F_2 plants			Ratio	X^2	*P* value
		High	Low	Total			
PP-06-MJ-HBR x PP-01-01514	1	26	8	34	3:1	0.04	0.9500 - 0.8000
					9:7	5.85	0.0250 - 0.0100
					13:3	0.51	0.6000 - 0.5000
					15:1	17.33	0.0010 - 0.0001
	2	27	8	35	3:1	0.09	0.9500 - 0.8000
					9:7	6.21	0.0200 - 0.0100
					13:3	0.39	0.7000 - 0.6000
					15:1	16.47	0.0010 - 0.0001
	Combined	53	16	69	3:1	0.12	0.4000 - 0.3000
					9:7	11.85	0.0005 - 0.0003
					13:3	0.89	0.4000 - 0.3000
					15:1	33.79	0.0010 - 0.0001
	Heterogeneity of X^2				3:1	0.13	0.8000 - 0.7000
					9:7	12.06	0.0005 - 0.0003
					13:3	0.90	0.4000 - 0.3000
					15:1	33.80	0.0010 - 0.0001

Table 4.5 Segregation pattern for root nodulation trait in pigeonpea F_2 populations ('cross 2') based on differences in leaf chlorophyll content of plants grown in a N-depleted medium and inoculated with the rhizobial strain R5 (*Rhizobium alamii*, 8b2-PP1).

Cross	Sub-set	Number of F_2 plants			Ratio	X^2	*P* value
		High	Low	Total			
PP-04-SST x PP-02-DC	1	30	27	57	3:1	15.21	0.0003 - 0.0001
					9:7	0.30	0.7000 -0.6000
					13:3	30.64	0.0010 - 0.0001
					15:1	164.47	0.0010 - 0.0001
	2	34	20	54	3:1	4.17	0.0500 - 0.0300
					9:7	0.99	0.4000 -0.3000
					13:3	11.85	0.0005 - 0.0003
					15:1	87.35	0.0010 - 0.0001
	Combined	64	47	111	3:1	17.80	0.0010 - 0.0001
					9:7	0.09	0.9000 -0.8500
					13:3	40.55	0.0010 - 0.0001
					15:1	246.78	0.0010 - 0.0001
	Heterogeneity of X^2				3:1	19.38	0.0010 - 0.0001
					9:7	1.29	0.4000 - 0.3000
					13:3	42.49	0.0010 - 0.0001
					15:1	251.82	0.0010 - 0.0001

4.2.2 Frequency distribution and segregation pattern for nodule dry weight

For nodule dry weight (NDW), the frequency distribution curve among the F_2 progenies of both 'cross 1' (P_6 x P_1) and 'cross 2' (P_4 x P_2) were skewed to the left (Fig. 4.8) which strongly suggested epistatis (Anbessa *et al.*, 2006). In addition, the NDW from 'cross 2' (P_4 x P_2) were slightly heavier than the nodules produced by the progenies which were derived from the 'cross 1' involving (P_6 x P_1). The segregation pattern of the F_2 plant populations in 'cross 1' indicated a segregation ratio of 3 heavy:1 light dry weight of nodules when the two sub-sets of data were combined (Table 4.6). Nonetheless, both the 13 heavy : 3 light and the 9 heavy : 7 light models also fitted the observed segregation patterns but as strongly as the monogenic inheritance model. However, the segregation pattern in 'cross 2' fitted best a 9 heavy:7 light dry nodule weight model (Table 4.7).

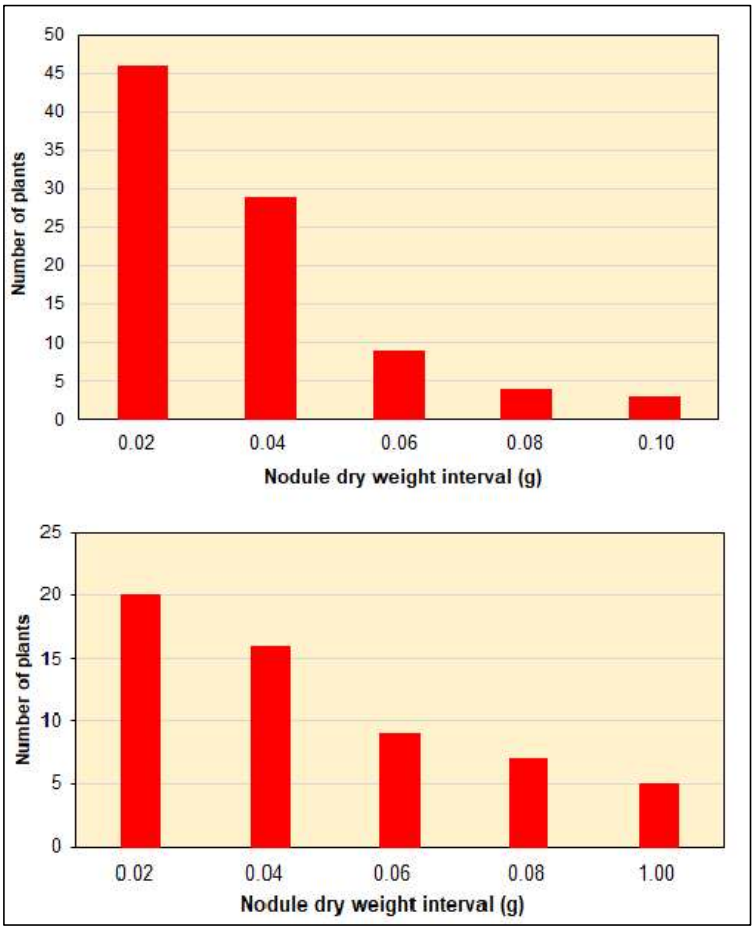

Fig. 4.8 Frequency distribution curve for leaf color score in pigeonpea F_2 populations derived from 'cross 1' (P_6 x P_1) (top) and 'cross 2' (P_4 x P_2) (bottom).

Table 4.6 Segregation pattern for root nodulation trait in pigeonpea F_2 populations ('cross 1') based on differences in nodule dry weight of plants grown in a N-depleted medium and inoculated with the rhizobial strain R14 (*Rhizobium* sp., 32b2-PP5).

Cross	Sub-set	Number of F_2 plants			Ratio	X^2	*P* value
		Heavy	Light	Total			
PP-06-MJ-HBR x PP-01-01514	1	10	18	28	3:1	1.71	0.3000 - 0.2000
					9:7	0.73	0.5000 - 0.4000
					13:3	5.23	0.0300 - 0.0200
					15:1	41.49	0.0010 - 0.0001
	2	18	11	29	3:1	2.59	0.1500 - 0.1000
					9:7	0.39	0.7000 - 0.6000
					13:3	7.00	0.0100 - 0.0050
					15:1	49.68	0.0010 - 0.0001
	Combined	28	29	57	3:1	20.36	0.0010 - 0.0001
					9:7	1.18	0.2000 - 0.1500
					13:3	38.62	0.0010 - 0.0001
					15:1	193.74	0.0010 - 0.0001
	Heterogeneity of X^2				3:1	4.30	0.0300 - 0.0200
					9:7	1.12	0.4000 - 0.3000
					13:3	12.23	0.0005 - 0.0003
					15:1	91.17	0.0010 - 0.0001

Table 4.7 Segregation pattern for root nodulation trait in pigeonpea F_2 populations ((('cross 2') of based on differences in nodule dry weight of plants grown in a N-depleted medium and inoculated with the rhizobial strain R5 (*Rhizobium alamii*, 8b2-PP1).

Cross	Sub-set	Number of F_2 plants			Ratio	X^2	*P* value
		Heavy	Light	Total			
PP-04-SST x PP-02-DC	1	18	28	46	3:1	4.89	0.0300 - 0.0200
					9:7	0.39	0.7000 - 0.6000
					13:3	12.54	0.0010 - 0.0005
					15:1	84.88	0.0010 - 0.0001
	2	12	33	45	3:1	0.07	0.9000 - 0.8000
					9:7	5.34	0.0300 - 0.0200
					13:3	1.85	0.2000 - 0.1500
					15:1	32.01	0.0010 - 0.0001
	Combined	30	61	91	3:1	3.08	0.0800 - 0.0500
					9:7	4.29	0.0300 - 0.0200
					13:3	12.07	0.0010 - 0.0005
					15:1	110.86	0.0010 - 0.0001
	Heterogeneity of X^2				3:1	4.96	0.0300 - 0.0200
					9:7	5.73	0.0200 - 0.0100
					13:3	14.39	0.0003 - 0.0001
					15:1	116.89	0.0010 - 0.0001

4.2.3 Frequency distribution and segregation pattern for shoot dry weight

In terms of shoot dry weight (SDW), the frequency distribution pattern among the F_2 progenies of both crosses produced normal distribution curves with no obvious discontinuities suggesting a polygenic mode of inheritance (Fig. 4.9). On average, the SDW in 'cross 2' (P_6 x P_1) were slightly (6.9%) heavier than those from 'cross 1' (P_4 x P_2). The segregation pattern of the F_2 progenies in both crosses consistently fitted a segregation ratio of 9 heavy : 7 light dry weight of nodules suggesting the involvement of epistasis gene action in control SDW (Table 4.8).

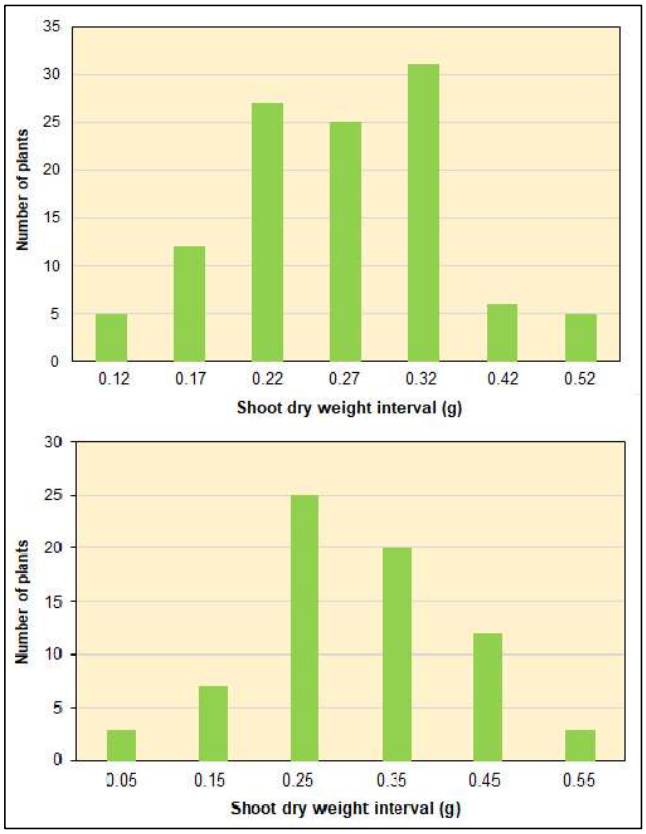

Fig. 4.9 Frequency distribution curve for shoot dry weight in pigeonpea F_2 populations derived from 'cross 1' (P_6 x P_1) (top) and 'cross 2' (P_4 x P_2) (bottom).

Table 4.8 Segregation pattern for root nodulation trait in pigeonpea F_2 populations ('cross 1') based on differences in shoot dry weight of plants grown in a N-depleted medium and inoculated with the rhizobial strain R14 (*Rhizobium* sp., 32b2-PP5).

Cross	Sub-set	Number of F_2 plants			Ratio	X^2	*P*-value
		Heavy	Light	Total			
PP-06-MJ-HBR x PP-01-01514	1	18	19	37	3:1	11.04	0.0010 - 0.0005
					9:7	0.36	0.7000 - 0.6000
					13:3	21.71	0.0010 - 0.0001
					15:1	113.51	0.0010 - 0.0001
	2	22	11	33	3:1	1.22	0.4000 - 0.3000
					9:7	1.46	0.3000 - 0.2000
					13:3	4.61	0.0500 - 0.0300
					15:1	41.31	0.0010 - 0.0001
	Combined	40	30	70	3:1	11.91	0.0005 - 0.0003
					9:7	0.02	0.9500 - 0.8000
					13:3	26.7	0.0010 - 0.0001
					15:1	160.09	0.0010 - 0.0001
	Heterogeneity of X^2				3:1	12.26	0.0005 - 0.0003
					9:7	1.82	0.2000 - 0.1500
					13:3	26.32	0.0010 - 0.0001
					15:1	154.82	0.0010 - 0.0001

Table 4.9 Segregation pattern for root nodulation trait in pigeonpea F_2 populations ('cross 2') based on differences in shoot dry weight of plants grown in a N-depleted medium and inoculated with the rhizobial strain R5 (*Rhizobium alamii*, 8b2-PP1).

Cross	Sub-set	Number of F_2 plants			Ratio	X^2	*P* value
		Heavy	Light	Total			
PP-04-SST x PP-02-DC	1	32	24	56	3:1	9.52	0.0020 - 0.0010
					9:7	0.02	0.9500 - 0.8000
					13:3	21.36	0.0010 - 0.0001
					15:1	128.08	0.0010 - 0.0001
	2	34	21	55	3:1	5.09	0.0300 - 0.0200
					9:7	0.69	0.5000 - 0.4000
					13:3	13.63	0.0003 - 0.0001
					15:1	95.71	0.0010 - 0.0001
	Combined	66	45	111	3:1	14.29	0.0003 - 0.0001
					9:7	0.46	0.6000 - 0.5000
					13:3	34.59	0.0010 - 0.0001
					15:1	222.75	0.0010 - 0.0001
	Heterogeneity of X^2				3:1	14.61	0.0003 - 0.0001
					9:7	0.48	0.6000 - 0.5000
					13:3	34.99	0.0010 - 0.0001
					15:1	223.79	0.0010 - 0.0001

4.2.4 Relationships between traits

The LCC showed a weak negative correlation with each of NDW and SDW in the F_2 progenies that were derived from cross P_6 x P_1 (Table 4.10). However, there was a positive but weak correlation between NDW and SDW in this set of progenies. In contrast, there was a highly significant ($P < 0.01$) positive correlation between NDW and SDW in 'cross 2' (Fig. 4.10). The LCC was positively correlated to both NDW and SDW in the F_2 progenies that were derived from the cross involving P_4 x P_2 (Table 4.10.

Table 4.10 Coefficient of correlation (*r*) among root nodulation attributes that are associated with nitrogen fixation in F_2 pigeonpea plant populations derived from 'cross 1' (P_6 x P_1) (bottom half) and 'cross 2' (P_4 x P_2) (top half, in bold print).

	LCC	NDW	SDW
LCC	1.0000	**0.1575**	**0.2191**
NDW	- 0.0818	1.0000	**0.3875 ****
SDW	- 0.0949	0.0938	1.0000

** = highly significant at the 1.0% probability level.

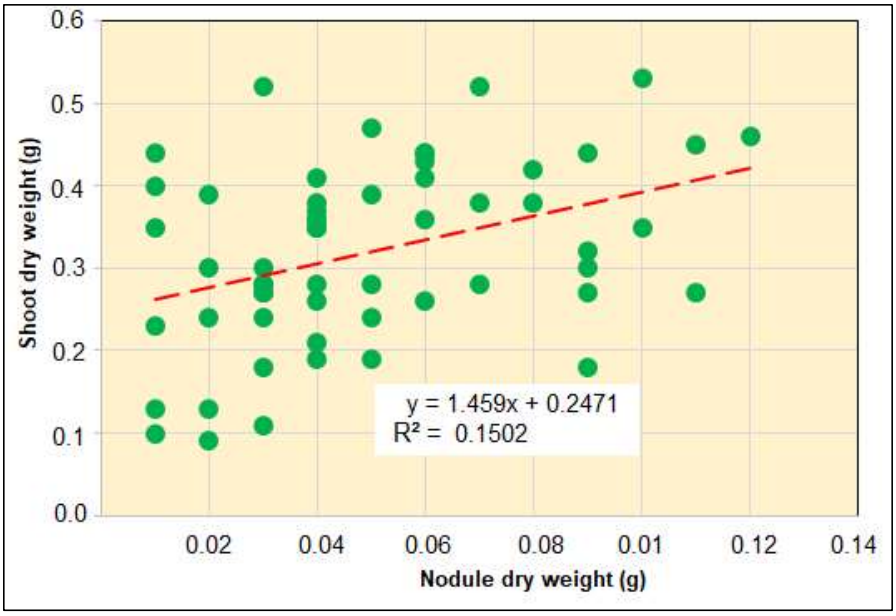

Fig. 4.10 The relationship between shoot dry weight (g) and nodule dry weight (g) in a segregating F_2 pigeonpea plant population derived from the cross P_4 x P_2.

5.0 CHAPTER FIVE: DISCUSSION

The method which was employed to phenotype both the parental genotypes and the F2 progenies was effective and enabled a distinction between the phenotypic classes in the context of the study. For instance, the classification of chlorotic plants and green plants posed minimal difficulty if any. Although the visual scoring of the leaf color can be subjective, the use of the chlorophyll meter presented an unbiased/objective method for determining the plant phenotype. The method was used previously in several crops (Castelli, et al., 1996) including corn (*Zea mays*) (Dwyer et al., 1991), rice (*Oryza sativa*) (Takebe and Yoneyama, 1989; Mahajan et al., 2014; Gosh et al.,2 020) and soybean (Gwata et al., 2003). In addition, the growth stage at which the leaf color scores were determined was consistent with the procedures that were employed in similar previous studies (Yamamoto et al., 2002; Gwata et al., 2004). A further advantage of using both methods was that they are non-destructive. In addition, the nitrogen depleted growth medium that was used consisting of sand and the Hoagland solution, coupled with individual rhizobial strains, created an efficient method for discriminating between the phenotypes that were compatible with the individual rhizobial strains resulting in effective fixation. Therefore, the approach that was used in the study was useful as a rapid, simple technique to identify contrasting parental genotypes for specific nodulation traits that can be used in genetic studies to understand the complex phenomenon of biological nitrogen fixation in pigeonpea and similar leguminous crops.

The LCC, NDW and SDW attributes were assumed to be the reliable indicators of the N fixation effectiveness in this study. In a study involving promiscuous nodulation in soybean, NDW was used as an indicator for N fixation effectiveness (Gwata et al., 2004). Similarly, the LCC is a key indicator of leaf greenness and is equivalent to using a leaf color score which was used as an indicator for N fixation effectiveness in the same study which examined promiscuous nodulation. The SDW was also used as an indicator N fixation in the study on the symbiotic effectiveness of *Bradyrhizobium* strains with adzuki bean (*Vigna angularis*) (Delic et al.,2010). Therefore, the use of each of these traits in examining genetic control is logical. Moreover, the X^2 approach was used widely to study the mode of inheritance and genetic control of various traits in F_2 segregating populations (Nevhudzholi et al., 2020; Sundaram et al., 2019; Mohammed et al., 2014; Bhor et al., 2014; Singh et al., 2011).

The results of the parental evaluation at the six-week growth stage also demonstrated the speed with which genotypes can be screened for N fixation attributes. However, only the leaf color score was a non-destructive method. The strong positive relationship between the NDW and SDW

suggested that the evaluation of only one of the variables will be sufficient in situations that are constrained by manpower availability or where rapid screening studies may be involved. The clear differences in the leaf color (yellow or chlorotic vs green) affirmed that the growth stage was optimum for fixation process under the greenhouse conditions that were utilized in the study. This is partly because the plants will still be growing actively and in need of nitrogen. A similar growth stage for assessing the fixation process was used in soybean (Gwata et al., 2005) although later reproductive stages have also been used (Roy et al, 2019).

The variation in the symbiotic effectiveness of the rhizobial strains strongly supported the observation of promiscuous nodulation which is associated with pigeonpea (Bopape et al., 2020). Secondly, the variation indicated the potential to select for rhizobial strain x host genotype combinations that attain optimum productivity in terms of the measured variables. Thirdly, both the variation and promiscuous nodulation indicated the need to evaluate individual strains against the host plant genotypes particularly where genetic studies are involved. In a study involving the genetics of promiscuity in soybean, a single rhizobial strain was used for inoculating the seed of the six different plant populations (Gwata et al., 2005). Similarly, a single strain was used to study the genetic control of nodule dry weight and leaf color score (Gwata et al., 2004) and RAPD marker analysis in soybean (Gwata et al., 2013). A differential impact of individual rhizobial strains on shoot nitrogen content was also reported in common bean (Mungai and Karubiu, 2011). In the current study, the strain 'R-8' could induce the differential responses in SDW between 'Gen-2' versus 'Gen-6' but attained lowest dry weight of the shoots among the parental genotypes suggesting that the ideal strain which results in high symbiotic efficiency with a set of test pigeonpea genotypes is not necessarily able to discriminate between contrasting parental genotypes for deriving segregating populations for genetic studies. In addition, this observation underscored the importance of identifying the correct contrasting parental genotypes for specific nodulation traits. Therefore, it was appropriate in this study to identify the rhizobial strains that exhibited contrasting effects on respective parental genotypes. In addition, the use of individual rhizobial strains in the study was consistent with the approaches that were used previously in similar studies. It is also important to recognize the possibility of inter-strain competition for nodule occupancy if more than one strain is involved (Rodríguez et al., 2010; Batista et al., 2015; Onishchuk et al., 2017). Nonetheless, co-inoculation with more than one strain in field production of legumes may still be useful if it enhances the productivity of the crop (Atieno et al., 2012., Iturralde et al., 2019). Currently, successful co-inoculations of various legume species are limited largely to rhizobial combinations with growth promoting fungi (Wang et al., 2021) or rhizobacteria

(Koriret al., 2017; Ju et al., 2019) although the benefits to the applicaion are still debatable (Primieri et al., 2022).

The GGE analysis that was used for providing more information on rhizobial strain performance and was useful particularly for obtaining a graphical picture of the interaction between the symbionts. The concept of the ideal rhizobial strain was based on its performance, as depicted by the concentric circles drawn to aid easy visualization and identification of the specific environment (i.e. in this case, the pigeonpea genotype) (Yan and Tinker, 2006). Moreover, the GGE analysis also allowed for determining genotypic stability (Pobkhunthod et al., 2022). Furthermore, the GGE biplot method could discriminate between the ideal and non-performing genotypes as well as the environments for individual N fixation variables. The method was widely used in crop performance (Zurweller et al., 2018; Yan and Hunt, 2001) and adapted for the first time in this current type of study.

The frequency distribution curves that cluster into discrete classes indicate qualitative characters that are governed by a small number of major genes but their segregation patterns may still depart from simple Mendelian inheritance. These situations arise because of interactions between two or more genes or linkage or the effects of modifier genes, thus often making it difficult to discern the relationship between genotype and phenotype (Botstein, 2015; Cockerton et al., 2022). In contrast, the frequency distributions of quantitative characters produce continuous gradations in expression which graphically resemble bell shapes (Muñoz and Abrams, 1971). However, in crop genetics, the analysis of these characters often considers the combined effect of all the genes governing their expression. The analysis of skewness and kurtosis provides information about the nature of gene action (Fisher et al., 1932) as well as the number of genes governing the traits (Robson, 1956). In this study, the frequency distribution curve for the F_2 plant population that was derived from ‘cross 2’ (P_6 x P_1) showed approximately a normal distribution curve but with a slight skew to the right suggesting the presence of epistatic gene action for the LCC trait. In other studies, non-additive integral gene interactions were associated with positive skewness (Bassuony et al., 2022). However, a contrasting pattern was observed for ‘çross 1’ which showed slight skew to left, thus suggesting the existence of duplicate epistatic gene action for LCC (Snape and Riggs, 1975). One of the implications for the skewness to the left is that the gain in selection is expected to be faster when the selection is moderate but slower when the selection is strong (i.e. high selection pressure) (Snape and Riggs, 1975). In addition, the negative skewness alluded to a connection with additive x additive duplicate gene interactions (Bassuony et al., 2022).

The segregation ratio of 9 high:7 low chlorophyll content in 'cross 1' (involving P_4 x P_2) suggested duplicate recessive epistasis in which there is complete dominance at both gene pairs; but, when either gene is homozygous recessive, it masks the effect of the other gene (Miko, 2008). It also suggested that there were nine combinations of alleles in the F_1 generation possessing at least two dominant alleles (one for each gene), which would produce a high chlorophyll content phenotype while, in contrast, potentially seven combinations that could produce a double recessive for each gene resulting in low chlorophyll phenotype. A similar observation was reported previously for the control of anthocyanin biosynthesis in the flower color of peas, for instance (Dooner et al., 1991).

The results also showed that the frequency distribution curves among the F_2 progenies of both crosses were skewed to the left which also suggested the existence of duplicate epistatic gene action for NDW. Furthermore, the segregation pattern in 'cross 2' fitted best a 9 heavy:7 light dry nodule weight, thus affirming the existence of duplicate epistatic gene action. For SDW, the results of also confirmed that the 9:7 ratio was the best fit. Therefore, the study revealed the dominance of heavy over light SDW. The segregation pattern, based on the LCC, of the F_2 population (constituted from at least 111 observations) in the 'cross 1' (P-04-SST x P-02-DC), best fitted the 9:7 ratio indicated the presence of one major gene along with inhibitory epistasis controlling this trait. However, the NDW in the same cross (in the subset 1 population) also best fitted the 9:7 but a 3:1 ratio (in the subset 2 population) indicating dominant alleles of two genes controlling the trait. The results showed that the 9:7 ratio was generally predominant for the traits that were studied thus indicating a high probability that more than one gene, with epistasis are involved in their genetic control. The segregation pattern for LCC fitted the 9:7 ratio for NDW and SDW, thus indicating that the mode of inheritance for both traits in this cross showed the presence of major gene along with inhibitory epistasis. In the cross (P-06-MJ-HBR x P-01-01514), the segregating F_2 generation based on differences in LCC best fitted 3:1 on subset 1 and 2 and when the data were combined the segregation pattern best fitted and 13:3 indicating two major genes (dominant and recessive) along with inhibitory epistasis in the mode of inheritance of this trait. However, the segregation pattern for NDW in the same cross revealed a 9:7 ratio which was previously interpreted as indicating the presence of duplicate recessive epistasis gene (Mohammad at el., 2020).

It is important to note that segregating F_2 populations provide the most appropriate and complete sample for studying complex genetic concepts, such as epistasis and dominance as well as linkage and the analyses of genetic interactions (Giménez et al., 2021). Moreover, the

genetic variability in such populations is important for the selection of superior genotypes (Priyanka et al., 2019). In this regard, the degree of variation that is heritable in the off-spring will contribute to the success of the genetic improvement program (Bello et al., 2012). However, in this study, determination of the heritability of the different traits was limited mainly by the requirement to include sufficient parental and F_1 genotypes in the simultaneous evaluation with the F_2 populations (Pfahler and Barnett, 1989). In approaches, which utilize the variances of just the two parental lines (instead of the F_1 and F_2 variances), it may be difficult to assume unequivocally that such genotypes are true breeding and homozygous (for nitrogen fixation traits) and their respective variances are the result of environmental influences (Gwata et al., 2004). Heritability values (in combination with selection intensity and phenotypic variance) can be used to predict the response to selection (Johnson et al.,1955; Liu, et al.,2011; Koebernick et al., 2019). Moreover, the genes that govern each of these nodulation traits do not function in isolation from the rest of the genome. For instance, the nitrogenase enzyme, which is key in catalyzing nitrogen fixation, consists of other proteins (such as the iron and molybdenum-iron proteins) that are necessary for electron transfer and its functioning (Dixon and Kahn, 2004). Several genes encode for these proteins forming integral components of the regulatory networks that govern nitrogen fixation. Nonetheless, the approach that was used in this study can accommodate in future the determination of heritability values for the traits when sufficient pre-requisite facilities become available.

6.0 CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

Based on the study findings, the pigeonpea genotypes showed genotypic and phenotypic variation in root nodulation traits based the leaf color content (LCC) and nodule dry weight (NDW) as well as shoot dry weight (SDW). The results revealed that there was compatibility between some rhizobia strains (R1, R7, R12, R18, R24, and R28) and pigeonpea genotypes (Gen-2 and Gen-3). The contrasting genotypes could provide some useful material for genetic studies involving nitrogen fixation traits. However, this study was the first one in which the genetics of root nodulation in pigeonpea alluded to the gene action exhibiting dominance of at least two gene pairs that are involved in controlling the LCC, NDW and SDW. Further validation studies of these results will be required.

The results of the parental evaluation at the six-week growth stage also demonstrated the speed with which genotypes can be screened for nitrogen fixation using a non-destructive method such as the leaf color score. The strong positive relationship between the NDW and SDW suggested that the evaluation of only one of the variables will be sufficient in situations that are constrained by manpower availability or where rapid screening studies may be involved. In addition, the variation in the symbiotic effectiveness of the rhizobial strains that was observed in this study strongly supported the conclusion that pigeonpea nodulates promiscuously. Moreover, the variation indicated the potential to select for rhizobial strain x host genotype combinations that attain optimum productivity in terms of the measured variables. Because of both the variability and promiscuous nodulation in this crop, it will be necessary to evaluate individual strains against the host plant genotypes particularly where genetic studies are involved. The GGE analysis method which was applied in this study elucidated the graphical picture of the interaction between the symbionts.

The segregation ratio of 9 high:7 low chlorophyll content in the cross ‘P4 x P2’ suggested duplicate recessive epistasis in which there is complete dominance at both gene pairs; but, when either gene is homozygous recessive, it masks the effect of the other gene. The results also showed that some frequency distribution curves among the F_2 progenies of both crosses were skewed to the left which suggested the existence of duplicate epistatic gene action for NDW. The determination of the heritability of the different traits was limited mainly by the lack of sufficient resources that would produce reliable estimations that can be exploited in predicting the response to selection for the nodulation traits. It is recommended that future studies should include the determination of heritability values that can be used in breeding programs aimed at the genetic improvement of nitrogen fixation in pigeonpea. It may also be necessary to combine classical

Mendelian genetics with modern genomics tools to gain a better understanding of the complex nature of nitrogen fixation in pigeonpea as well as its genetic manipulation (Valentine et al., 2018; Black et al., 2012; MacLean et al., 2007; Weidner et al., 2003).

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Appendix

Appendix 2.1

Composition of the nitrogen free Hoagland solution which was used in the study.

Chemical		Weight
KCl		100.0g
$Ca_3 (PO_4)_2$		100.0g
$CaSO_4.2H_2O$		100.0g
$MgSO_4.7H_2O$		156.0g
Nafe solution		400.0ml
Trace element		40.0ml
	Nafe solution	
	N_2EDTA	15.6g
	$FeSO_4.7H_2O$	15.0g
	Trace element solution	
	LiCl	0.2g
	$CuSO_4.5H_2O$	1.0g
	$ZnSO_4.7H_2O$	1.0g
	TiO_2	1.0g
	H_3BO_3	1.0g
	$AL_2(SO_4)_3$	1.0g
	$SnCl._2.2H_2O$	0.5g
	$MnCl_2.4H_2O$	7.0g