GENETIC DIVERSITY OF INDIGENOUS *BRADYRHIZOBIUM* STRAINS NODULATING DUAL-PURPOSE SOYABEAN (*Glycine max L.* Merr.) GENOTYPES AND THEIR POTENTIAL TO FIX NITROGEN IN KENYA

VICTOR WAFULA WASIKE

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DECLARATION AND RECOMENDATION

DECLARATION

This is my original work and has not been presented for award of a degree or diploma in any other university.

Signed: ---------- Date 6/4/2012

Victor Wafula Wasike

RECOMMENDATION

We hereby declare that the work has been presented with our approval as supervisors.

Signed: ---------- Date 10/4/2012

Dr. Nancy W. Mungai
Department of Crops Horticulture and Soils

Signed: ---------- Date 6/4/2012

Prof. Francis N. Wachira
Department of Biochemistry and Molecular Biology

Signed: ---------- Date 04.2012

Prof. Louis M. Mumera
Department of Crops Horticulture and Soils
Egerton University

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DEDICATION

This thesis is dedicated to my parents, Hilary and Rozina Wasike and to my wife, Sara, for their love, patience and support.

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ABSTRACT

Dual-purpose / promiscuous soyabean [*Glycine max* (L.) (Merrill)], have recently been introduced in Kenya. The objectives of this study were to determine the natural nodulation of promiscuous soyabean varieties under different inputs, assess the genetic diversity, effectiveness and competitiveness of indigenous *Bradyrhizobium* strains and determine the soyabean N credit to maize of managed fallows. Varieties were grown under phosphorus, phosphorus + lime and nitrogen + phosphorus + lime in two highland sites and under DAP (18:46:0) in three lowland sites. Genetic diversity was assayed using the PCR-RFLP markers by amplifying the 16S-23S rDNA IGS region and sequencing the 16S rRNA gene. Indigenous strains were compared with those deposited in the GenBank through BLAST. Selected strains were tested for effectiveness and competitiveness in sand and soil respectively. Competitive strains were inoculated on released promiscuous soyabean varieties and maize grown at three N rates (0, 30, 60 kg ha^{-1}) to estimate soyabean N credit to maize in a split plot design field trial replicated three times at two sites. Data from greenhouse and field studies were analyzed using ANOVA and means separated using LSD and DMRT at $p<0.05$. In presence of P, promiscuous varieties showed improvements in nodulation (102 to 280 nodules per 0.5 m) than the local variety (3 to 167 nodules per 0.5m). In absence of P, none of the varieties performed better than the local control. PCR-RFLP analysis distinguished 18 intergenic spacer groups (I-XVIII) in the highlands and eight (A-H) IGS groups in the lowlands sites. The IGS groups were specific to sites and treatments but not varieties. All indigenous strains belonged to *Bradyrhizobium* genus. *Bradyrhizobium japonicum, Bradyrhizobium elkanii and Bradyrhizobium* spp related strains were predominant in highland and lowland sites accounting for 37.5%, 30.0% and 25.0% respectively, while *Bradyrhizobium yuanmigense* related strains accounted for 7.5%. Seventy percent of the tested indigenous strains were more effective than USDA 110. TSBF 531 was the most competitive indigenous strain. Maize grain yield following promiscuous soyabean (SB19) inoculated with TSBF 531 increased by 38.30% over that following maize, 20.60% over that following local soyabean, 18.50% over that following fallow and by 12.96% over that following 1:1 mixture of TSBF 531 and TSBF 442 strains. Phosphorus should be applied when cultivating introduced promiscuous soyabeans in Kenya. Indigenous elite *Bradyrhizobium* strain, TSBF 531, is effective and competitive and can be used in soyabean inoculants. It is recommended that maize be grown in sequence with promiscuous soyabeans inoculated with TSBF 531 in order to achieve economic maize grain yields.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
BNF	Biological Nitrogen Fixation
BLAST	Basic Local Alignment Search Tool
CEC	Cation Exchange Capacity
CIAT	International Centre of Tropical Agriculture
CL	Coastal Lowlands
DNA	Deoxyribonucleic acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
GTZ	German Technical Cooperation
IGS	Intergenic Spacer
IITA	International Institute for Tropical Agriculture
ISFM	Integrated Soil Fertility Management
KAFPROD	Kenya Accelerated Food production
KARI	Kenya Agricultural Research Institute
KEPHIS	Kenya Plant Health Inspectorate Service
NCBI	National Centre for Biological Information
NPT	National Performance Trial
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
TSBF-CIAT	Tropical Soil Biology and Fertility Institute of CIAT
USDA	United States Department of Agriculture

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background Information

Soyabean [*Glycine max* L. (Merrill)] also known as soybean, Chinese pea and Manchuria bean, is a native crop of East Asia. It is one of the most popular oilseeds in the world because of factors related to its composition and productivity. It has the highest protein content of all pulses (40%) double that of commonly used pulses in Africa (cowpea and common beans), and a high fat content comparable to that of groundnuts. After oil extraction, soya cake forms an important ingredient in animal feeds. Most of the soyabean in Africa is produced by South Africa, Nigeria, Uganda, Rwanda, Zimbabwe, Democratic Republic of Congo (DRC) and Malawi (FAO STAT, 2008).

In the 1970s, the International Institute for Tropical Agriculture (IITA) identified soyabean as an alternative source of inexpensive high quality protein for improving nutrition and health and livelihoods of rural communities in Africa (Sanginga and Okogun, 2003). Research was initiated to develop management practices that would optimize yields and varieties that were adapted to the African environment. In Nigeria, for example, increased soyabean production was stimulated through increases in home consumption and increased demand by food and feed millers (Chianu *et al.*, 2011). Recipe development using soyabean to enhance nutritional value and taste of traditional Nigerian dishes without increasing cooking time and cost spurred increased local consumption of soyabeans and its products and also stimulated both local production and industrial processing. This approach is expected to apply in Kenya.

A common problem with soyabean is that the nitrogen fixation process is not always working optimally or often not at all due to the absence of effective N fixing bacteria. Additionally, although inoculation would potentially resolve this problem, experience with farm conditions had not been successful (Sanginga and Okogun, 2003). The challenge then was to develop soyabean varieties capable of producing nodules with indigenous rhizobia in African soils (promiscuous nodulation) to fix atmospheric nitrogen without the need to inoculate seed with purchased inoculum (Sanginga and Okogun, 2003). These varieties have been developed and successfully deployed for cultivation in Nigeria (Kueneman *et al.*, 1984; Abaidoo *et al.*, 2000). In order to optimize soyabean yields under farm conditions, and to

avoid the need to inoculate soyabean with *Bradyrhizobium japonicum,* soyabean breeders at IITA developed new soyabean germplasm known as Tropical Glycine Cross (TGx.) which nodulate with *Bradyrhizobium* spp. populations indigenous to African soils (Kueneman *et al.,* 1984; Pulver *et al.,* 1985; Abaidoo *et al.,* 2000). However, TGx soyabean genotypes in some locations, are reported to develop nitrogen deficiency symptoms (Okereke and Eaglesham, 1993), suggesting that they may require inoculation with superior *Bradyrhizobia* at some location if high soyabean yields are to be realized (Kueneman *et al.,* 1984; Abaidoo *et al.,* 2000). TGx soyabean varieties have been tested in Eastern and Southern African region as promiscuous varieties (Javaheri *et al.*, 1994; Wasike, 1998; Kasasa, 1999; Mpepereki *et al.*, 2000) with mixed results. In Kenya the cultivation and use of soyabeans is still low but increasing. The use of promiscuous soyabeans with its potential benefits in soil fertility improvement is still limited. Due to poverty, farmers in Kenya grow continuous maize in their fields often resulting in low grain yields. It has however been known that when maize is grown after soyabeans, there is N savings made to realize optimum yields. This is called soybean N credit. N credit is defined as the amount of nitrogen needed for optimum yield in a soyabean-maize rotation as that needed for maize following maize (Bergerou *et al.,* 2004). In Kenya this N credit for elite indigenous strains of rhizobia has not been determined.

1.2 Statement of the Problem

Soil fertility especially N is critical to crop production in Kenya. While legumes such as soyabean are known to improve soil fertility through N fixation, traditional soyabean varieties require inoculation with exotic rhizobia in order to nodulate and fix nitrogen. The use of introduced exotic rhizobia is often logistically cumbersome, and raises quality issues especially when they are delivered to far flung rural areas in harsh environmental conditions. Promiscuous soyabean genotypes were bred at IITA to nodulate with indigenous populations of *Bradyrhizobium* strains in African soils. However, introductions of these varieties in Kenya have given inconsistent results. In some locations, nodulation has been low or even absent while profuse in others. Indigenous rhizobia are also known to be diverse in effectiveness and nodulation within and between hosts and locations. Poor nodulation and nitrogen fixation has been attributed to the low persistence and low numbers of the rhizobia in the soil, and low competitive ability of introduced *Bradyrhizobium* for infection sites. Few previous studies have been conducted in Kenya to assess the diversity of indigenous rhizobia nodulating promiscuous soyabeans and to select more efficient strains. Assessing the diversity of indigenous rhizobia would facilitate the identification and selection of efficient

strains. These strains would form a suitable, cheap and sustainable substitute for inorganic nitrogenous inputs in the promotion and use, as inoculants, of more efficient indigenous *Bradyrhizobium* strains and promiscuous soyabean varieties suitable for different agro-ecological zones of Kenya. Little research has been done in Kenya to determine the extent of natural nodulation of newly introduced promiscuous soyabean varieties, the genetic diversity, effectiveness and competitiveness of indigenous rhizobia nodulating promiscuous soyabean varieties. Besides, there is scant information on the soyabean N credit to maize when grown in sequence with promiscuous soyabean varieties inoculated with elite indigenous strains of rhizobia in Kenya. There is therefore need to identify, characterize and select effective and competitive indigenous *Bradyrhizobium* strains and to quantify the nitrogen credit to maize by soyabean inoculated with elite indigenous strains of rhizobia.

1.3 Objectives

1.3.1 Broad Objective

To contribute to food and nutritional security through development of sustainable soyabean inoculants technologies for improved soil fertility management in Kenya.

1.3.2 Specific Objectives

(i) To determine the natural nodulation of promiscuous soyabean varieties bred in West Africa in the two selected Kenyan agro-ecological zones under different inputs.

(ii) To assess the genetic diversity of indigenous *Bradyrhizobium* strains nodulating promiscuous soyabean varieties in different sites in Kenya.

(iii) To determine the best-bet promiscuous soyabean variety-*Bradyrhizobium* strain combination (s) for nodulation and biomass production.

(iv) To determine the competitiveness of selected indigenous rhizobia in promiscuous and specific soyabean variety nodules.

(v) To determine the soyabean N credit to maize from promiscuous soyabean inoculated with elite indigenous strains of rhizobia.

1.4 Hypotheses

The following hypotheses were tested in the study:

(i) Soyabean varieties developed under West African conditions retain their promiscuity and dual purpose nature under different Kenyan ecological conditions.

(ii) Indigenous *Bradyrhizobium* strains nodulating promiscuous soyabean varieties in selected sites in Kenya are genetically diverse.

(iii) There are best-bet promiscuous soyabean variety-*Bradyrhizobium* strain combinations (s) which result in improved nodulation and biomass production / nitrogen fixation.

(iv) Indigenous *Bradyrhizobium* strains nodulating promiscuous soyabeans in Kenya are more competitive than the introduced *Bradyrhizobium japonicum* strain USDA 110 currently used in inoculants.

(v) Maize grown in sequence with nodulated soyabean derives better soyabean N credit relative to maize-maize sequence.

1.5 Justification

Options that provide farmers with immediate benefits are required to reverse the declining soil fertility status resulting from monocropping and lack of rotation in substantial areas in highlands and coastal parts of Kenya. These zones are characterized by biotic and abiotic constraints. Available options include dual-purpose, promiscuous soyabeans (*Glycine max* L. Merr.) that produce a substantial amount of grains and leaf biomass and do not require inoculation with specific *Bradyrhizobium* strains. These varieties nodulate with indigenous rhizobia to fix nitrogen, consequently precluding or reducing the use of inorganic nitrogen sources. They also produce a substantial amount of biomass. These varieties have the potential to increase resilience of farming while providing income to farmers in Kenya. These varieties are a potential entry point for soil fertility improvement in Kenya, provided they retain their promiscuity and dual-purpose character in this new environment. The distribution, genetic diversity, effectiveness, competitiveness and nitrogen equivalence of elite indigenous rhizobia isolates in the country's soyabean growing zones has previously not been reported. Indigenous populations of *Bradyrhizobium* strains adapted to local conditions are ubiquitous and persistent and therefore represent an important reservoir from which superior strains adapted to environmental stresses such as drought, low pH, and aluminium toxicity and low phosphorus levels can be selected. The utilization of these strains and promiscuous soyabean varieties will contribute to improvement of soil fertility and nutrition. Adoption and

cultivation of promiscuous soyabean varieties is also likely to promote and sustain environmental integrity through reduced fertilizer nitrogen application while contributing to food and nutrition security.

1.6 The Scope and Limitations of the Study

This study determined the natural nodulation of indigenous *Bradyrhizobium* strains with a set of introduced promiscuous soyabean varieties bred in West Africa under Kenya conditions. Further, investigations were conducted to determine the genetic diversity of these indigenous rhizobia using polymerase chain reaction-restriction fragment length polymorphism of the 16S-23S rDNA interenic region and sequencing of the 16rDNA gene. A sample of strains from the monomorphic and predominant IGS groups were selected and tested for nodulation and biomass production with introduced varieties in sand under greenhouse conditions. Although TSBF 442, TSBF 101 and TSBF 531 recorded the highest amount of biomass, TSBF 442 and TSBF 531 were selected due to their preponderance in the soils. Competitiveness of these strains in nodules was tested on three varieties in three soils and TSBF 531 identified to be more competitive than any other tested indigenous strains including introduced strain USDA 110. Maize grain yield increased substantially following promiscuous soyabean inoculated with indigenous strain TSBF 531 over that following maize, local soyabean, fallow management, and 1:1 strain mix of TSBF 531 and TSBF 442 rotations. While the N credit in this experiment is indicated, actual amounts of N fixed by inoculated precursor treatments were not measured due to logistical and budgetary constraints. Estimation of N in this thesis was inferred by the use of dry shoot biomass, an indirect measure for N_2 fixation as described by Abaidoo *et al.*, 2000. Another limitation of this thesis was the use of nodule fresh weight instead of nodule dry weight to delineate an indirect measure of N_2 fixation. However, this limitation was overcome by using nodulation data to support inferences on nitrogen fixation in chapter three.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Role of Legumes in Soil Fertility

In response to increasing population pressure, agricultural production is intensifying in humid and sub-humid parts of Africa (Burney *et al.*, 2010). In these parts, relatively new crops such as tea, coffee, sugar cane and cotton are replacing sorghum, millet and groundnuts in traditional agriculture. As a result, soil degradation and nutrient depletion have gradually increased and now pose serious threats to food production. It has been reported that soils supporting maize (the most common cereal crop in rain-fed agriculture) must supply 50-60 kg N ha^{-1} (usually as nitrate) and 30 kg P ha^{-1} (in form of plant available P) for each ton of grain produced per hectare (Weber, 1996). Eroded soils in many parts of Africa cannot supply the quantities of N required and levels decline rapidly once cropping commences. Nitrogen depletion is reported to be in the range of at least 36 kg N ha^{-1} and may even approach 80 kg N ha^{-1} in some farmers' fields in Nigeria (Sanginga, 2003). Depletion in organic matter was estimated at 4% per year resulting in very low organic carbon levels after 15-20 years of cultivation. Below 0.5 % carbon, the soil supplies less than 50% kg N ha^{-1} and sufficient for only about 1 ton per hectare of maize grain at normal N use efficiency (Carsky and Iwuafor, 1999). Nitrogen derived from indigenous organic matter must hence be augmented from other sources. Such sources include expensive and often unavailable inorganic fertilizers leading to none-use, or more frequently, use of less than recommended rates of N leading to low yields.

The low rates of N use can be attributable to high fertilizer costs as result of removal of subsidies and inefficient marketing systems. Because of the poor and highly eroded soils and relatively high cost of inorganic fertilizer, it is imperative that the inorganic sources be supplemented with organic matter. Of the plant nutrients, N is unique in that its supply and replenishment of soil capital need not entail the direct application of external inputs. Legume nodulated roots as well as their above ground crop residues partially replenish soil organic N. The benefits from biologically nitrogen fixation (BNF) of legumes are both direct, due to intrinsic value of legumes, as well as indirect, as inclusion of legumes affords greater yield stability in adverse growing conditions and benefit companion or crops following non-legumes. Legumes contribute to the N economy of subsequent crops through their symbiotic association with rhizobium therefore adding nitrogen to the system. The amount of nitrogen

added to the system is a function of legume yield, composition and amount of N_2 fixed. Growing of legumes in crop mixtures usually results in the legume fixing most of its N (80-95%) when moisture and other plant nutrients are not limiting. The uptake of soil nitrogen by associated cereal is thought to keep the supply of inorganic N low favouring biological nitrogen fixation (BNF). It would therefore be worthwhile to grow legumes in mixed cropping with cereal rather than in pure stands where biological nitrogen fixation (BNF) would decline due to the increase in soil N supply as legume litter accumulates and releases its N as it is mineralized.

The N_2 fixed by the legume in a mixed crop is available to the associating cereal through direct transfer as exudates from fixing legume roots and nodules, decaying root and nodules or decomposing litter. Exudates from legume roots will contain small amounts of amino acids, ammonia and nitrates (Paynel *et al.,* 2001). The N exudates may be available for uptake by cereal in a mixed cropping situation. However, the amounts would be small. Legumes continually shed old roots and nodules, as a response to environmental stress, which decay and release N which subsequently is available for cereal uptake. Although root and nodule turnover could be high it has been indicated that their contribution to the N supply to the cereal component in mixed cropping is low (Osunde *et al.,* 2004; Paynel *et al.,* 2001).

Depending on the degree of utilization, the senescence of the above ground biomass can add a substantial amount of litter to the soil. The litter decomposes and releases N into the soil. The rate and extent of decay and release of N from litter will depend on its quality (Prescott, 2005). Immediate net mineralization (inorganic N release into the soil) occurs if the N content of the recently added plant litter is higher than the microbial requirements for their own growth. If the N content is lower a net immobilization occurs as microorganisms take up N for their growth and the release of N into the soil will depend on the microorganism' turnover rate. The carbon: nitrogen (C:N) of the recently added plant litter can be used as an indicator of whether immediate net mineralization or immobilization will occur. If the C:N ratio is large then net immobilization occurs, and if small then net mineralization is the result (Mafongoya *et al.,* 2007).

In mixed farming, legumes are used in rotation with cereals. The supply of N to subsequent crops when a legume is included in the rotation is through above ground litter and below ground root and nodule decay. MacColl (1990) compared the N contribution of forage legumes, grasses and non-legumes to the subsequent maize crop. The study reported that the legume *Desmodium uncinatum* can supply between 30-40 kg N per ha per year to a maize crop. In these systems, forages were cut but residues were not removed from the plot.

In a farming system where most of the legume dry matter is removed from the plot, the pathway through which the fixed N contributes to the soil N economy is through leaf fall, root exudates and nodule/root decay. Therefore legumes with a lower harvesting index or lower utilization contributes more to the N economy of the system through leaf fall than those with a high harvest index (Thomas, 1992). The assumption is that the legume is growing well and forms viable symbiotic association with rhizobium and therefore a large proportion of its N requirement is derived from N_2 fixation.

2.2 Soyabean in Kenya

Soyabean [*Glycine max* L. (Merr.)] is an annual legume that is commonly used as human food, feed, in industrial use application as well as a source of bio-energy (Chianu *et al.*, 2009). It contains cholesterol free oil, high in calcium, phosphorus, fibre and low levels of saturated fatty acids (Chianu *et al.*, 2009). Soyabean was introduced to Kenya from Uganda in 1904. Cultivation on a small-scale commenced in the early sixties in Nyanza and Western provinces and by large scale farmers for fodder in Trans-Nzoia, Uasin Gishu, Laikipia, and Nakuru (Njuguna, 1985). Production of soyabean has however stagnated over the years because past efforts were aimed at promoting the crop as a cash crop against a background of limited utilization awareness, research and extension constraints. Besides, there was no real need for soyabean until the economic situation in Kenya began to deteriorate causing the search for alternative sources of proteins to be explored (Kaara *et al.*, 1998). Total soyabean production had risen from 1,000 metric tonnes in 1993 to 6,000 metric tonnes in 1998 mainly from Western, Central, Rift Valley and Nyanza provinces, against a demand of about 30,000 metric tonnes (Kaara *et al.*, 1998). Most of the soyabean is utilized in the livestock feed industry as well as mass feeding programmes in the form of unimix (3 parts of maize milled with 1 part soyabean) (Lokuruka, 2011).

Between 1993 and 1998, aggressive and sustained promotion of soyabean cultivation, marketing and marketing information, utilization and processing, was undertaken by an array of players including the German Technical Cooperation (GTZ) Soyabean Project and the Kenya Agricultural Food Productivity Project (KAFPROD). During this period, a relatively high degree of soyabean awareness was achieved. In an impact study, Kaara *et al.*, (1998), reported an awareness of 70-80% of those interviewed in eastern and western regions of Kenya. This awareness resulted in the creation of markets for certified seed and bean for human food and livestock feed.

In a diagnostic survey conducted in 1993, covering seven agro-ecozones in main soybean producing districts (Meru, Embu, Nakuru, Busia, Kisii and Kakamega), considerably low (452 kg ha^{-1}) soyabean yields were reported (Wasike and Karanja, 1993). There was limited information available to farmers on utilization. Limited marketability of soyabean also affected farmers' decision to grow the crop. It was observed that soyabean was also being grown in varying cropping patterns; from pure stand to intercropping with cotton, sugarcane, cassava, tomatoes, tobacco, citrus and bananas. Average yields of 821 kg ha^{-1} for the pure stand and 332 kg ha^{-1}, 337 kg ha^{-1},186 kg ha^{-1} for intercrops with maize, sugarcane, and cotton respectively, were reported. These low yields were attributed to low plant stands and generally poor crop management. None of the farmers applied fertilizer on soyabean but most did apply it on the main crops. Farmers reported no pest or disease incidences. The three main priority constraints reported were lack of adaptable high yielding varieties, use of sub-optimal fertilizer rates and inoculants and lack of suitable varieties for intercropping with other crops (Wasike and Karanja, 1993). Despite many promotional efforts, current, soyabean production is estimated at about 5000 tonnes against an annual demand of over 100,000 tonnes. The reasons for the failure of previous promotional efforts include: lack of awareness of processing techniques, lack of market, low yields, lack of policy support, and lack of coordination among various agencies supporting soyabean promotion, (Chianu *et al.,* 2011). These constraints largely remain.

A six year (1993-1998) varietal screening exercise in the Rift Valley, Western, Eastern, Nyanza and Central provinces, (364 entries at 13 sites) resulted in the identification and release of six high yielding soyabean varieties (Wasike, 1998). Efforts at identifying promiscuous (dual-purpose) varieties were largely unsuccessful despite screening presumably promiscuous varieties from Nigeria and Southern Africa (Zambia and Zimbabwe). Varieties (TGx. 1627-3F, TGx. 1636-7F, TGx. 1707-4E, TGx.1681-3F, TGx.1681-1F, Magoye, Kaleya, and Hernon 147) were tested specifically because they were reported to have the ability to nodulate with indigenous rhizobia in their countries of domicile. Of these introductions, none gave conclusive results and as a result, the search for promiscuous soybean varieties for Kenya was temporarily suspended.

In recent years, however, a number of varieties developed at IITA and screened in limited areas of western Kenya have shown promise in both promiscuity and ability to produce a large amount of biomass under adequate P conditions, (Wanjekeche, 2004; Chemining'wa *et al.,* 2004; Misiko, 2007). These are named as dual-purpose (promiscuous) varieties. Dual-purpose varieties not only provide grain but also leave a net amount of N in

the soil which subsequent cereal crops can benefit from. Inclusion of these varieties in existing cropping systems in Kenya will assist small-scale resource poor farmers to obviate the need for applying inorganic and often expensive N sources. Promiscuous nodulation would allow soyabean to be introduced into a range of environments where lack of suitable inoculants would otherwise preclude farmers from growing the crop. Small-scale farmers would need only access to seed to be able to grow soyabean which brings multiple benefits in improved household nutrition from the high protein and oil content, cash income from the local soyabean sales, and inputs in nitrogen which enhance soil fertility and contribute to the sustainability of their cropping systems (Chianu *et al.,* 2011). Promiscuous soyabean varieties therefore represent a highly appropriate technology for small-holder farmers in Kenya.

While research on promiscuous varieties has been limited in Kenya, the search for and use of promiscuous soyabean varieties in cropping systems in other African countries has been reported extensively (Javaheri *et al.,* 1994; Kasasa *et al.,* 1999; Giller *et al.*, 2000; and Mpepereki *et al.,* 2000). The potential of promiscuous soyabean to produce a significant amount of grain yield (1.5-2.0 t ha^{-1}) without inoculation has been demonstrated in Zambia with varieties Magoye and Hernon 147 (Javaheri *et al.,* 1994). Significant contributions to soil fertility improvement through biological nitrogen fixation by promiscuous soyabean varieties with relatively low harvest indices have also been reported (Giller and Wilson, 1991).

2.3 Specificity and Promiscuity in Legume Rhizobial Symbiosis

Leguminous plants whether grown as pulses for grain, as pastures, in agro forestry or in natural environments provide a major N input into the ecosystem as a result of their ability to convert atmospheric N_2 to a form that is assimilated by the plant. This ability arises as a result of a symbiosis between the legume and the soil borne N_2 fixing bacteria of the genera *Allorhizobium, Azorrhizobium, Mesorrhizobium, Bradyrhizobium, Rhizobium* and *Sinorhizobium* referred to in this thesis as Rhizobia or *Rhizobium.*

Legume hosts and rhizobia are known to differ in the range of partners with which they can form symbioses. Legumes which nodulate with a restricted number of rhizobial strains (or species), or rhizobial strains which nodulate with a restricted range of host plants are termed as specific such as the symbiosis between *Rhizobium galagae* and *Galega orientalis* or *Galega officinalis* (Lin *et al.*, 1989). Conversely, promiscuity is the ability of a legume host to nodulate with a wide diversity of rhizobial strains or the ability of rhizobial strain to nodulate with a wide diversity of legume host plants. Cowpea (*Vigna unguiculata),*

is so far the most promiscuous legume to have been studied, nodulating with a wide variety of range of fast and slow-growing rhizobia. *Rhizobium* strain NGR234, isolated from a nodule of *Lablab pupureus* in Papua New Guinea, is the best documented case of a promiscuous *rhizobium* strain nodulating legumes from more than 112 genera (Pueppke and Broughton, 1999). Promiscuous genotypes of soyabean, also called naturally nodulating, are nodulated by slow growing, *Bradyrhizobium japonicum* (Jordan, 1982), *Bradyrhizobium elkanii,* (Kuykendall *et al.,* 1992) and *Bradyrhizobium liaoningense* (Xu *et al.,* 1995), as well as the fast growing S*inorhizobium freidii* (Scholla and Elkan, 1984; Chen *et al.*, 1988) rhizobia. Variability in soyabean nodulation specificity of soyabean genotypes has long been recognized. Although soyabean has been known to be specific in its rhizobial requirements, there were early reports of soyabean nodulating in even the North American soils with no history of soyabean inoculation (Sanginga *et al.,* 1996). Even then, marked differences in nodulation ability were observed between different cultivars of soyabean growing in the same field (Sanginga *et al.,* 1996; Mpepereki *et al.,* 2000). Some studies, however, have reported that N_2 fixation is dependent on both the plant and nodulating bacteria (Sanginga *et al.,* 1996; Kishinevsky and Zur, 1997).

2.4 The Potential of Promiscuous Soyabeans Varieties in Kenya

Declining soil fertility is a major limitation to crop production in small holder farms in Kenya (Cheruiyot, *et al*., 2001; Chemining'wa *et al.,* 2004). This has had serious implications on food security and livelihoods. The decline in small-scale crop production systems in Sub-Saharan Africa including Kenya can be attributable to no or inadequate application of fertilizers especially N. Reasons for no or inadequate fertilizer use include poverty or cost of fertilizer (Place *et al.,* 2005), accessibility, infrastructure and attitude (Misiko, 2007). Additional factors which cause inadequate application of nutrients include leaching of nutrients especially N in high rainfall areas, erosion, denitrification and crop off-take without recapitalization (Vanlauwe and Giller, 2006). The decline in crop productivity can be reversed by implementation of integrated soil fertility management (ISFM) paradigm (Vanlauwe and Giller, 2006). Integrated soil fertility management refers to socially acceptable sustainable soil management practices that integrate biological, chemical, physical, social, cultural and economic processes regulating soil fertility (Vanlauwe *et al*., 2002). This includes the application of a combination of fertilizer, crop rotation, N rich green manures or inclusion of legumes including promiscuous soyabean varieties in the cropping system, among other options (Vanlauwe *et al*., 2002).

Promiscuous soyabean varieties can adapt to a range of climatic conditions prevalent in most arable agro-ecological zones (upper midlands to lower midlands) in Kenya (Jaetzold and Schmidt, 1983). These zones covering central, eastern, central rift and Nyanza provinces form the breadbasket of Kenya and are characterized by bimodal rainfall regimes, humid conditions and well drained slightly acidic soils. Maingi *et al.* (2006) reported the presence of indigenous population of rhizobia able to nodulate promiscuous varieties in the lower midlands (LM_{5-6}) and upper midland (UM_{2-3}) agroe-cological zones in Kenya. In this study, the population of *Bradyrhizobia* specific to promiscuous soyabean varieties at the lower midland was between $7.81x10^2$ and $5.67x10^3$ while the population at the upper midland site was between $2.37x10^2$ and $1.73x10^3$ cells per gram of soil and were able to nodulate promiscuous soyabean variety TGx 1869-31E. Elsewhere, Wanjekeche *et al.* (2004) demonstrated that promiscuous soyabean varieties produced significantly higher biomass than local varieties in the north Rift. This biomass could be used as livestock fodder and for soil fertility improvement in small land holdings (< 2 ha) typical in the study areas of western, eastern and coastal Kenya. Crop rotation is not feasible for such farmers who have the challenge of satisfying the demand for food for family and for cash income. Application of green manures is also an option available to farmers. However, it has not been adopted on a wide scale due to the drudgery of having to plant and turn under the green manure plants without the benefit of harvested grains (Vanlauwe *et al.*, 2002). The inclusion of a legume, such as promiscuous soyabean, in the cropping system offers an attractive option as it will supplement the protein needs at the home level in addition to N_2 fixation and/or N sparing effect. The amount of atmospheric N_2 fixed depends on the yield of the variety and the proportion of N_2 derived from fixation. The value of inclusion of promiscuous soyabean in a cropping system can only be determined by accurate measurements of the N fixed in the field. In recent years, promiscuous soyabean varieties were introduced and tested in western Kenya. In a farmer evaluation, these varieties were preferred over the local checks due to their superiority in grain yield (500-1500 kg ha^{-1}), biomass production, weed and striga suppression, low P requirements and their nodulation (Misiko, 2007).

2.5 Genetic Diversity of *Bradyrhizobium* strains

Biological diversity refers to all species of plants, animals and microorganisms existing and interacting within an ecosystem (Vandermeer and Perfecto, 1995). It is also defined as the variety and variability of all living organisms including the diversity of plants,

animals, fungi, algae, bacteria and other microorganisms, their genetic variability, the natural communities in which they live, and the processes and interactions that weave the biological and physical elements of the planet into a complex web (Altieri, 1994). Biological diversity benefits mankind in many ways including performing many ecological services such as recycling nutrients, control of micro-climate, regulation of the abundance of undesirable organisms and detoxification of noxious chemicals (Altieri, 1999).

Genetic diversity refers to variation in genetic makeup among individuals of the same species and occurs within and between populations. Variation within the gene pool increases the chance that a species will adapt to changing environmental conditions. Natural and human-induced change in biodiversity is inevitable. Factors that bring about change in biological diversity range from local disturbances such as pollution, to shifts in global climatic conditions (Altieri, 1994). Although bacteria are known to constitute a small proportion of the soil biomass, they are characterized by high genetic diversity (Delong, 1997; Hugenholtz *et al.,* 1998). Genetic diversity is caused by changes in the genetic makeup of the bacteria and although the phenotype may appear similar the genetic makeup could be completely different.

The changes in genetic makeup of microbial populations could be caused by such mechanisms as point mutations, gene duplication, gene rearrangements and loss, horizontal (lateral) gene transfer (Virdi and Sachdeva, 2005). Genetic diversity of microorganisms may have far-reaching implications for soil fertility. For example, variance in *Bradyrhizobia* effectiveness in N_2 fixation, their resilience in survival in polluted soils and their capacity to biodegrade plastics and effluent detoxification. The study of diversity may also enhance the understanding of evolution, taxonomy and pathogenicity. Genotypic variation in rhizobial bacterial populations can be exploited to select for superior N_2 fixing strains in association with legumes. So far, amongst the microorganisms, diversity of bacterial pathogens infecting human or animal hosts is the most studied. Much less is known about the genetic diversity of plant associated bacteria, though basic concepts would be the same as applicable to human or animal hosts (Virdi and Sachdeva, 2005).

A variety of methods have been used to study genetic diversity in microorganisms. Some of these are the restriction fragment length polymorphism (RFLP) (Laguerre *et al.,* 1994); randomly amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), variable number of tandem repeats (VNTR) (Nakamura *et al.,* 1987), Intergenic Simple Sequence Repeat motifs (ISSR) (Lanham and Brennan 1999). Other methods include the repetitive sequence element-based

strategies like REP (repetitive extragenic palindrome)-PCR, (Stern *et al.*, 1984), ERIC (enterobacterial repetitive intergenic consensus)-PCR (Hulton *et al.*, 1991) and BOX-PCR based fingerprinting multi locus enzyme electrophoresis (MLEE), single locus and multilocus sequence typing (MLST) including multi-virulence locus sequence (Virdi and Sachdeva, 2005). In all these methods, the generated PCR fingerprints are unique to each isolate and are used to group them at strain level. The choice of which method to use is determined by the availability of equipment and the discriminating power between strains typing methods as these influence the picture of the diversity obtained. An enormous amount of data on the genetic diversity of a number of common pathogenic and non-pathogenic microorganisms has been generated using these techniques. An understanding and knowledge of genetic diversity of micro organisms can therefore serve as a rich source for addressing questions related to evolution or development of a pathogen, or symbiont and important lessons derived from this for future use including revealing the existence of variants having varying degrees of pathogenicity in pathogens and infectivity and effectiveness in symbionts and nitrogen fixation.

In the study of diversity of bradyrhizobia from *Faidherbia albida* and various *Aeschynomene* species, AFLP analysis, a high-resolution genotypic fingerprinting technique based on the selective amplification of restriction fragments from a digest of total genomic DNA was used (Vos *et al.*, 1995). The AFLP technique was shown to provide an insight into the extent of genotypic diversity of *Bradyrhizobium* isolates. As a grouping method for new isolates, it has been found to be superior to restriction analysis of amplified 16S rDNA because of its taxonomic resolution (Willems *et al.*, 2000). Since the AFLP procedure is rather laborious, the preferred, alternative rapid technique for grouping of *Bradyrhizobium* isolates, is the use of partial or complete 16S rDNA sequence analysis, but because of the small sequence divergence among *Bradyrhizobium* strains, it offers little scope for assessing diversity among these closely related strains. The spacer region between the 16S and 23S rDNA in bacteria is reported to generally show much more variation, both in length and in sequence (Gurtler and Stanisch, 1996). Recently, restriction analysis of the spacer region has been preferred for grouping bradyrhizobia (Vinuesa *et al.*, 1998; Doignon-Bourcier *et al.*, 2000). Since *Bradyrhizobium japonicum is* reported to have only one rDNA operon (Kundig *et al.*, 1995), amplification and direct sequencing of this region could potentially provide information useful for deducing relationships between these highly related rhizobial organisms /strains. This approach has proved valuable in the study of other highly related

organisms such as slow growing mycobacteria (Roth *et al.*, 1998), *Nocardioides* and related taxa (Yoon *et al.*, 1998) and was used in this study.

2.6 Factors Influencing Biological Nitrogen Fixation

Bacteria of the genus, *Rhizobium* and *Bradyrhizobium* form a symbiotic relationship with various leguminous plants resulting in the development of nitrogen fixing root nodules. The symbiosis involves cell to cell communication between the host plant and the infecting bacterium. Plant roots secrete flavonoids or isoflavonoids which induce the expression of rhizobial *nod* (nodulation) genes (Schlaman *et al.,* 1998; Zhang *et al.,* 2009). This results in the production of bacterial lipo-oligosaccharides *nod* (nodulation) factors, which are usually oligomers of four or five B 1-4 linked N acyl glucosamine residues carrying an N linked acyl group on the terminal (non reducing end) (Downie, 2010). Nod factors are the principal determinants of the host range of a given *Rhizobium* species and specificity is determined by nod gene products that modify the nod factor by the addition of various sustituents such as acetate, sulphate or sugars such as fructose or arabinose (Downie, 2010). The genes for Nod factor production and nitrogen fixation are carried on large symbiotic (Sym) plasmids in some strains (Schlaman *et al.,* 1998).

Factors that influence N_2 fixation also include poor legume growth and nodulation, lack of efficient strains of appropriate rhizobia (Zaman-Allah *et al.,* 2007), salt stress (Delgado *et al.,* 1984), Nod factors determinants by the addition of various substituents such as acetate, sulphate or sugars such as fructose or arabinose (Downie, 2010). Other factors include soil acidity, (pH) (Graham *et al.,* 1994; Giller, 2001), deficiency of phosphorus (Cassman *et al.,* 1981); nutrients (apart from N), temperature, water stress (Aranjuelo *et al,* 2007) and high soil N level (Arreseigor *et al.*, 1997). Soil acidity is a major constraint to crop production in many parts of the world (Hue, 1992). While it has been reported that soil acidity, manganese and aluminium toxicity, negatively affect nitrogen fixation by delaying or reducing nodulation and or reducing the efficiency of symbiosis (Unkovitch *et al.,* 1996), adding lime has shown benefits on growth and nodulation of legumes grown on acid soils (Mapaona *et al.*, 1995). Sanginga *et al.* (1996) suggested that the poor growth of the host resulted in lack of nodulation and ineffective N fixation in a number of soils in West Africa. Sanginga *et al.* (1996) suggested that in west Africa, the use of appropriate rhizobial strains could improve N_2 fixation and consequent growth of the host in N deficient soils or without adequate rhizobial populations. Nutrient deficiencies may restrict the development of a population of free living rhizobia in the rhizosphere, limit the growth of the host plant,

restrict nodulation itself and result in impaired nodule function. Other factors influencing nitrogen fixation include soil factors such as low CEC and soil mineralogy (Boddey *et al.*, 1997).

2.7 Interactions of Mycorrhizae and Phosphorus Nutrition on Nitrogen Fixation

Efficient legume N_2-fixation is highly dependent on an adequate supply of phosphorus. Legumes are thus often more susceptible to P deficiency than cereals or grasses and are more dependent on mycorrhizas for P uptake. When legumes are intercropped with cereals or are grown in mixed swards in pastures, N_2-fixation and production of the legume can be determined by the P availability and mycorrhizal colonization of the legume root (Zain, 1999). Mycorrhizal fungi occur widely in various environmental conditions, and are found in association with a number of leguminous crops and trees. Mycorrhizal fungi are a group of important soil micro-organisms, ubiquitous throughout the world. They are known to improve plant growth by increasing nutrient uptake, increasing the absorbing surface area, mobilizing sparingly available nutrient sources, or by secretion of chelating compounds or ecto-enzymes and may also protect roots from soil pathogens (Perrin, 1990), thereby increasing root growth and nutrient acquisition by the host root. Some studies on vesicular-arbuscular mycorrhiza (VAM)-*Rhizobium* interactions suggest that colonization with efficient endophytes significantly improves phosphorus nutrition and consequently nodulation and nitrogen fixation (Osonubi *et al.*, 1991). While the principal effect of mycorrhiza on nodulation is undoubtedly phosphate mediated, mycorrhiza may have other secondary effects. Potentially limiting factors may include the supply of photosynthates, trace elements or plant hormones. It has been reported that simultaneous inoculation of legumes with *Rhizobium* and vesicular arbuscular mycorrhizae (VAM) causes synergistic beneficial effects (Bagyaraj *et al.*, 1979).

2.8 Methods for Quantifying Amounts of Atmospheric N_2 Fixed by Legumes

Many methods to estimate N_2 fixation under field conditions have been developed since research in this area was initiated over a century ago (Azam and Farooq, 2003). Under conditions where yield is limited by N the dry matter accumulation is positively correlated with the amount of N fixed. Similarly, the number and mass of legume nodules may provide a rough estimate of N_2 fixation (Azam and Farooq, 2003). However, the most sensitive measures of N_2 fixation are the isotope- based methods. Direct exposure of plants to ^{15}N with enrichment greater than the natural abundance of the isotope in the air (0.3663%) has often

been applied as an ambiguous proof of N_2 fixation (Herridge *et al.*, 1990). However, this method is not suitable in the field.

The isotope dilution method, which involves growing both the fixing and non-fixing plant in soil that is enriched with ^{15}N by addition of labeled inorganic or organic fertilizer, has been widely applied (Hardason and Danso, 1993). Measurement of the extent to which the N_2 fixing plant dilutes the ^{15}N enriched N taken up from the soil allows discrimination of the fixed N_2. The non fixing plant often called the "reference plant" may be a cereal or non fixing genotype of a legume crop under test and is used to take up the enrichment of the soil N. This measurement provides the basis from which the contribution of fixed N in the legume can be quantified. The assumptions in these methods are that the fixing and non fixing plant take up N from the same soil volume, i.e. that the distribution of roots and patterns of nutrient uptake from the soil are similar and that they take place at roughly the same time (Herridge *et al.*, 1990).

The other method used to estimate nitrogen fixation is the determination of dry matter production. This is the simplest and cheapest method used to estimate biological nitrogen fixation. Since legumes meet 90 % of their N requirements, through BNF and the fact that biomass yield of crops is dependent on N content, dry matter accumulation by legume plants is usually used to measure efficiency of fixation by different varieties (Azam and Farooq, 2003). However, reliable quantitative estimates of the fixed N are compromised by inherent genetic differences in cultivars in exploring native soil N. This method also pre-supposes the existence of indigenous effective rhizobia. No single flawless method has been identified to determine absolute amounts of fixed N. However, the choice of methods to use is informed by availability of facilities, study objectives and how easy it is to meet assumptions which underpin each method. Since 90% of legumes meet their N needs from rhizobial fixation, and the fact that there is a close correlation between plant N and biomass yield, biomass production method, the simplest and inexpensive method, to determine N fixation was used in this study.

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CHAPTER THREE

NATURAL NODULATION OF PROMISCUOUS SOYABEAN GENOTYPES IN HIGHLAND AND LOWLAND AGRO-ECOLOGICAL ZONES IN KENYA

Abstract

Current interest in soyabean in Kenya is due to its potential role as food, feed, and soil fertility improvement and income generation. However, to fully exploit this potential, there is need to assess the ability of soyabean varieties to nodulate with indigenous rhizobia so as to improve yields and enhance soil improving characteristics. The objectives of this study were to determine the natural nodulation of promiscuous soyabean varieties bred in West Africa in two selected agroecological zones under different inputs. In the presence of P, most promiscuous soyabean varieties showed more substantial improvements in nodulation (8 to 338 nodules per 0.5 m row) than the local variety (3 to 167 nodules per 0.5 m). Increases in nodulation and nodule fresh weights were mainly observed after application of P fertilizer. In the absence of P, few of the varieties performed better than the local control for any of the measured characteristics. P application is therefore required for nodulation and optimal growth of promiscuous soyabean varieties in Kenya.

3.1 Introduction

Soyabean [*Glycine max* L. (Merrill)] has recently gained prominence in Kenya due to its potential as food, livestock feed, for soil fertility improvement and income generation for smallholder farmers. As human food, it is rated as one of the most important sources of high quality protein, edible oils and vitamins (GTZ, 1996). Soyabean cultivation is reported to have picked up in earnest on a small-scale in the early sixties in Nyanza and Western provinces and by large-scale farmers for fodder in Trans-Nzoia, Uasin Gishu, Laikipia and Nakuru (Njuguna, 1985). However, soyabean production has only reached a fraction of the demand of food processors and animal feed millers, while demand is expected to increase to about 150,000 tons per year over the next ten years (TSBF, 2003).

Soyabean varieties currently grown by farmers produce low yields and have low soil improving potential unless inoculated with *Bradyrhizobium* bacteria to enhance their nodulation and nitrogen fixing ability. Most parts of western, central and eastern Kenya regions receive adequate rainfall, have well drained soils, moderate to high fertility with pH ranges of 5.5-7.5 and are considered to have the highest potential for soyabean production and future expansion (TSBF, 2003). Most of the soils in these areas have a high phosphorus (P) fixation capacity necessitating the addition of P for adequate nodule establishment and function (Sinclair and Valdez, 2002). Inoculation success depends on abiotic factors such as pH, temperature and biotic stresses (Brockwell *et al.,* 1991; Kahindi *et al.,* 1997; Zahran, 1999; Musiyiwa *et al.,* 2005a).

Compatible populations of specific *Bradyrhizobium* species necessary for nodulation of soyabean are seldom present in soils where the crop has not been previously grown. *Bradyrhizobium japonicum* inoculation is therefore required in order to achieve adequate and effective nodulation of the crop when first introduced to many tropical soils that may even contain high cowpea rhizobial populations (Caldwel and Vest, 1968). The need to inoculate was considered unfeasible because many African countries were not adequately equipped to deal with problems associated with *Bradyrhizobium* inoculant use in the tropics (Ayanaba, 1977). This constraint remains an important bottleneck in the production of soyabean genotypes with specific *Bradyrhizobium* requirements.

To circumvent this problem, the soyabean breeding program at the International Institute for Tropical Agriculture (IITA), Nigeria, developed soyabean genotypes, designated TGx (tropical glycine cross), which nodulate effectively with indigenous *Bradyrhizobium* spp populations. These promiscuous varieties have been tested in many African countries without

N fertilizer or *B. japonicum* inoculation. Results from these trials indicated that indigenous *Bradyrhizobium* populations do not always meet the N demand of the tested TGx genotypes in many locations in Nigeria (Okereke and Eaglesham 1993; Sanginga *et al.*, 1996) and Eastern and Southern Africa (Mpepereki *et al.*, 2000). The presence of effective indigenous soil bradyrhizobia in sufficient populations can facilitate TGx. varieties to derive nitrogen (N) through biological nitrogen fixation (BNF) and to determine whether or not they will respond to added rhizobia or N fertilizer (Turk *et al.*, 1993). In some soils, as few as 10 competitive indigenous *Bradyrhizobium* cells per gram of soil can act as an efficient barrier to introduced strains (Thies *et al.*, 1991; Abaidoo *et al.*, 2007). Indigenous rhizobia associated with leguminous crops are diverse and exhibit this diversity in their genetic constitution as well as competiveness and effectivity with and between hosts (Padzemik *et al.*, 1977; Hansen, 1994; Pueppke and Broughton, 1999).

The objective of this study was to determine the natural nodulation of seven introduced promiscuous soyabean varieties with indigenous *Bradyrhizobium* strains under phosphorus and lime application in two highland sites and under diamonium phosphate in three lowland sites.

3.2 Materials and Methods

3.2.1 Experimental Sites

Field experiments were conducted in two highland sites; Mitunguu (N 00° 06' 00.5", E 037° 47' 39.2" ; 959 m above sea level) and Bungoma (N 00° 76' 68.0", E 034° 67' 05.7"); 1648 m above sea level) both of which have high potential for soyabean production in eastern and western Kenya, respectively. The predominant soils at Mitunguu (LM_3) (Jaetzold and Schmidt, 1983) and Bungoma (LM_1) are classified as Ferralsols (FAO-UNESCO 1990). The two sites had no known recorded history of soyabean cultivation or inoculation with *Bradyrhizobium* strains. At the lowland sites, field experiments were superimposed on a soyabean National Performance Trial (NPT) conducted by the Kenya Plant Health Inspectorate Service (KEPHIS). The aim was to trap soyabean nodulating indigenous strains from the lowland coastal sites. The lowland sites were Mtwapa (S 03° 56' 02.4", E 039° 44' 24.4", 15 m.a.s.l), Msabaha (S 03° 15' 38.4", E 040° 30' 34.2", 20 m.a.s.l and Chonyi (S 03° 38' 29.8", E 039° 41' 44.2", 256 m.a.s.l). The predominant soils at Mtwapa, Chonyi and Msabaha are classified as orthic Ferralsols, verto-luvic Arenosols and ferralic Arenosols respectively (FAO-UNESCO 1990; Jaetzold and Schmidt, 1983). These sites represent the major and potential soyabean growing agro-ecological zones in Kenya.

3.2.2 Soil Sampling and Analysis

In each of the five sites, top soil (0-15cm) was collected following a 'W' format before fields were prepared for planting. Eight cores per replicate per site were randomly sampled using a 3.5 cm diameter soil auger, bulked and sub-sampled. Organic carbon (OC) content, extractable P and exchangeable calcium were determined as described by Anderson and Ingram, (1993). Soil solution pH was measured using a glass electrode in a suspension of 5.0 g soil in 10 ml water after equilibrating for 60 minutes.

3.2.3 Land Preparation, Planting and Treatment Application in Highland and Lowland sites

Land was ploughed twice using the mould board plough and harrowed once before planting. Seed was surface sterilized with 96% ethanol for 30 seconds and rinsed with sterile water, then surface sterilized with 3.3% w/v $Ca(OCl)_2$ for 3 minutes, and again rinsed with sterile distilled water five times to remove excess disinfectant. In the highland sites, disinfected seed was then hand drilled in rows, 0.45 m apart and later thinned to 0.1 m distance between plants after emergence. A seeding rate of 60 kg ha^{-1} was used during planting. Varieties were planted in a strip plot design replicated three times at each site. Seven promiscuous soyabean varieties namely TGx 1871-12E (SB 4), TGx 1835-10E (SB 8), TGx 1895-33F (SB 9), TGx 1889-12F (SB15), TGx 1893-10F (SB 17), TGx 1740-2F (SB 19), TGx 1448-2E (SB 20) and a local variety (Nyala) were used in this study. TGxs are crosses between non-promiscuous North American and promiscuous Asian soyabean varieties (Kueneman *et al.,* 1984). The varieties were allocated in the main plot and management levels in the sub-plots. Main plots were 2.5 m x 7.2 m while sub-plots were 2.5 m x 1.8 m consisting of 4 rows 2.5 metres long and 0.45 metres wide with within row spacing of 0.1 m. Combinations of varieties and management (control, Phosphorus, Phosphorus + lime and Phosphorus + Lime + Nitrogen) were assigned in three replicates in a strip plot design. Phosphorus was applied at 40 kg ha^{-1}, lime at 1 ton ha^{-1}. Phosphorus, lime and nitrogen were supplied in the form of triple super phosphate (TSP), calcium carbonate ($CaCO_3$) and urea respectively. P and lime were applied before planting by broadcasting and incorporating into the soil. Forty five kg of N in the form of urea was applied at planting while the rest was applied at top dressing. Top dressing was done 25 days after planting by banding and incorporating the fertilizer near the soyabean lines.

In the lowland sites, plots measuring 6 m x 1.8 m with 4 rows of 6 metres each were laid out in a randomized complete block design and replicated three times at each site. Diammonium Phosphate (18:46:0) fertilizer was applied at 123.5 kg ha^{-1}. The fertilizer was drilled in furrows and mixed well with the soils before planting. Cultural practices were similar to those applied in the highland sites

3.2.4 Crop Management and Data Collection

Two hand weedings were done to manage weeds. No pest and disease management measures were undertaken as there were no challenges from pathogens. At full podding (R3) (Fehr *et al.,* 1971), plants from a 0.5 m row section were randomly sampled leaving at least 0.5m from each end of any of the two net plot rows. The entire root system was dug out up to 50 cm depth and the roots mass and nodules were collected. Nodules were separated from the roots, cleaned and weighed. A sub-sample was immersed in 95% ethanol and temporarily kept in glycerol at 30 °C. The rest of the nodules were sliced with a blade and the colour determined. Pink, red, or brown nodules were considered active in fixing N_2 while white or light green nodules were considered non active in fixing N_2 (Somasegaran and Hoben, 1994).

3.2.5 Statistical Analysis

All data was subjected to analysis of variance using the following model for the highland sites:

$$Y_{ijklm} = \mu + E_i + R_{j(i)} + M_k + EM_{ik} + V_l + VE_{il} + VR_{jl(i)} + VM_{kl} + VME_{ikl} + \xi_{ijklm}$$

Where E_i = Effect due to i^{th} environment,

$R_{j(i)}$ = Effect due to j^{th} replicate in the i^{th} environment.

M_k = Effect due to management in the j^{th} replicate,

EM_{ik} = the interaction between i^{th} environment and k^{th} management,

V_l = Effect due to l^{th} variety in the j^{th} replicate,

VE_{il} = Effect due to interaction between i^{th} environment and l^{th} variety,

$VR_{jl(i)}$ = The interaction effect between l^{th} variety and j^{th} replicate,

VM_{kl} = the interaction effect of k^{th} management, and l^{th} variety,

VME_{ikl} = the interaction effect between i^{th} environment, k^{th} management and l^{th} variety while

ξ_{ijklm} is the random error.

The PROC GLM procedure of the SAS system (SAS, 1996) using 'variety' and 'P application' as fixed factor following a strip plot design ('P application' as main plot and 'variety' as sub-plot) and 'replicate within site' and 'variety replicate within site' as random factors was used to determine the significance of the treatment effects.

In the lowland sites, data was subjected to analysis of variance using the following model:

$$Y_{ijk} = \mu + E_i + R_{j(i)} + V_k + EV_{ik} + \xi_{ijkl;}$$

Where E_i = Effect due to i^{th} environment,

$R_{j(i)}$ = Effect due to j^{th} replicate in the i^{th} environment.

V_k = Effect due to variety in the j^{th} replicate,

EM_{ik} = the interaction between i^{th} environment and k^{th} variety, while

ξ_{ijkl} is the random error.

Means were separated using Duncan multiple range test at $p<0.05$.

3.3 Results

3.3.1 Soil Properties

There were variable soil characteristics observed in the sites. In general, soils at the coastal lowlands had relatively lower pH than the highland sites. Soil pH was 6.1 and 6.9 at Bungoma and Mitunguu, respectively, while it was 5.9, 5.5 and 5.7 in Chonyi, Mtwapa and Msabaha respectively. Extractable phosphorus was generally low in all sites and was lowest in Bungoma (2.39 mg P kg^{-1}) and Chonyi (2.53 mg P kg^{-1}) (Table 3.1).

Table 3.1: Soil chemical characteristics of the various environments from which promiscuous soyabean varieties were grown in 2006.

Site	pH (water)	Organic C ($g\ kg^{-1}$)	Total soil N ($g\ kg^{-1}$)	Extract. P ($mg\ kg^{-1}$)	Exch. Ca ($cmol_{(+)}\ kg^{-1}$)	Excha. Mg ($cmol_{(+)}\ kg^{-1}$)	Exch.K ($cmol_{(+)}\ kg^{-1}$)
Chonyi	5.9	1.04	0.11	2.53	7.02	4.4	0.51
Mtwapa	5.5	0.55	0.04	3.89	2.08	0.52	0.19
Msabaha	5.7	0.44	0.03	6.09	1.46	0.70	0.50
Bungoma	6.1	1.01	0.07	2.39	6.23	1.80	0.15
Mitunguu	6.9	2.46	0.25	9.25	17.2	4.60	1.00

+ Values are means of three replicates samples.

3.3.2 Nodulation of Promiscuous Soyabean Varieties in Highland Sites

Nodules were observed on all varieties at the two highland sites and for all treatments. However, the number of nodules varied according to site, variety and treatment (Tables 3.2 and Table 3.3). There were more nodules per 0.5 m and nodule fresh weights at Mitunguu than Bungoma. When averaged over treatments, nodule numbers per 0.5 m ranged from 5 on Nyala to 136 on SB 15, while nodule fresh weight per 0.5 m ranged from 0.26 g for Nyala to 9.71g for SB 8 at Bungoma (Table 3.2). At Mitunguu, nodule numbers per 0.5 m ranged from 124 on SB 17 to 275 on SB 4 while nodule fresh weight per 0.5 m ranged from 9.66 g for SB 17 to 22.40 g for SB 4 (Table 3.3). There was a significant interaction ($p<0.05$) between treatments and varieties on nodule numbers and nodule fresh weights at both Bungoma and Mitunguu (Table 3.2 and Table 3.3). In general, the application of phosphorus resulted in a positive response in nodule numbers and nodule fresh weights in some varieties (SB 15, SB 19, SB 20, SB 4, SB 8 and SB 9) but not others (Nyala and SB 17) in Mitunguu (Table 3.3), while in Bungoma, the application of P resulted a general increment in nodule numbers in all except two varieties, SB 17 and SB 4 (Table 3.2). The application of phosphorus resulted in a significant ($p<0.05$) increment in nodule numbers and fresh nodule weights in SB 15, SB 19, SB 20, SB 4 and SB 9 at Mitunguu and only in SB 15 at Bungoma (Table 3.2 and Table 3.3). The application of P on SB 4 and Nyala resulted in a reduction in nodule numbers and fresh weights at Bungoma although these reductions were not significant. There was also high variability in nodulation among promiscuous varieties resulting in high standard errors (Table 3.2).

Table 3.2: The effect of Phosphorus and lime application on number of nodules per plant and nodule fresh weight of seven TGx soyabean varieties grown in Bungoma, western Kenya in 2006

	Number of Nodules			Nodule fresh weight (g)		
Varieties	Control	Phosphorus	Phosphorus+Lime	Control	Phosphorus	Phosphorus+Lime
Nyala	3 ± (2)a	7± (6)a	5± (4)a	0.10± (0.05)a	0.09 ± (0.09)a	0.58 ± (0.58) a
SB 15	13 ± (9)b	344± (7)a	50± (23)b	1.15 ± (0.77)b	15.80±(0.35)a	4.24 ± (1.72) b
SB 17	41± (11)a	12 ± (5)a	39± (17)a	2.37 ± (0.39)a	1.62± (0.48)a	4.05± (1.96) a
SB 19	18 ± (10)a	36 ± (18)a	77± (9)a	1.90± (1.09)b	2.64± (1.50)b	6.87 ± (0.62) a
SB 20	80 ± (41)a	123 ± (23)a	99 ± (23)a	3.23± (1.59)a	5.83 ± (1.06)a	6.36 ± (1.97) a
SB 4	34 ± (15)ab	8 ± (4)b	84 ± (21)a	3.32 ± (1.09)a	0.96 ± (0.58)a	5.82± (1.68) a
SB 8	46 ± (7)a	103 ± (38)a	81 ± (7)a	5.93 ± (2.06)b	10.72 ±(1.20) a	12.47± (2.10) a
SB 9	53 ± (11)b	88 ± (29)b	150 ± (25)a	4.79 ± (0.73)b	9.03 ± (2.64) a	12.33 ± (0.49) a

Values indicate the means (SE), n = 3. Means followed by the same letter in a row for each variety are not significantly different from each other ($p<0.05$)
Nodules were transformed using the formula: square root (Nodule No.+ 1).

Table 3.3: The effect of phosphorus and lime application on number of nodules per plant and fresh nodule weight of seven TGx soyabean varieties grown in Mitunguu site, eastern Kenya, 2006

	Number of Nodules			Nodule fresh weight (g)		
Treatments	Control	Phosphorus	Phosphorus+Lime	Control	Phosphorus	Phosphorus+Lime
Varieties						
Nyala	167 ± (26)ab	96 ± (38)b	241 ± (11)a	11.95 ± (2.3)a	14.02 ± (2.6)a	17.27 ± (0.77)a
SB 15	177 ± (18)b	278± (22)a	247 ± 53ab	12.74 ± (1.30)b	20.03 ±(1.58)a	17.82 ± (3.78)ab
SB 17	170 ± (19)a	171 ± (9.0)a	30.0 ± (7)b	13.43 ± (1.69)a	13.23 ±(0.69)a	2.31 ± (0.53)b
SB 19	136 ± (29)b	274 ± (53)a	276 ± (9)a	9.32± (1.97) b	18.71 ± 3.63)a	16.89 ± (2.56) a
SB 20	162 ± (12)c	338 ± (36)a	249 ± (47)b	12.95 ± (0.97)c	26.43 ± 2.83)a	19.59 ± (3.66)b
SB 4	205 ± (29)b	338 ± (62)a	261± (17)ab	17.14 ± (2.44)b	28.23 ± 5.17)a	21.82 ± (1.43)b
SB 8	168 ± (12.8)a	229 ± (54)a	211.0 ± (40)a	9.17± (0.69)a	12.49 ±(2.98)a	11.56 ± (2.20)a
SB 9	162 ± (17)b	338± (22)a	324.0 ± (11)a	8.03 ± (1.24)b	24.12 ± 1.59)a	23.09 ± (0.80)a

Values indicate the means (SE), n = 3. Means followed by the same letter in a row for each variety are not significantly different from each other ($p<0.05$).
Nodules were transformed using the formula square (Nodule No.+ 1).

3.3.3 Nodulation of Promiscuous Soyabean Varieties in the Lowland Sites

Number of nodules on 0.5 m at lowland sites ranged from 4 (Nyala) to 54 (SB 19) at Chonyi, while at Mtwapa and Msabaha, nodule numbers ranged from 1 (Nyala) to 64 (SB 19), and 2 (SB 3) and Nyala to 28 (SB 19) respectively (Table 3.4). Variety SB 19 recorded the highest number of nodules at all the lowland sites, while Nyala had the lowest (Table 3.4).

Table 3.4: The effect of site on number of nodules on plants in a 0.5 m row length and fresh nodule weight of six promiscuous soyabean varieties grown in 2006

	Lowland sites (<900 m.a.s.l)					
	Chonyi		Mtwapa		Msabaha	
Varieties	Number of Nodules	Nodule Fresh weight (mg)	Number of Nodules	Nodule Fresh weight (mg)	Number of Nodules	Nodule Fresh weight (mg)
Nyala	4 c [+]	3.02 cd	1 b	1.8 c	2 c	1.9 a
SB15	13 bc	9.8 bcd	5 b	4.2 c	7 bc	3.8 a
SB17	16 bc	10.3bcd	12 b	8.2 c	4 bc	1.6 a
SB19	54 a	32.12 a	64 a	62.6 a	28 a	14.5 a
SB20	33 ab	27.1ab	50 a	45.6 ab	16 ab	7.6 a
SB8	22 abc	15.4 abcd	24 ab	16.3 bc	20 ab	13.4 a
SB9	28 ab	23.2 abc	5 b	3.4 c	10 abc	3.7 a
SB3	5 c	0.89 d	7 b	4.3 c	2 c	3.3 a

[+] Values indicate the mean number of nodules and nodule fresh weights for each variety at each site. Means followed by the same letter in a column for each variety are not significantly different from each other at $p<0.05$ according to Duncan's Multiple Range Test.

3.4 Discussion

In this study, sites from which soils were sampled varied in organic carbon, pH, extractable phosphorus, total N and exchangeable bases. The sites in lowlands were in general more acidic and had lower P content, organic carbon and exchangeable calcium than those at the highland sites. This trend is mirrored in the number of nodules where in general the varieties were better nodulated in highland sites, which had higher pH and phosphorus, than those at the lowland sites. This confirms previous reports which showed that acidity negatively affects nodulation and nitrogen fixation (Giller, 2001). The TGx soyabean varieties developed by IITA breeding program for promiscuity nodulated with indigenous rhizobia at both sites where they were introduced for the first time. The presence of these indigenous *Bradyrhizobium* in both Nigeria and Kenya soils separated by a tropical forest in

central Africa would suggest a common evolutionary path of bacteria influenced by comparable biotic and abiotic conditions in both countries. It could also be due to genetic exchange of *nif* genes through a variety and combination of events such as strain dispersion, genomic combination and horizontal gene transfer among indigenous *Bradyrhizobium* communities along the Nigerian and Kenya contiguous path through the Congo forest. Previous studies (Mulongoy and Ayanaba, 1986) have reported the presence of *Bradyrhizobium japonicum* in some African soils even though soyabean was not commonly grown. Kasasa (1999) and Musiyiwa *et al.* (2005b) reported the presence of indigenous rhizobia nodulating promiscuous soyabean varieties in many soils in Zimbabwe. Some of the isolates were similar or superior in N_2 fixation effectiveness to commercial inoculant strains under greenhouse conditions.

The present study shows that P improved nodulation across tested varieties at both highland sites although the magnitude of this response was higher in Bungoma which had a low inherent soil P status than Mitunguu. In presence of P, nearly all improved varieties had more nodules than the local variety at both highland sites and most of the nodules contained leghaemoglobin indicating active N_2 fixation. Application of phosphorus has previously been shown to increase soyabean nodulation traits (nodule number, nodule mass and size). The significant interaction between varieties and treatments in nodulation response at the sites suggests that some promiscuous soyabean varieties may be less pH sensitive and may require relatively less P than others for optimal nodulation (Munns *et al.,* 1981). Phosphorus requirement has previously been shown to vary among soyabean genotypes (Gunawardena *et al.,* 1993) while the degree of nodulation has also been reported to depend on plant genotype and field site (Sanginga *et al.,* 2000). Promiscuous varieties in Bungoma showed improved growth and biomass yield in response to N fertilizer application as compared to Mitunguu. This could be attributable to the low population of background indigenous bradyrhizobia (Thies *et al.,* 1991). As a result, N_2 fixation induced by the indigenous bradyrhizobial community supplied less than optimal amounts of N. This indicates that in some cases there is need to apply a rhizobial inoculum on the varieties poorly nodulated by indigenous bradyrhizobia.

Conclusion

This study has shown that soyabean varieties bred under lowland West African conditions retain their promiscuity under Kenya conditions. Results also suggest that, there is need to supply P in order to realize the full potential of promiscuous varieties in terms of N_2

fixation and soil fertility improvement. The expression of the promiscuity trait differed according to site. The most promiscuous varieties at Mitunguu site were SB 9, SB 4, SB 20 and SB 15, while at Bungoma, they were SB 15, SB 20, SB 8. This has important implications in the successful introduction of promiscuous soyabean with its multiple benefits including soil fertility improvement in Kenya. While these introduced varieties have been shown to be adaptable and nodulate with indigenous bradyrhizobia in Kenyan soils, there is need to quantify the amounts of N fixed by these varieties since nodule number alone is not considered a substantive indicator for N_2 fixation (Sanginga *et al.,* 1997).

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CHAPTER FOUR

GENETIC DIVERSITY OF INDIGENOUS *BRADYRHIZOBIUM* STRAINS NODULATING PROMISCUOUS SOYABEAN (*Glycine max* L. Merr.) VARIETIES IN KENYA

Abstract

While soyabean is an exotic crop introduced in Kenya early in the last century, promiscuous (TGx) varieties which nodulate with indigenous rhizobia have only been recently introduced. Since the majority of farmers in Kenya cannot afford or access inorganic fertilizer, the identification of effective indigenous *Bradyrhizobium* strains which nodulate promiscuous soyabean could be useful in the development of inoculant strains. The objective of this experiment were to assess the genetic diversity and phylogeny of indigenous *Bradyrhizobium* strains nodulating introduced promiscuous soyabean varieties grown in contrasting sites in the Kenya and to determine genetic relatedness of the indigenous rhizobia with type and other strains in the GenBank.

Genetic diversity was assayed using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the 16S-23S rDNA intergenic spacer region and 16S rRNA gene sequencing. The differences in fingerprints were used to group strains into intergenic spacer (IGS) groups. Strains nodulating varieties in lowland sites were grouped into 8 while those in highland sites were grouped into 18 IGS groups respectively. Predominant groups were A, B and D and I, III and II in the lowland and highland sites respectively. The IGS groups were specific to sites but not varieties. Phylogenetic analysis of the 16S rDNA gene sequences showed that all indigenous strains belonged to the genus *Bradyrhizobium.* Sequencing of 16S rDNA gene also showed that 37.5% of the strains nodulating soyabean in all sites were related to *Bradyrhizobium elkanii,* 30% to *Bradyrhizobium japonicum*, 25% to *Bradyrhizobium* spp and 7.5% to *Bradyrhizobium yuanmingense.* The polymorphism in *Bradyrhizobium* populations from these sites represents a valuable genetic resource that has potential variability for the selection of more effective and competitive strains for use as inoculants to facilitate soyabean production at low cost.

4.1 Introduction

Indigenous rhizobia associated with leguminous crops are diverse. They exhibit this diversity in their genetic constitution as well as competitiveness and effectiveness with and between hosts (Padzemik *et al.,* 1977; Pueppke and Broughton, 1999). A variety of methods exist for the assessment of genetic diversity in closely or distantly related bacterial species. Traditionally, variation has been determined using characteristics such as growth rate, colony morphology (size, shape, color, texture and general appearance) and antibiotic resistance methods (Graham *et al.,* 1991; Somasegaran and Hoben, 1994). However, these methods are not sufficiently discriminative to account for all the variation exhibited in the target species. They cannot delineate sources of observed phenotypic variation into its components that may be due to environmental factors or underlying genetic factors. In recent years, DNA techniques have been used to detect sequence polymorphisms within and between strains of bacteria (Stern *et al.,* 1984; Nakamura *et al.,* 1987; Williams *et al.,* 1990; Hulton *et al.,* 1991; Laguerre *et al.,* 1994; Vos *et al.,* 1995; Lanham and Brennan, 1999; Virdi and Sachdeva, 2005).

The application of PCR-RFLP analysis of the 16S-23 rDNA intergenic region and sequence analysis of the 16S RNA gene are vital tools in clustering genetically related rhizobia. These have been frequently used in microbial taxonomy to determine inter and intra specific relationships (Vinuesa *et al.,* 1998; Abaidoo *et al.,* 2000; Doignon-Bourcier *et al.,* 2000; Sarr *et al.,* 2007). In these methods, the generated PCR fingerprints are unique to each isolate and are used to group them at strain level. Previous studies on the diversity of bradyrhizobia from soyabean have used the PCR-RFLP analysis, a high-resolution genotypic fingerprinting technique based on the restriction of amplified fragments from total genomic DNA (Laguerre *et al.,* 1994). The PCR-RFLP technique was shown to provide an insight into the extent of genetic diversity of indigenous *Bradyrhizobium* isolates nodulating cowpea (Krasova-Wade *et al.,* 2003). Indigenous rhizobia associated with leguminous crops are diverse and usually exhibit this diversity in their genetic constitution as well as competiveness and effectiveness with and between hosts (Hansen, 1994). Previous studies have shown great diversity of strains isolated from nodules of the same legume in both competiveness and effectiveness (Hansen, 1994). Genetic diversity of rhizobia nodulating promiscuous soyabean varieties has not been determined in Kenya. Determination of genetic diversity of indigenous *Bradyrhizobium* populations in Kenyan soils is a valuable step in the development of cost effective strategies to optimize biological nitrogen fixation and thus increase soyabean yields. The objective of this experiment were to assess the genetic

diversity and phylogeny of indigenous *Bradyrhizobium* strains nodulating introduced promiscuous soyabean varieties grown in contrasting sites in the Kenya and to determine genetic relatedness of the indigenous rhizobia with type and other strains in the GenBank.

4.2 Materials and Methods

4.2.1 Isolation of *Bradyrhizobium* Strains from Nodules

Nodules were harvested at reproductive (R1) stage (Fehr *et al.*, 1971) for each variety as described in chapter three. Ten nodules per treatment for each variety were used for analysis. Before analysis each nodule was surface sterilized with 96% ethanol for 30 seconds and rinsed with sterile water, then surface sterilized with 3.3% w/v $Ca(OCl)_2$ for 3 minutes, and three times rinsed with sterile distilled water. From this stage the nodules were manipulated aseptically, in a lamina flow. Each nodule was crushed in 300 µl of sterile water with plastic micro pestles sterilized in 96% ethanol in a 1.5 ml eppendorf tube.

4.2.2 DNA Extraction

Total genomic DNA was extracted according to the procedure described by Krasova-Wade *et al.*, 2003 with modifications made on centrifugation times. DNA from 10 nodules per treatment was extracted by adding 150 µl of 2X CTAB/PVPP buffer [0.2 M Tris HCL, pH 8.0; 0.04 EDTA pH 8.0; 2.8M NaCl; 4% CTAB and 2% (w/v) PVPP] to the nodule suspension and incubating the mixture in a water bath at 65°C for 60 minutes with intermittent shaking at 15 min intervals. This was followed by centrifuging for 15 minutes at 13000 rpm at room temperature and subsequently pipetting the supernatant into a sterile eppendorf tube. To this supernatant, 250 µl of phenol: chloroform: isoamylalcohol (25:24:1 v/v/v) was added and centrifuged at 13000 rpm at room temperature. The supernatant was pipetted and to it added 150 µl of chloroform:isoamyl alcohol (24:1 v/v) and centrifuged for 5 minutes at 13000 rpm at room temperature. The supernatant was pipetted into a sterile eppendorf tube and 100 µl of ice cold isopropanol added and placed at -20°C overnight for DNA precipitation. The mixture was centrifuged for 5-10 minutes at 13000 rpm at 4°C. The resulting DNA pellet was washed with 70% v/v ethanol by centrifugation for 10 minutes at 13000 rpm at room temperature, air dried and re-suspended in 50 µl of sterile double distilled water. Ten µl of RNase (40 µg/ml) were added to the DNA extract and incubated at 37°C for 30 min. DNA was also extracted from the reference strain USDA 110 using the same procedure.

4.2.3 PCR amplification of 16S-23S rDNA Region

The intergenic region between the 16S and 23S rDNA from 289 nodules was amplified by PCR with primers derived from the 3' end of the 16S rDNA (FGPS 1490-72; 5'-TGCGGCTGGATCCCCTCCTT-3') corresponding to positions 4 (1521-1541) of *E. coli* gene (Navarro *et al.* 1992) and from the 5' end of the 23S rDNA (FGPL 132-38; 5'-CCGGGTTTCCCCATTCGG-3') corresponding to positions 6 (114-132) of *E. coli* gene (Ponsonnet and Nesme, 1994) within the rDNA operon close to the 16S-23S intergenic spacer. PCR amplification was carried out in a 25 µl reaction volume containing 2µl of pure total DNA extract, freeze dried beads (Ready-to-Go PCR beads, Pharmacia Biotech, Uppsala, Sweden) containing 2.5 U of Taq DNA polymerase, 200 µM Tris-HCL, (pH 9 at RT), 50 mM KCL, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1.0 µM of each primer. PCR amplification was performed in a Primus 96 ^{plus} thermal cycler (MWG AG BIOTECH) adjusted to the following program: initial denaturation for 5 minutes at 94°C, 35 cycles of denaturation (30 sec at 94°C), annealing (30 sec at 58°C) and extension (30 sec at 72°C) and a final extension (7 min at 72°C). PCR amplified DNA was visualized by electrophoresis of 3 µl of the amplified DNA on 1% (w/v) horizontal agarose gel in TBE buffer (1.1 w/v Tris-HCL; 0.1% w/v $Na_2EDTA\ 2H_2O$; 0.55% w/v Boric acid), pre-stained with 3.5µl of ethidium bromide (1µg/ml). The gel was photographed under UV illumination with Gel Doc (BIO-RAD) Software (USA).

4.2.4 Restriction of 16S-23S rDNA Region

Aliquots (10 µl) of PCR products (46 nodules from lowland sites and 289 nodules from highland sites) were digested with 1 µl of the restriction endonuclease *Msp* I in 2 µl double distilled water and 2 µl of buffer in a total volume of 15 µl for 3 hours at 37°C. The restriction fragments were separated by horizontal gel electrophoresis in 1X TBE buffer on 3% (w/v) agarose (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) pre-stained with 3.5 µl of ethidium bromide (1µg/ml). The gels were run at 100 V for 3 hours and photographed under UV illumination with Gel Doc (BIO-RAD, USA) software.

4.2.5 Sequence Analysis of the 16S rRNA Gene

A sample of thirty nine 39 *Bradyrhizobium* isolates (29 from highland sites and 11 from lowland sites respectively) isolated from different varieties grown at different sites and treatments were selected for 16S rRNA gene sequencing at Macrogen in South Korea. The forward primer 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) corresponding to positions 27-46 of the *E. coli* of the 16S rRNA gene sequence and the reverse primer 1492R (5′-GGTT TAC CTT GTT ACG ACT T-3′) corresponding to positions 1525-1506 of *E. coli* (Lane, 1991) were used to amplify the 16S rRNA gene. The 1500 bp PCR products were sequenced for the DNA region coding for the 16S rRNA gene in an ABI 377 (PE-Applied Biosystems sequence analyzer. The generated sequences were submitted to the national council for biological information (NCBI) GenBank database through basic local alignment search tool (BLAST) to search for significant 16S rRNA alignments. A phylogenetic tree was constructed based on the partial 16S rRNA gene sequences of the TGx soybean nodule isolates and rhizobial reference strains from the GenBank. The sequences of the rhizobial strains were aligned pair wise and compared to type strains in the GenBank database. A dendogram was inferred with Neigbour-Joining Algorithm (Saitou and Nei, 1987) using ClustalX software (Thompson *et al.,* 1997) and the phylogenetic tree reconstructed with PHYLIP (Felsestein, 1993), package and a bootstrap analysis using 100 replications. Shannon's index of diversity (Ho) was estimated based on the number of strains belonging to each genus / species (Shannon and Weaver, 1949) using the formula:

$H_o = -\Sigma P_i \ln P_i$; where;

I = an index number for each strain (restriction profile) present in the sample

$P_i = n_i / N$ = the number of strains within a sample (n_i) divided by the total number of strains (N) present in the entire sample with similar restriction profiles

ln = the natural log

4.3 Results

4.3.1 Isolation of DNA

Whole genomic DNA was extracted from all the sampled nodules. It was of high molecular weight and was suitable for further processing through polymerase chain reaction (Figure 4.1).

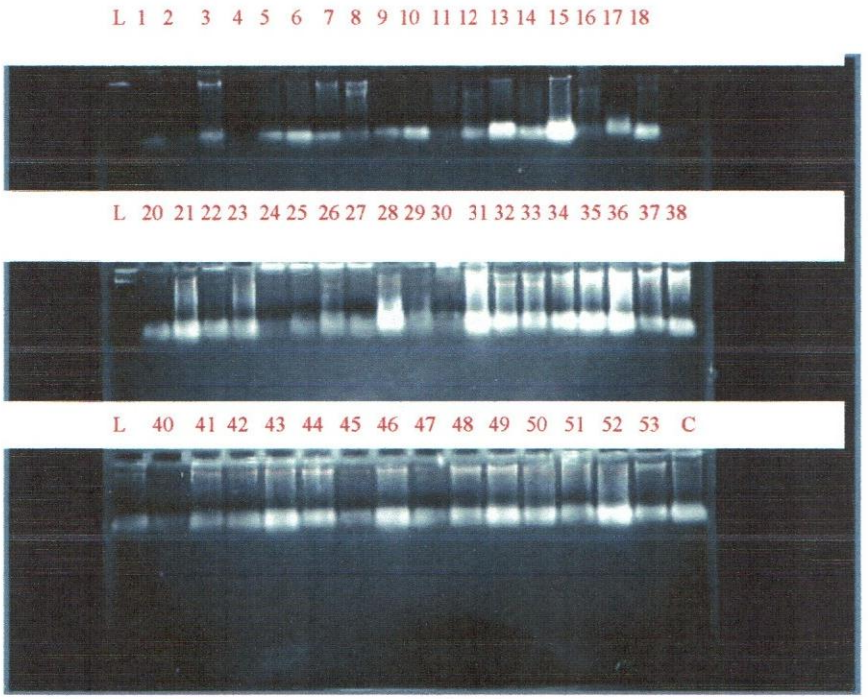


Figure 4.1: DNA extracted from *Bradyrhizobium* strains in nodules from varieties grown in different treatments. +Numbers represent the strains. L indicate the 100 base pair marker while C is the USDA 110 control

4.3.2 PCR-RFLP of 16S-23S rDNA IGS Region

Single IGS PCR products ranging from 930-1100 bp were obtained from all the analyzed nodules and one reference strain (USDA 110) (Figure 4.2). Each nodule presented a single profile in all the nodules analyzed in lowland and highland sites. The PCR amplicons generated from the nodules were further processed by restriction with *Msp* I enzyme. The recognition site for *Msp* I is C:CCG or GCC:C. Profiles generated revealed different IGS phenotypes (Figure 4.3).

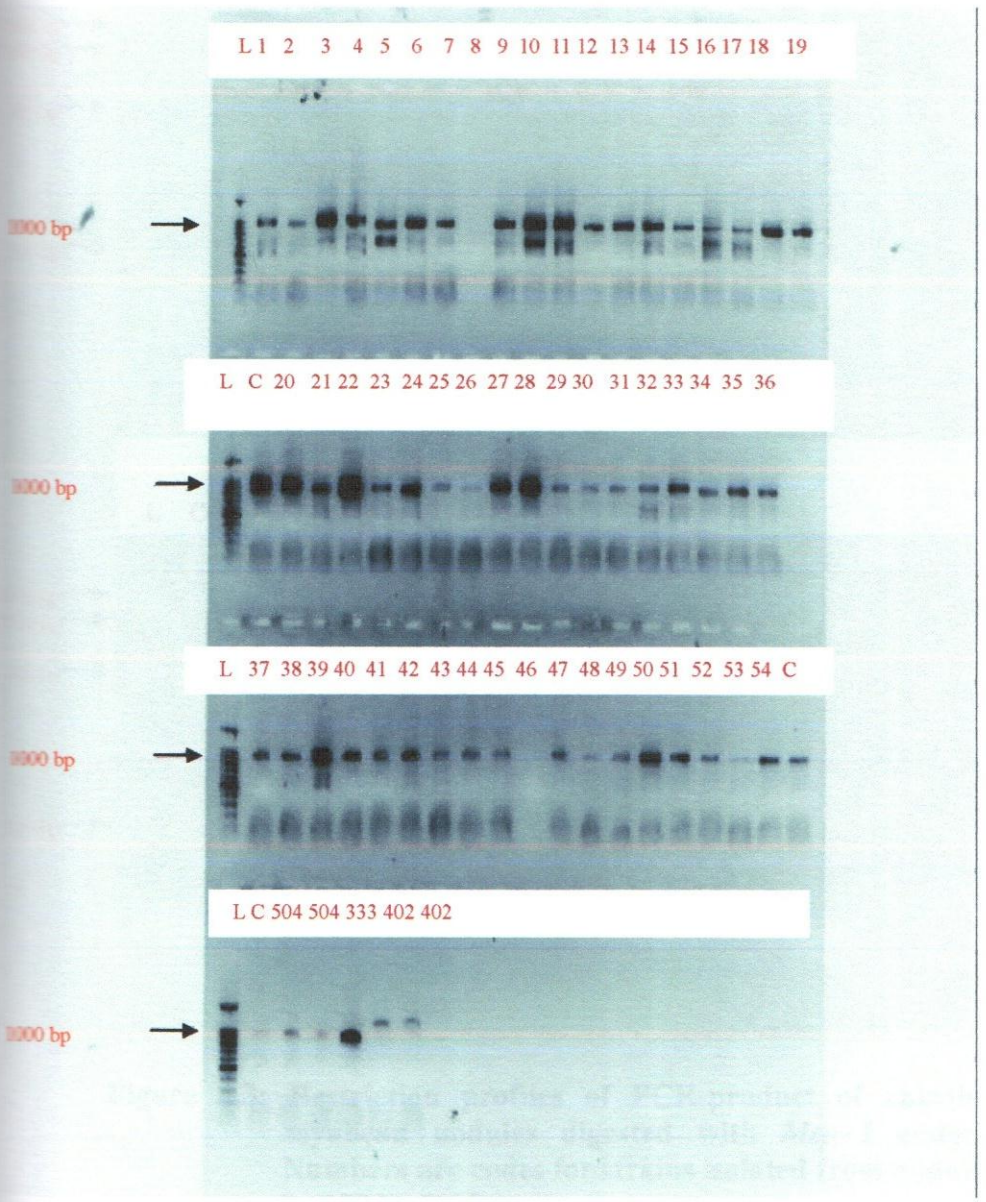


Figure 4.2: PCR amplified products of different rhizobia strains in soyabean nodules. Numbers are strain codes, C-USDA 110 and L-100bp ladder

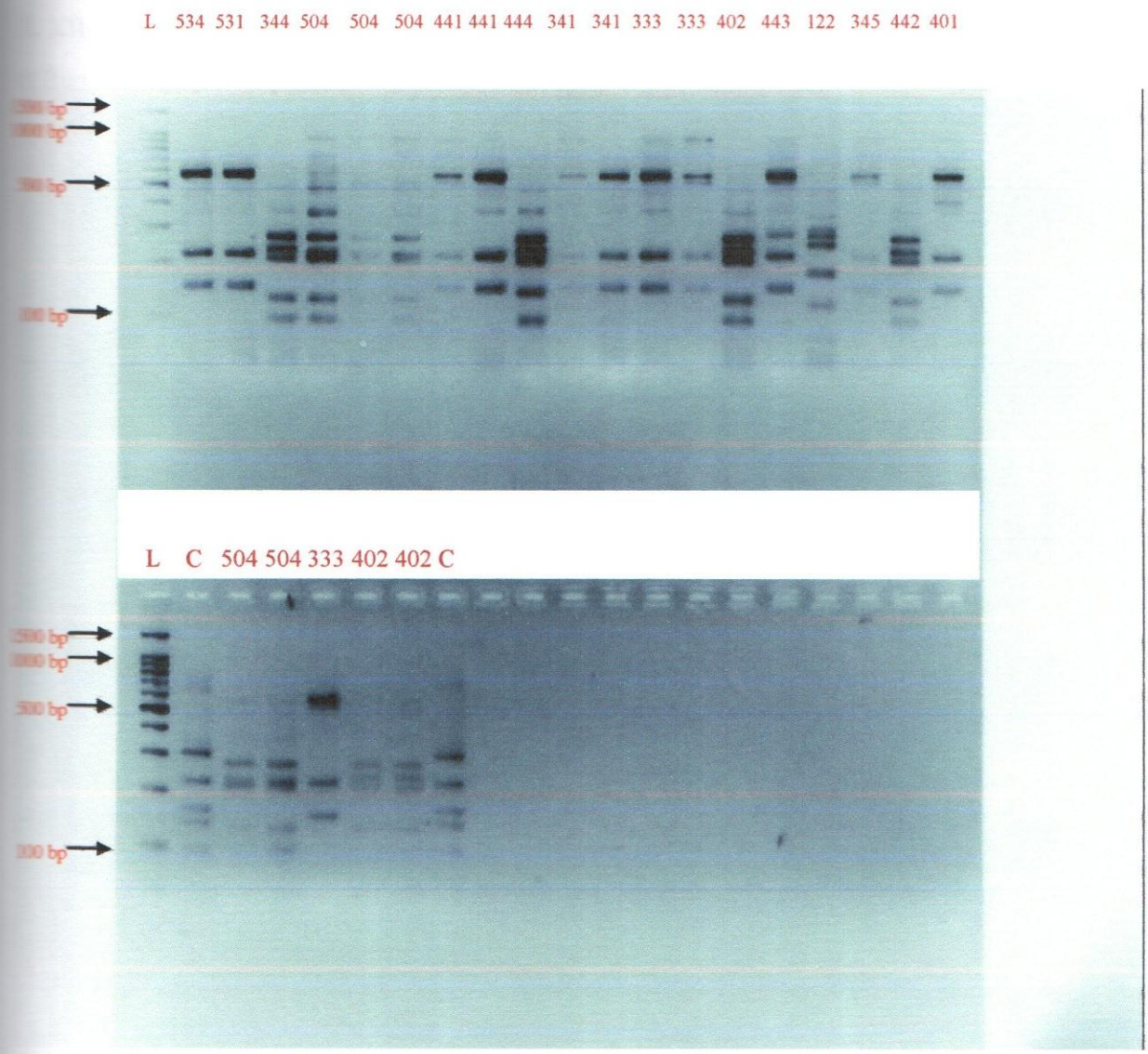


Figure 4.3: Restriction profiles of PCR-product of rhizobia strains from soyabean nodules digested with *Msp* I endonulease enzyme. Numbers are codes for strains isolated from nodules, C-USDA 110, L- 100 bp ladder

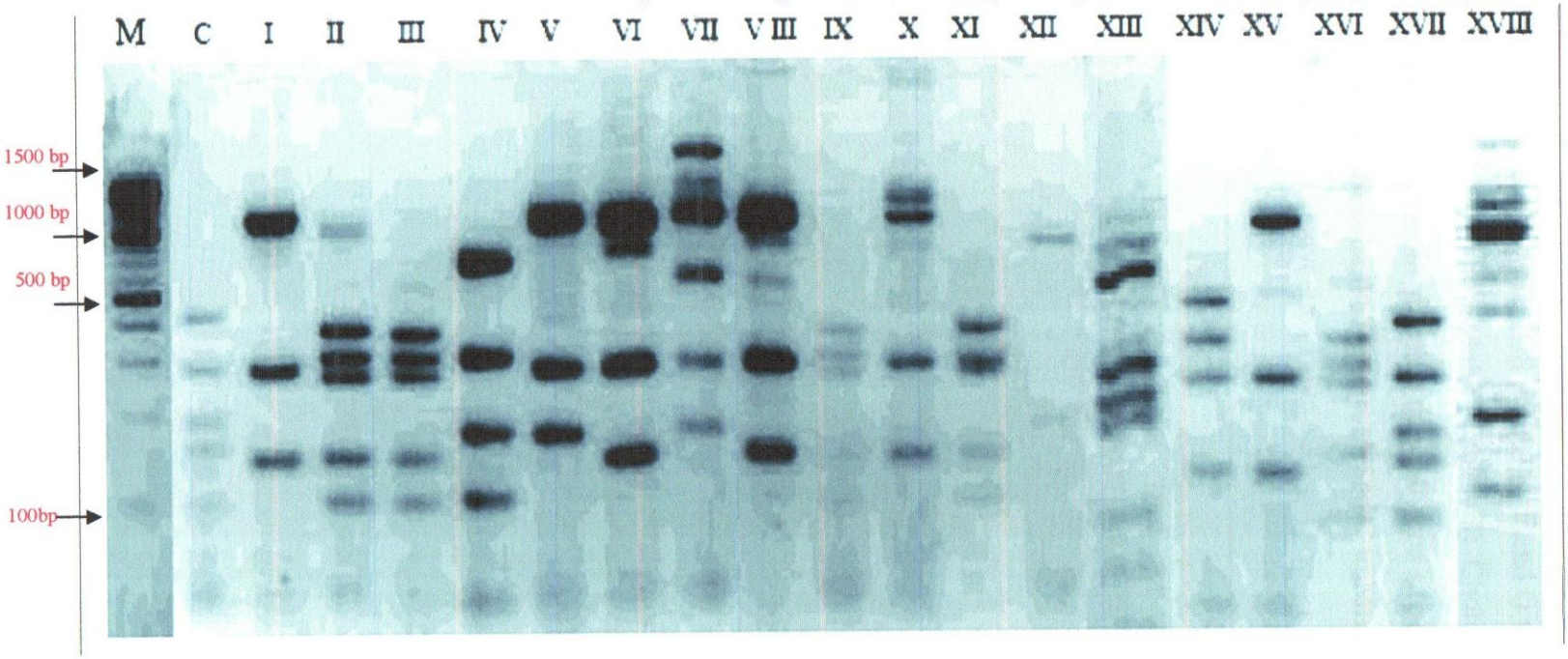
In the highland sites, Bungoma and Mitunguu, strains were classified into 18 IGS groups (Figure 4.4). The five most predominant IGS groups were I, III, II, IV and VI which constituted, 43.9%, 24.6%, 8.3% 7.6% and 6.9% respectively of all the analyzed nodules from the two sites, while IGS groups VII, VIII, IX, X, XI, XII, XIV, XVI, XVII, XVIII each constituted 1% or less (Table 4.1). Both sites had relatively similar numbers of different indigenous *Bradyrhizobium* strains and IGS groups. Mitunguu had 141 strains disaggregated into 12 IGS groups while Bungoma had 148 strains disaggregated into 13 IGS groups. Some IGS groups were specific to sites and treatments but not varieties (Table 4.1). While five IGS

roups (IX, XIII, XVI, XVII, and XVIII) were specific to Bungoma, six groups (V, VII, VIII, XII, XIV) were detected only in Mitunguu. The Shannon-Weaver (H_o) indices were higher Bungoma (H_o = 1.9) compared to Mitunguu (H_o = 1.7) (Table 4.2).

Table 4.1: Distribution of *Bradyrhizobium* strains among different IGS groups across varieties in highland sites by *Msp* I enzyme restriction

Sites	Bungoma				Mitunguu					
Treatments	Control	P	P+Lime	Sub-total	Control	P	P+Lime	Sub-total	Total	% of total
IGS group										
I	18	17	16	51	28	17	31	76	127	43.9
II	6	8	4	18	1	2	3	6	24	8.3.
III	8	12	9	29	10	24	8	42	71	24.6
IV	6	6	6	18	3	1	0	4	22	7.6
V	0	1	2	3	0	0	0	0	3	1.0
VI	6	4	8	18	2	0	0	2	20	6.9
VII	0	0	1	1	0	0	0	0	1	0.3
VIII	1	2	0	3	0	0	0	0	3	1.0
IX	0	0	0	0	0	1	0	1	1	0.3
X	0	1	0	1	0	0	0	0	1	0.3
XI	1	0	0	1	0	0	1	1	2	0.7
XII	1	0	0	1	0	0	0	0	1	0.3
XIII	0	0	0	0	2	1	0	3	3	1.0
XIV	0	0	1	1	0	0	0	0	1	0.3
XV	2	0	1	3	1	2	0	3	6	2.1
XVI	0	0	0	0	0	1	0	1	1	0.3
XVII	0	0	0	0	1	0	0	1	1	0.3
XVIII	0	0	0	0	0	1	0	1	1	0.3
Total	**49**	**51**	**48**	**148**	**48**	**50**	**43**	**141**	**289**	

Values indicate the number of strains in each IGS groups for each treatment, n= 289. Treatments: Treatments were control (none), + P (40 kg ha^{-1}), + lime (1t ha^{-1}), + N (90 kg ha^{-1}, split applied + lime + P). No nodules formed in the + N treatment and is not reported in this table.



Key: M- 100bp ladder; C – Control strain (USDA 110)

Figure 4.4: IGS groups obtained from *Msp* I restricted products of indigenous *Bradyrhizobium* strains isolated from promiscuous soyabean varieties in Bungoma and Mitunguu sites in Kenya

In the lowland sites (Chonyi, Mtwapa and Msabaha), restriction with *Msp* I revealed 8 IGS groups while restriction with *Hae* III revealed 9 IGS groups (Figure 4.5). IGS groups A, E, B and C were the most predominant profiles representing 41.3%, 21.7%, 19.6%, 17.4%, and 10.9% respectively while IGS groups H, F and G were the least predominant constituting 4.3%, 2.2% and 2.2% of all nodules (Table 4.3). Chonyi site had higher ($p<0.05$) diversity (6 IGS groups) of indigenous rhizobia than Mtwapa site (2 IGS groups). IGS group A, B and D were specific to Msabaha, IGS groups A and C to Mtwapa, while IGS groups C, E, F, G and H were specific to Chonyi site (Table 4.3).

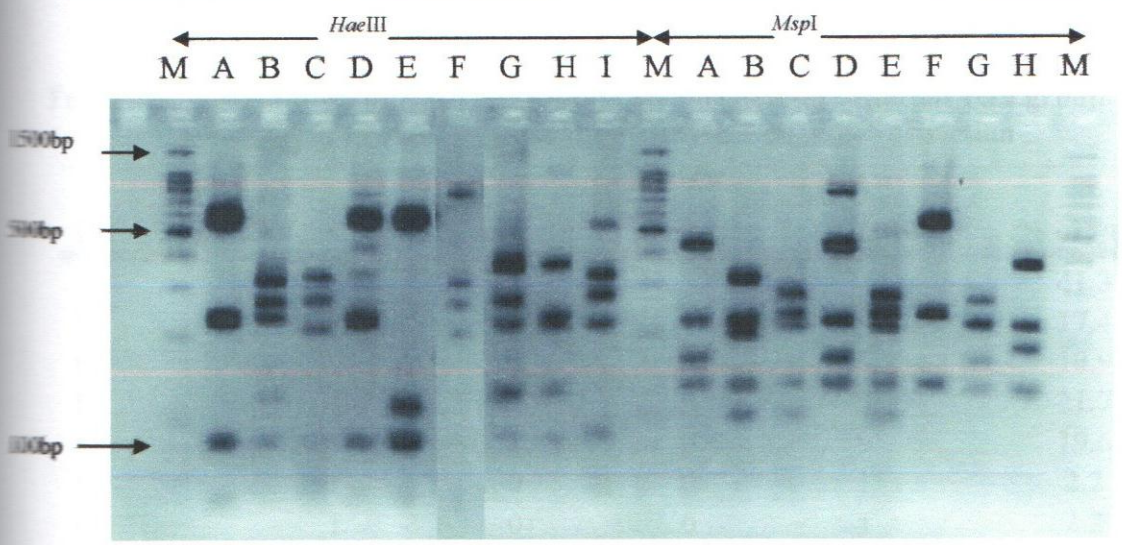


Figure 4.5: RFLP patterns obtained by restriction of different PCR-amplified 16S-23S rDNA IGS region using endonucleases *Hae* III and *Msp* I from crushed nodules isolated at the coastal lowland sites

Table 4.2: Shannon index (Ho) of diversity of *Bradyrhizobium* populations from highland sites based on 16S rRNA gene sequencing

Sites	Mitunguu	Bungoma
Number of individual species	16	13
Shannon index (H_o)	1.7	1.9
Evenness	0.31	0.49

Table 4.3: Distribution of Bradyrhizobium strains among different IGS groups across varieties in lowland coastal sites by Msp 1 enzyme restriction

IGS group	Chonyi	Msabaha	Mtwapa	Total	% of total
A	0	16	3	19	**41.3**
B	0	8	0	8	**17.4**
C	2	0	3	5	**10.9**
D	10	0	0	10	**21.7**
E	5	4	0	9	**19.6**
F	1	0	0	1	**2.2**
G	1	0	0	1	**2.2**
H	2	0	0	2	**4.3**
	12	**28**	**6**	**46**	

Values indicate the number of strains in each IGS group for each treatment, n= 46. All varieties received 9 kg ha^{-1}kg N and 23 kg ha^{-1} P as basal application treatment.

4.3.3 Sequence Analysis of the 16S rRNA Gene for Highland Sites

All the selected 29 isolates produced a single PCR product of approximately 1500 bp. The partial sequences of the 16S rRNA gene of these selected isolates of indigenous bradyrhizobia were deposited in the GenBank and given accession numbers EU625518 to EU625546 (Table 4.4). Alignments of partial sequences of the TGx isolates with related 16S rRNA gene sequences in GenBank database revealed that the 29 strains were all related to the *Bradyrhizobium* genus and were group in clusters A, B, C, D, E and F (Figure 4.6). *Bradyrhizobium elkanii, Bradyrhizobium* spp and *Bradyrhizobium japonicum* related strains were the most predominant and accounted for 37.9%, 34.5% and 20.7%, respectively, while *Bradyrhizobium yuanmigense* related strains accounted for 6.9% of all nodules analyzed. Eleven strains (TSBF 161, TSBF 402, TSBF 344, TSBF 444, TSBF 404, TSBF 442, TSBF

260A, TSBF 336A, TSBF 488, TSBF 137 and TSBF 530) were related to *Bradyrhizobium elkanii,* ten strains (TSBF 531, TSBF 523, TSBF 534, TSBF 331, TSBF 341, TSBF 333' TSBF 381, TSBF 504, TSBF 438 and TSBF 101A) to *Bradyrhizobium* spp, six strains (TSBF 345, TSBF 336, TSBF 131, TSBF 216, TSBF 101 and TSBF 102 to *Bradyrhizobium japonicum* while two strains (SBF-441 and TSBF-160) to *Bradyrhizobium yuanmingense* (Table 4.1). A phylogenetic tree derived from the partial sequences of the 16S rRNA gene by neighbor-joining analysis (Figure 4.6) confirmed the greater relationship of indigenous strains of *Bradyrhizobium* to reference strains of *B. elkanii, B. japonicum* and *Bradyrhizobium* spp. The *Rhizobium* and S*inorhizobium* reference strains constituted an outside group in the phylogenetic tree.

The BLAST results of the partial 16S rDNA gene sequences from highland and lowland sites indicated that 11 isolates were related to *Bradyrhizobuim japonicum;* 15 isolates related to *Bradyrhizobium elkanii;* 3 isolates related to *Bradyrhizobium yuanmigense* while 10 isolates were related to *Bradyrhizobium* spp (Table 4.4). Across all five sites, the most predominant strains were *Bradyrhizobium elkanii, Bradyrhizobium japonicum, Bradyrhizobium* spp and *Bradyrhizobium yuanmigense* representing 37.5%, 30.0%, 25% and 7.5% respectively. However, site differences exist and certain strains predominate in certain sites and not others. *Bradyrhizobium* spp was most predominant in Mitunguu, *B. elkanii* in Bungoma and Mtwapa, *B. japonicum* in Msabaha while *B. elkanii* and *B. japonicum* equally prevalent in Chonyi (Table 4.5). A phylogenetic tree showed that the TGx soyabean isolates clustered together with *Bradyrhizobium* genus strains from the GenBank while the other genus *Rhizobium*, *Mesorhizobia* and *Sinorhizobia,* clustered away (Figure 4.6 and Figure 4.7). Majority of the test strains, however, clustered according to geographical region (Figure 4.7).

Figure 4.6: Phylogenetic relationship between highland strains and GenBank reference strains based on aligned sequences of 16S rRNA gene, constructed as unrooted tree using the nearest neighbour-joining method

Table 4.4: Relationship between the partial 16S RNA gene sequences of genbank strains and isolated strains from Bungoma, Mitunguu, Mtwapa, Chonyi and Msabaha sites in Kenya

Isolate ID.	Genbank Acc. No.	Site*	Altitude (m.a.s.l) **	Variety	Treatment	Sequence Length (bp)	Species affiliation	% Similarity
TSBF-438	EU625543	1	> 900 m	SB 20	P	1373	*Bradyrhizobium* spp KO3G	99
TSBF-666	AY649431	5	> 900 m	SB 17	DAP	794	*Bradyrhizobium elkanii* strain BR 3277	95
TSBF-701	EF549397	3	< 90 m	SB 8	DAP	774	*Bradyrhizobium elkanii* strain CCBAU 83387	94
TSBF-694	AY649431	3	< 90 m	SB 17	DAP	806	*Bradyrhizobium elkanii* strain BR 3277	94
TSBF-260A	EU625535	2	> 900 m	SB 9	P	1044	*Bradyrhizobium elkanii* isolate TSBF 694	93
TSBF-444	EU625531	2	> 900 m	SB 20	P+Lime	1125	*Bradyrhizobium elkanii*	93
TSBF 102	EU625546	2	> 900 m	SB 19	P	898	*Bradyrhizobium japonicum* isolate TSBF 734	92
TSBF-531	EU625518	1	> 900 m	SB 9	P+Lime	924	*Bradyrhizobium* spp	92
TSBF-523	EU625519	1	> 900 m	SB 9	P	898	*Bradyrhizobium* spp	91
TSBF-488	EU625540	1	> 900 m	SB 8	Control	1083	*Bradyrhizobium elkanii* strain USDA 61	91
TSBF-534	EU625522	1	> 900 m	SB 9	P+Lime	917	*Bradyrhizobium* spp	91
TSBF-336A	EU625536	1	> 900 m	SB 15	Control	902	*Bradyrhizobium elkanii* isolate TSBF 694	91
TSBF-331	EU625523	2	> 900 m	SB 15	Control	1024	*Bradyrhizobium* spp	91
TSBF 333	EU625526	1	> 900 m	SB 15	Control	996	*Bradyrhizobium* spp PAC 41	91
TSBF-402	EU625529	1	> 900 m	SB 19	P	815	*Bradyrhizobium elkanii* strain USDA61	89
TSBF-131	EU625527	2	> 900 m	SB 20	P	725	*Bradyrhizobium japonicum* isolate 734	88
TSBF-442	EU625533	2	> 900 m	SB 20	P+Lime	870	*Bradyrhizobium elkanii*	88
TSBF-60	EU625542	2	> 900 m	SB 15	P+Lime	809	*Bradyrhizobium yuanmingense* isolate TSBF 627	88
TSBF-336	EU625524	1	> 900 m	SB 15	Control	837	*B. japonicum* strain TSBF 734	88
TSBF-341	EU625525	2	> 900 m	SB 15	P	867	*Bradyrhizobium.* spp SjCL5 (MS 867)	88
TSBF-627	EU170554	4	< 90 m	SB 19	DAP	815	*Bradyrhizobium yuanmigense* strain CCBAU33230	88
TSBF-345	EU625520	1	> 900 m	SB 15	P	850	*Bradyrhizobium japonicum* isolate TSBF-607	88
TSBF-101	EU625541	2	> 900 m	SB 19	P	863	*Bradyrhizobium japonicum* isolate TSBF 734	87
TSBF-607	AF530466	4	> 900 m	SB 8	DAP	810	*Bradyrhizobium japonicum* isolate WC4	86
TSBF 216	EU625538	2	> 900 m	SB 8	Control	942	*Bradyrhizobium japonicum* isolate JZ 1	86
TSBF-374	EU625537	1	> 900 m	SB 17	P	942	*Bradyrhizobium* japonicum isolate JZ 1	86
TSBF-381	EU625528	1	> 900 m	SB 17	P+Lime	676	*Bradyrhizobium* spp MAF 210190	86
TSBF-734	AF530466	3	< 90 m	SB 19	DAP	815	*Bradyrhizobium japonicum* isolate W4	85

Isolate ID.	Genbank Acc. No.	Site*	Altitude (m.a.s.l) **	Variety	Treatment	Sequence Length (bp)	Species affiliation	% Similarity
TSBF-441	EU625521	1	> 900 m	SB 20	P+Lime	592	*Bradyrhizobium yuanmingense* isolate TSBF-627	85
TSBF-344	EU625530	1	> 900 m	SB 15	P	680	*Bradyrhizobium elkanii*	84
TSBF-717	AF208512	3	<90 m	SB 15	DAP	813	*Bradyrhizobium elkanii* strain USDA 31	83
TSBF-695	EF394144	3	< 90 m	SB 17	DAP	809	*B. japonicum* strain CCBAU 53152	82
TSBF-640	AY624129	4	< 90 m	SB 20	DAP	788	*Bradyrhizobium* spp PAC 41	80
TSBF-404	EU625532	1	> 900 m	SB 19	P	295	*Bradyrhizobium elkanii* SEMIA 6425	80
TSBF-101A	EU625534	2	> 900 m	SB 19	P	289	*Bradyrhizobium* spp MAF 210190	78
TSBF-718	AF530466	3	< 90 m	SB 19	DAP	749	*Bradyrhizobium japonicum* isolate W4	78
TSBF-530	EU625545	1	> 900 m	SB 9	P	355	*Bradyrhizobium elkanii* isolate TSBF-734	77
TSBF-639	DQ26711	4	< 90 m	SB 20	DAP	780	*Bradyrhizobium* spp CCBAU 35186	76
TSBF-137	EU625544	2	> 900 m	SB 20	P	284	*Bradyrhizobium elkanii* isolate TSBF 717	73
TSBF 161	EU625539	2	> 900 m	SB 4	P	183	*Bradyrhizobium elkanii* isolate TSBF 717	69
Control	BA000040			-	-	760	*B. japonicum* strain USDA 110	67

*Sites notation: 1= Mitunguu, 2 = Bungoma, 3= Chonyi, 4= Msabaha, 5=Mtwapa ; ** m.a.s.l. - metres above sea level

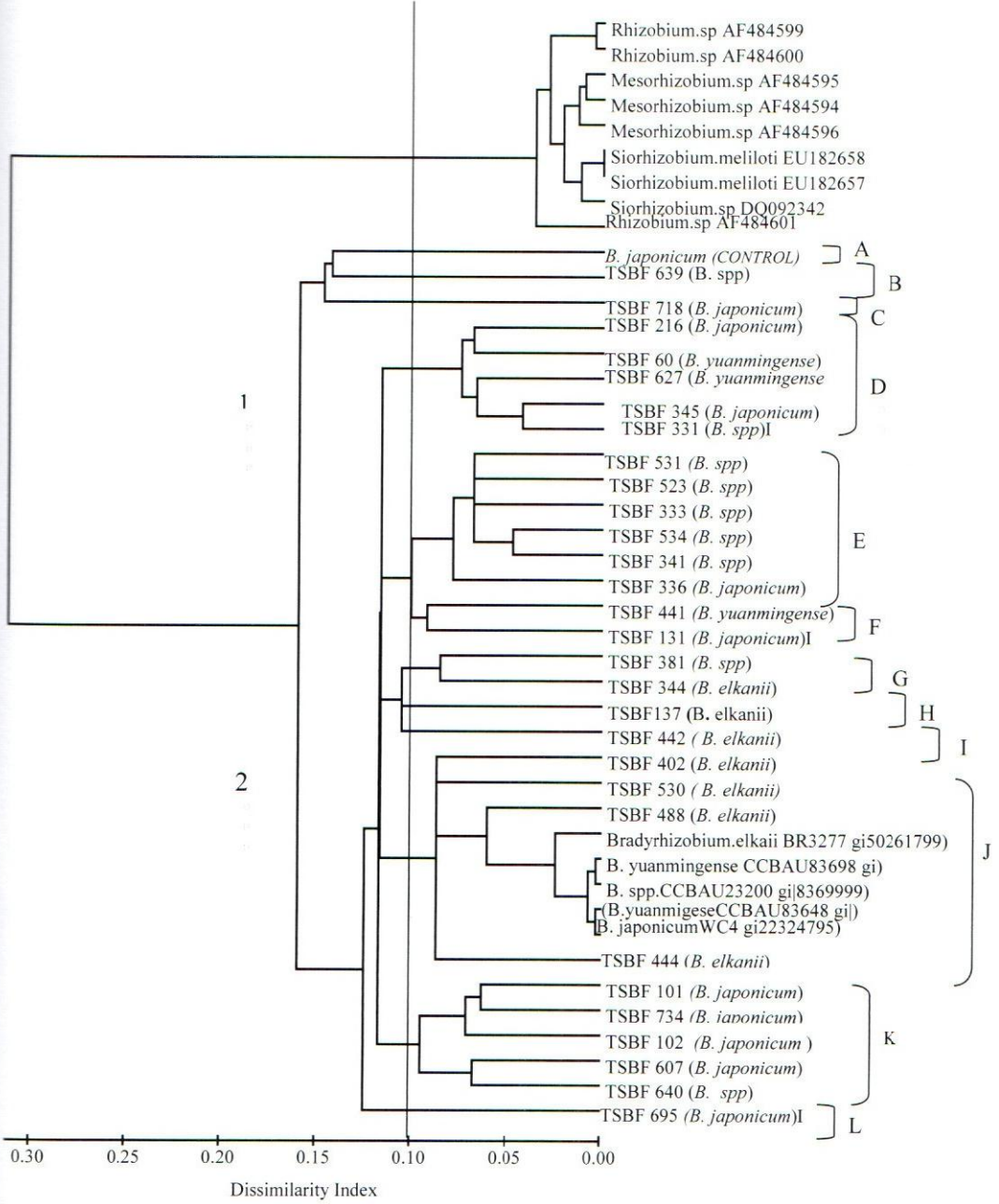


Figure 4.7: Phylogenetic relationships among strains isolated from promiscuous soyabean varieties grown in five sites in Kenya

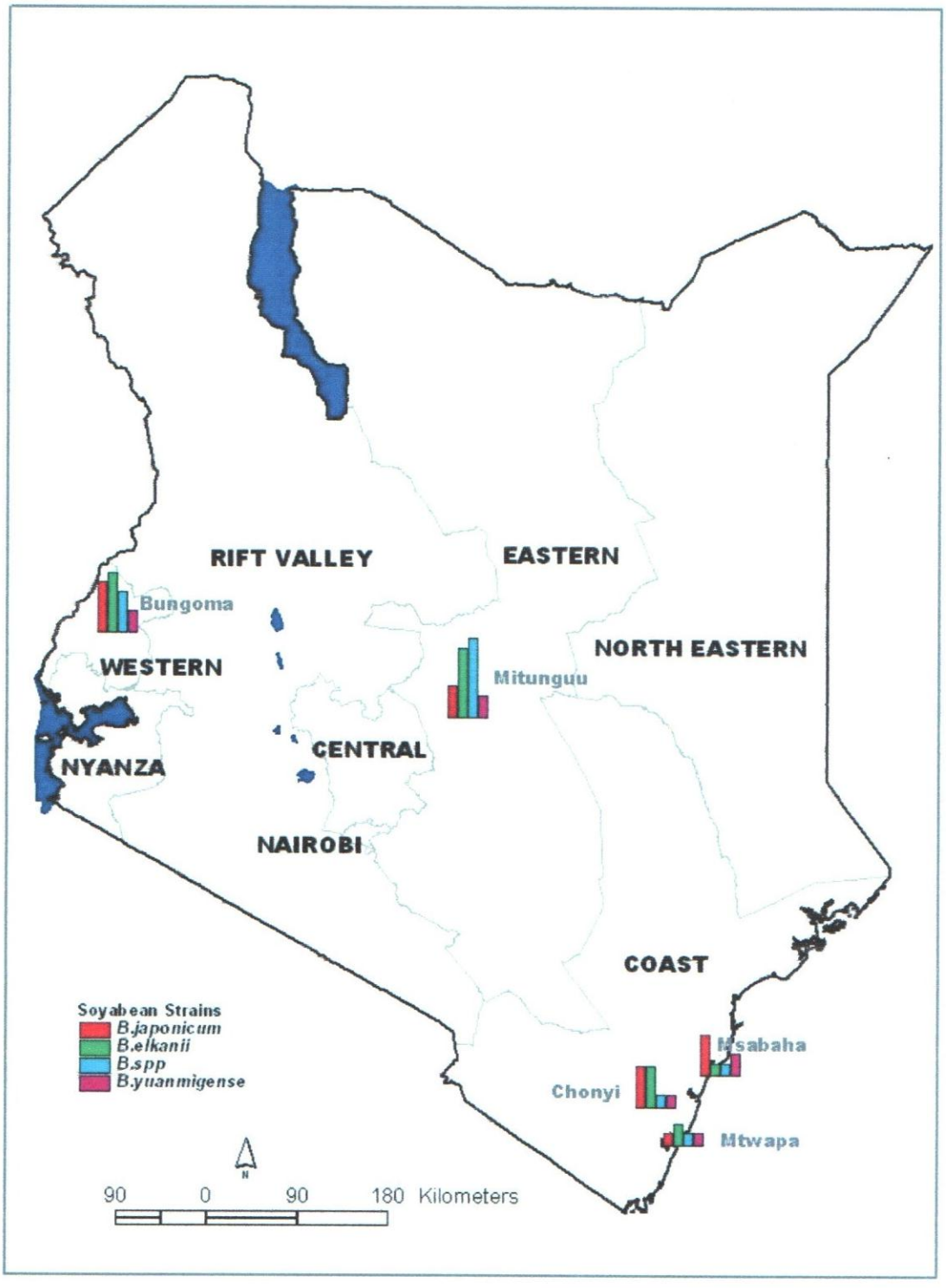


Figure 4.8: Distribution and prevalence of indigenous *Bradyrhizobium* strains nodulating promiscuous soyabean varieties in lowland and highland sites in Kenya

Table 4.5: Relative proportion (%) of strains nodulating seven TGx soyabean varieties at five sites in Kenya

	Highland sites		Lowland sites			
	Mitunguu	Bungoma	Msabaha	Mtwapa	Chonyi	Overall
Strain	%	%	%	%	%	%
B. japonicum	12.50	30.77	75.00	0.00	50.00	30.00
B. elkanii	37.50	38.46	0.00	100.00	50.00	37.50
B. spp.	43.75	23.08	0.00	0.00	0.00	25.00
B. yuanmigense	6.25	7.69	25.00	0.00	0.00	7.50

4.4 Discussion

The TGx soyabean varieties developed by the breeding program at IITA for promiscuity nodulated with diverse indigenous rhizobia populations in both sites where they had been introduced for the first time. The presence of these indigenous *Bradyrhizobium* in both Nigeria and Kenya soils separated by a tropical forest in central Africa suggests a common evolutionary path of bacteria influenced by comparable biotic and abiotic conditions. It could also be due to genetic exchange of *nif* genes through a variety and combination of events such as strain dispersion, genomic combination and horizontal gene transfer among indigenous *Bradyrhizobium* communities along the Nigerian and Kenya contiguous path through the Congo forest. In related studies, Mulongoy and Ayanaba (1986) reported the presence of *Bradyrhizobium japonicum* in some African soils even though soyabean was not commonly grown. Kasasa (1999) and Musiyiwa *et al.*, (2005a) similarly reported the presence of rhizobia nodulating promiscuous soyabean varieties in many soils in Zimbabwe. Some of the isolates were as good or superior in N_2 fixation effectiveness to commercial inoculant strains under greenhouse conditions.

The phylogenetic tree clearly shows that the Kenyan isolates form a distinct group. Thus the indigenous strains of *Bradyrhizobium* nodulating TGx varieties are distinct from *Bradyrhizobium* that nodulate North American soyabeans varieties. In Nigeria, similar results were obtained by Abaidoo *et al.*, (2000) with the TGx varieties. This was not unexpected because the indigenous *Bradyrhizobium* from the two sites, with no previous history of soyabean

cultivation and hence no introduction of exotic *Bradyrhizobium* strains, had been genetically isolated and consequently had evolved independently. The phylogenetic tree nevertheless shows that there is adequate genetic variation among the indigenous strains of *Bradyrhizobium*. This study showed that *Bradyrhizobium* strains nodulating promiscuous soyabean genotypes grown under lime and phosphorus application in two contrasting sites in Kenya were highly diverse. This diversity could be linked to the fact that *Bradyrhizobium* strains may have different capacities to utilize P in their metabolic activities which influence nodule initiation and effectiveness (Gunawardena, *et al.,* 1993). There was a positive relationship between diversity assessed as number of IGS groups and abundance of bradyrhizobia strain population at the two sites. Bungoma had 13 IGS groups comprising 148 strains while Mitunguu had 12 IGS groups comprising 141 strains. The relatively higher diversity in Bungoma (H_o=1.9) compared to Mitunguu (H_o=1.7) could be attributed to a combination of factors such as the overall improved environmental soyabean growing conditions in Bungoma (humid) as compared to semi-humid conditions at Mitunguu. Elsewhere, several authors have reported similar genetic diversity indices of rhizobia nodulating soyabean (Sikora and Redzepovic, 2003; Chen *et al.,* 2004; Giongo *et al.,* 2008) and *Phaseolus vulgaris* (Andrade *et al.,* 2002) using molecular markers.

In this study, IGS groups were specific to sites and treatments but not varieties. This finding is in accordance with results described by Wei Tao Zang *et al.,* (2007) who showed that geographical location affects composition and biodiversity of indigenous rhizobia. This behavioural interaction between strains and geographical location is attributed to relative strain competitiveness or saprophytic competence under the conditions existing at specific sites (Wei Tao Zang *et al.,* 2007). Lime application has previously been reported to increase diversity of IGS groups in *Phaseolus* nodulating rhizobia in Brazil (Andrade *et al.,* 2002). Strains restricted to a geographical location generally develop special phenotypic and genotypic characteristics (Xu *et al.*, 1995; Vinuesa *et. al.,* 1998). In contrast, Chen *et al.,* (2004) and Thiao *et al.,* (2004) found no relationship between IGS groups and geographical location.

In Kenya, few studies have investigated the genetic diversity of indigenous rhizobia nodulating legumes (Anyango *et al.,* 1995; Odee *et al.,* 2002). The preponderance of *Bradyrhizobium* spp related strains in Mitunguu and *B. elkanii* related strains in Bungoma sites may be attributed to their saprophytic competence at the respective sites (Anyango *et al.,* 1995, Batista *et al.,* 2006). Results corroborate those of Abaidoo *et al.,* (2002) which showed that TGx

varieties in Nigeria were nodulated by *Bradyrhizobium* spp. They also suggest that *Bradyrhizobium* spp, *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* required for effective nodulation and cultivation of soyabean in Africa are endemic in eastern, western and coastal sites in Kenya.

4.5 Conclusion

This study has revealed genetic diversity among *Bradyrhizobium* nodulating seven promiscuous soyabean varieties grown at two contrasting sites. Results show that the tested promiscuous soyabean varieties in the two sites are nodulated by populations of *Bradyrhizobium* strains which are genetically diverse and are closely related to *B. japonicum, B. elkanii, Bradyrhizobium* spp and *B. yuanmingense.* The results also showed that the strains were restricted to geographical location and confirm the observation that strains generally develop special phenotypic and genotypic characteristics to adapt to local conditions. This implies that selection of strains for use under certain soil conditions should be isolated from soils with similar characteristics. However, these results need to be confirmed by analysis of a larger sample of strains from more sites in order to fully assess the diversity inherent in Kenyan soils and to select more competitive, efficient and adapted strain(s) at each site for potential use as inoculants to optimize biological nitrogen fixation.

4.6 References

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CHAPTER FIVE

EFFECTIVESS OF INDIGENOUS *BRADYRHIZOBIUM* ISOLATES ON PROMISCUOUS SOYABEAN GENOTYPES

Abstract

Small-holder farmers frequently cannot cope with the high and ever increasing prices of inorganic fertilizers. Consequently, low yields, chronic food and nutritional insecurity are frequent phenomena. Cheaper and sustainable alternatives to improving soil fertility especially for N are being sought. The use of indigenous strains of *Bradyrhizobium* inoculants offers a potential economic and sustainable option in meeting soyabean nitrogen needs. Inoculation of soyabean in Kenya has often relied on introduced rhizobia. This is because elite indigenous strains have not been isolated, tested and selected for use under local conditions. This study was conducted to assess the nodulation and nitrogen fixation of nine indigenous *Bradyrhizobium* strains inoculated on three promiscuous soyabean varieties grown in sand under greenhouse conditions. Three soyabean and nine indigenous *Bradyrhizobium* strains genotypes were evaluated in a randomized complete block design. There was a significant ($p<0.01$) indigenous strain effect on dry shoot biomass and nodulation ($p<0.05$). Inoculation with TSBF 442 resulted in 66.9% and 154.7% more shoot biomass than inoculation with USDA 110 and negative control respectively; while inoculation with TSBF 531 elicited 59.4% and 143.2% more shoot biomass than USDA 110 and negative control respectively. All tested indigenous strains nodulated significantly better than the introduced strain (USDA 110) on all promiscuous soyabean varieties. Inoculation with TSBF 442 resulted in 110% more nodules than USDA 110 while inoculation with TSBF 531 resulted in 850% more nodules than USDA 110. There was a significant interaction between soyabean genotypes and indigenous rhizobia strains for nodule dry weight. Inoculation of SB 8, SB 9 and SB 19 with TSBF 531, TSBF 101 and TSBF 344, respectively, resulted in high nodule dry weight. This study indicates that potential exists to deploy indigenous rhizobia strains to replace/supplement introduced USDA 110 currently used in soyabean inoculants in Kenya. Competitiveness of these strains, however, needs to be tested before deployment.

5.1 Introduction

Symbiosis between legume plants and bacteria commonly referred to as rhizobia is of considerable environmental as well as agricultural significance (Ogutcu *et al.*, 2003). This symbiosis is responsible for an estimated 180 x 10^6 tons per year of biological nitrogen fixation world wide which is equivalent to generation of a resource equivalent to US $ 160-180 billion (Sanghal and Johri, 2003). The symbiotic component alone contributes about 20 x 10^6 tons per year to global N economy which represents 65% of nitrogen used in agriculture and several fold larger than the input of N fertilizer estimated at 65 x 10^6 tons per annum (Sanghal and Johri, 2003). Soyabean is the most important legume crop in the world in terms of trade.

Part of the problem of insufficient food production is low soil fertility and high cost of farm inputs especially fertilizers. There is a general consensus on the need to address the problem of low soil fertility in order to improve agricultural productivity and ensure food security. Small-scale farmers, who constitute about 75% of the farming community in Kenya, are resource constrained and majority live on less than $1 dollar per day. As a result, they cannot afford to increase their fertilizer use rate (currently estimated at about 25 kg ha^{-1}) leading to low crop production, food insecurity and malnutrition. With the high cost of inorganic fertilizers and distribution constraints of agricultural inputs, farmers continually find they are unable to apply any of the external inputs often leading to low crop yields. The situation is unlikely to improve given continuous land fragmentation and the increasing poverty levels in Kenya, often exceeding 50% in most districts. A suitable, cheap and sustainable supplement/substitute for some of the N fertilizer inputs is the selection, promotion and use, as inoculants, of more efficient indigenous *Bradyrhizobium* strains and promiscuous soyabean varieties suitable for different agro-ecological zones of Kenya. This has been achieved in Brazil (Hungria *et al.*, 2005) where benefits resulting from the use of inoculants with selected superior indigenous strains are equivalent to about $ 3 billion per cropping season that would otherwise go to purchase, transportation and application of nitrogenous fertilizers. Currently the cost of Diamomnium Phosphate (DAP) commonly used in crop production in the country is in excess of $ 1644 per ton at farm level. Hence the renewed interest in promiscuous soyabean in Kenya due to its capacity to nodulate and fix N with populations of indigenous bradyrhizobia. As farmers generally cannot afford or access inorganic sources of N, identification of effective locally adapted *Bradyrhizobium* strains which nodulate

promiscuous soyabean could be useful in the development of inoculant strains. As shown in Chapter 4, there is considerable genetic diversity of indigenous *Bradyrhizobia* strains nodulating seven introduced promiscuous soyabean varieties grown in two highland and three lowland sites in Kenya. This diversity in indigenous *Bradyrhizobium* populations is a valuable genetic resource that has potential utility for the selection of more competitive and effective strains to improve biological nitrogen fixation and enable crop production at low cost. However, great diversity of strains isolated even from nodules of the same legume in both competiveness and effectiveness has been reported before (Okereke *et al.*, 2001; Tien *et al.*, 2002; Mahna *et al.*, 2006). Consequently, better N_2 fixation can be achieved by selecting superior rhizobia strains.

The objective of this experiment was to determine the best-bet soyabean variety x *Bradyrhizobium* strain interaction on nitrogen fixation in greenhouse studies using shoot dry biomass and nodule dry weight as indicators for nitrogen fixation potential.

5.2 Materials and Methods

5.2.1 Planting Medium

River sand was obtained from Athi river-Machakos district, Kenya. It was washed several times with distilled water, dried and bagged in 2 kg sterilized polythene bags. The content of nitrogen in the sand was determined following standard methods (Anderson and Ingram, 1993). The amount of water needed to bring the sand to field capacity was determined. Field capacity was determined by weighing 2 kg of sand, wetting it with double distilled water, letting it settle for 24 hours in a greenhouse and then getting differences in weights.

5.2.2 Soyabean Seed Preparation

Fresh harvested seed was obtained from TSBF-Maseno station. Three promiscuous soyabean varieties (SB 8, SB 9 and SB 19) bred in IITA and recommended (recommended and released in 2010) for cultivation in Kenya were used. Seeds of each of these genotypes were surface sterilized in 3% (v/v) calcium hypochlorite for 2 minutes followed by immersion in 70% ethanol for 3 minutes and then washed six times with double distilled water. They were allowed to imbibe water for 30 minutes (Somasegaran and Hoben, 1994) and pre-germinated for 5 days in 95% water agar in a growth chamber maintained at 28 °C. Pre-germinated seedlings were transferred to sterilized polythene bags containing 2 kg each of sand.

5.2.3 Indigenous *Bradyrhizobium* Strains

Tested strains were isolated from Bungoma (four strains) and Mitunguu (five strains) belonging to predominant intergenic spacer groups (IGS) groups (I, III, and IV) and clusters A, B, C, E, and F (Table 5.1) identified in Chapter 4. Strain USDA 110, a strain currently used in soyabean commercial inoculants in Kenya was used as a control.

Table 5.1: Selected *Bradyrhizobium* strain origin, IGS group and phylogeny as described by PCR-RFLP and partial 16S rRNA gene sequence analysis

Sno.	Code	Site*	Cluster**	Species affiliation	IGS group
1	TSBF-404	1	B	*B. elkanii SEMIA 6425*	III
2	TSBF-101	2	C	*B. japonicum* isolate *TSBF 734*	IV
3	TSBF-131	2	C	*B. japonicum* isolate *734*	I
4	TSBF-531	1	C	*Bradyrhizobium sp.*	I
5	TSBF-534	1	C	*Bradyrhizobium sp.*	I
6	TSBF-331	2	F	*Bradyrhizobium sp.*	I
7	TSBF-442	2	A	*B. elkanii*	III
8	TSBF-344	1	E	*B. elkanii*	III
9	TSBF-336A	1	B	*B. elkanii* isolate *TSBF 694*	III
10	USDA 110	-	-	*Bradyrhizobium japonicum*	-
11	- ve control	-	-	-	-
12	+ ve control (70 µg g^{-1}N)	-	-	-	-

*1 = Mitunguu, 2 = Bungoma

** = These are the major clusters of strains isolated from the Bungoma and Mitunguu sites in chapter 4.

5.2.4 Bradyrhizobial Culture

Pure colonies of *Bradyrhizobium* strains were cultured from nodule suspension. The nodule suspension was obtained from crushing each nodule in 300 µl of sterile water in a 1.5 ml eppendorf tube with disposable plastic micro pestles (Sigma@, Z3014) sterilized in 96% ethanol

serially streaked on yeast manitol agar (YEMA) Sigma@ on peri plates to obtain pure colonies. A sample was obtained from this procedure as described in section 4.2.1. These were then made into broth culture as described by Vincent (1970) and shaken at 28°C at 275 rpm in a rotary shaker for 5 days until (turbid) logarithmic phase was attained

5.2.5 Seedling Inoculation of Cultures

Three seedlings for each variety were transplanted into each polythene bag using sterilized forceps. Three days after transplanting, seedlings were thinned to one seedling per bag. Five days after transplanting, seedlings were inoculated with 3ml (10^8 cell mL^{-1}) of each rhizobium culture strain at the base of each seedling. Polythene bags were then arranged in a randomized complete block design replicated five times in a greenhouse at the International Centre for Insect Physiology and Ecology (ICIPE) campus at Kasarani. A negative (no inoculation) was used to check for cross contamination. A positive (70 µg g^{-1} N) control and strain (USDA 110) were included for comparison. The experiment was established in a greenhouse at approximately 12/12 light and 25 °C/32 °C. The seedlings were watered to field capacity by alternate day watering with double distilled water and Broughton (Broughton and Dilworth, 1970) solution until harvest. Plants were harvested after 45 days at R1 stage (Fehr *et al.*, 1971) for each of the genotypes by cutting at the first node level separating the shoot and the root system.

Nodules were carefully separated from roots, and fresh biomass separately bagged and weighed. All samples were dried in an oven at 70° C for 48 hrs and nodule dry weight (NDW) and shoot dry weights (SDW) recorded. The SDW produced in this nitrogen free growth system was used as a proxy for nitrogen fixation (Abaidoo *et al.*, 2000). The mean SDW (X) was used to calculate an index of effectiveness (E) as defined by Ferreira and Marques (1992):

$$E_J = \frac{X_J - X_{TO}}{X_{TN} - X_{TO}}$$

Where, J is the shoot dry weight of inoculated test strain, T_O the uninoculated control and T_N is the nitrogen control. Strains were arranged in ascending order and grouped into classes of effectiveness using the method by Beck *et al.*, (1994).

5.2.6 Statistical Analysis

Statistical analysis was performed on all the parameters measured using the following model.

$$Y_{ijkl} = \mu + \beta_i + I_j + J_k + IG_{jk} + \xi_{;jkl}$$

where,

μ = is the general mean;

β_I = Effect due to *i*th block where (i =1.....5);

I_j = Effect due to the j^{th} strain, where (j=1.....12);

J_k = Effect due to k^{th} variety in the i^{th} replicate (k =1....3),

IG_{jk} = Effect due to interaction between j^{th} strain and k^{th} variety)

$\xi_{;jkl}$ = is the random error;

Statistical analysis was conducted using the ANOVA procedure of SAS (1996) and means separated using Duncan multiple range test (DMRT) whenever treatment effects were significant at 95% confidence level (Steel and Torrie, 1977).

5.3 Results

Significant strain effects were observed on shoot dry weight, number and weight of nodules. However, interactive variety and strain effects were only observed on dry weight of nodules ($p<0.05$).

5.3.1 Nodulation

Strains TSBF 442, TSBF 344, TSBF 534 and TSBF 531 produced highest numbers of nodules compared to the rest while TSBF 131 and USDA 110 produced the least amount of nodules per plant. TSBF 442 produced 1100 % more nodules than USDA 110 while TSBF 344 and TSBF 531 produced 950% and 850% more than USDA 110 respectively (Figure 5.1).

5.3.2 Shoot Dry Weight

Effects due to strains were highly significant for shoot biomass ($p<0.01$). Shoot dry weight ranged from 0.438 mg per plant for negative control to 0.731 mg per plant for TSBF 442. TSBF 442 had about 1.7 times more shoot dry biomass than the negative control while it recorded at least 2.5 times more shoot biomass than the control strain (USDA 110). The control

strain USDA 110 produced significantly ($p<0.05$) less shoot dry biomass and nodules than TSBF 442 strains in all three varieties.

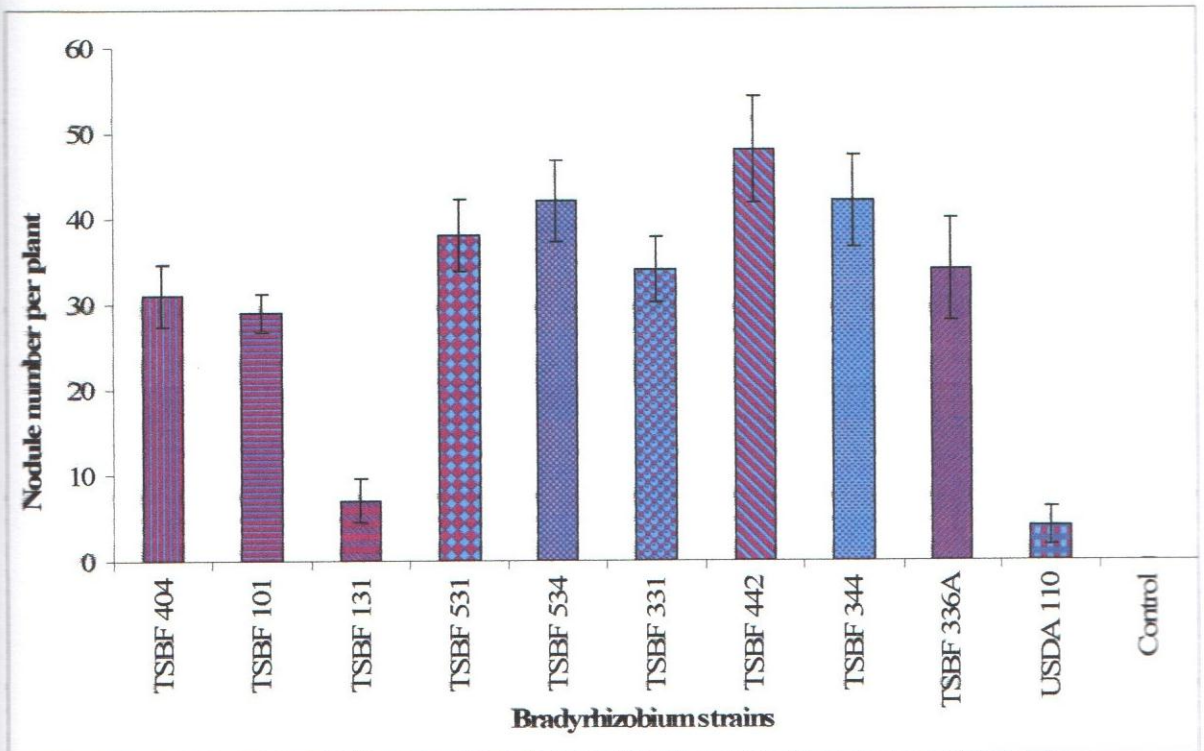

Figure 5.1: Effect of different *Bradyrhizobium* strains on the nodule number of promisucous soyabean varieties.

Table 5.2: Mean nodulation and shoot weight of three promiscuous soyabean varieties inoculated with different *Bradyrhizobium* strains

Strain	Isolate	IGS group	Origin of Strain	No. Nodule	Shoot dry weight (g)	¥Effectiveness %
TSBF- 442	*B.* spp	III	Bungoma	48 a	0.731 b	30.97
TSBF- 344	*B. elkanii*	III	Mitunguu	42 ab	0.666 bc	26.95
TSBF-336A	*B. elkanii*	III	Mitunguu	34 bc	0.585 bc	23.34
TSBF- 531	*B.* spp	I	Mitunguu	38 abc	0.698 bc	29.24
TSBF- 331	*B.* spp	I	Bungoma	34 bc	0.567 bc	22.40
TSBF- 101	*B. japonicum TSBF 734*	IV	Bungoma	29 c	0.713 bc	30.03
TSBF- 534	*B.* spp	I	Mitunguu	42 ab	0.609 bc	24.60
TSBF- 131	*B. japonicum*	I	Bungoma	7 d	0.255 d	6.11
TSBF- 404	*B. elkanii* SEMIA 6425	III	Mitunguu	31 bc	0.493 bcd	18.53
Negative		-		0 e	0.287 d	7.78
USDA 110		-		4 de	0.438 cd	
Positive		-		0 e	2.053 a	

Means followed by the same letter in a column are not significantly different from each other ($p<0.05$)
¥ = denotes effectiveness.

5.3.3 Nodule Dry Weight

There was a significant ($p<0.05$) interaction between strains and varieties for nodule dry weight. When inoculated on variety SB 8, TSBF 531, TSBF 442 and TSBF 101 resulted in the highest nodule weights while the least was by USDA 110, TSBF 344, TSBF 131 and negative control. On SB 9, the highest nodule dry weights was as a result of inoculation with TSBF 101, TSBF 344, TSBF 534 and TSBF 404 while on variety SB 19, inoculation by TSBF 344, TSBF 531 TSBF 336A and TSBF 442 while the least was as a result of inoculation by USDA 110, TSBF 440 and TSBF 131 (Table 5.3).

Table 5.3: Interaction between varieties and *Bradyrhizobium* strains on nodule dry weight of three promiscuous varieties inoculated with different *Bradyrhizobium* strains in green house, ICIPE

Strain	Isolate	IGS group	Origin of Strain	Nodule dry weight of varieties (g) SB 8 (TGx 835-10E)	SB 9 (TGx 889-12F)	SB 19 (TGx. 1740-2F)
TSBF-442	*B.* spp	III	Bungoma	0.074 (0.027)	0.045 (0.001)	0.075 (0.030)
TSBF-331	*B.*spp	I	Bungoma	0.063 (0.028)	0.045 (0.014)	0.027 (0.004)
TSBF-101	*B. japonicum TSBF 734*	IV	Bungoma	0.066 (0.002)	0.165 (0.053)	0.055 (0.022)
TSBF-131	*B.japonicum*	I	Bungoma	0.011 (0.007)	0.019 (0.007)	0.007 (0.004)
TSBF-344	*B. elkanii*	III	Mitunguu	0.024 (0.010)	0.088 (0.022)	0.139 (0.084)
TSBF-336A	*B. elkanii*	III	Mitunguu	0.063 (0.016)	0.021 (0.017)	0.098 (0.046)
TSBF-531	*B.* spp	I	Mitunguu	0.078 (0.020)	0.033 (0.016)	0.010 (0.047)
TSBF-534	*B.* spp *B. elkanii* SEMIA 6425	I	Mitunguu	0.057 (0.016)	0.056 (0.031)	0.019 (0.010)
TSBF- 404		III	Mitunguu	0.065 (0.019)	0.048 (0.018)	0.009 (0.004)
USDA 110		-		0.009 (0.005)	0.006 (0.003)	0.000 (0.000)
Negative control		-		0.0120 (0.005)	0.000 (0.000)	0.000 (0.000)
Positive Control		-		0.012 (0.006)	0.00 (0.000)	0.037 (0.018)

Values in brackets are standard errors (SE) of respective means.

5.4 Discussion

The first criterion for a *Rhizobium* strain to be used in legume inocula is that it must be highly effective in fixing nitrogen (Matos and Shroeder, 1989). Effectiveness is defined as the amount of N_2 fixed by a particular strain of rhizobia existing at a location and is manifested by increased shoot biomass produced and leaf greenness (colour). In this study, inoculation of all tested strains formed nodules on promiscuous varieties, increased dry shoot weight relative to the control indicating active nitrogen fixation. Two (22.2%) of the strains, TSBF 131 and USDA 110, formed less than 10 nodules per plant while the rest, (78.8 %) formed nodules in excess of 20 nodules per plant. Majority (70.0%) of tested strains were significantly more effective than introduced strain USDA 110, indigenous strains TSBF 404 and TSBF 131. This is in conformity with studies by Mungai and Karubiu (2010) (*elearning.jkuat.ac.ke/journals/ojs/index.php/*) who found that indigenous bean rhizobia are more effective than introduced rhizobia in two soils in Kenya. In this study, TSBF 442 TSBF 101, TSBF 344 and TSBF 531 were the most effective. These results corroborate previous studies by Meghvansi *et al.,* (2005) where significant differences among strains for various parameters such as shoot dry weight and nodule dry weight were reported under greenhouse conditions. Inoculation of strains enhanced vegetative growth and N uptake in soyabeans in the greenhouse (Meghvansi *et al.,* 2005). In this study, variable effects of strains on shoot dry weight were observed on soyabean genotypes. These results confirm previous studies which showed significant differences among rhizobial strains under growth room, greenhouse and field conditions in Chickpea (Icgen *et al.,* 2002). The increased yield due to inoculation by *Bradyrhizobium* strains have previously been reported by Egamberdiyeva *et al.,* 2004. They reported an increase in oil and protein content as a result of inoculation. This study reported an average 48% shoot yield advantage over uninoculated control. The significant interactive effects between strains and varieties on nodule dry weight imply specificity between promiscuous varieties and indigenous strains for this trait. Whereas TSBF 531 produced more nodule dry weight when inoculated to SB 8, TSBF 101 and TSBF 344 produced better shoot biomass when inoculated to SB 9 and SB 19 respectively.

5.5 Conclusion

Indigenous strains TSBF 442, TSBF 101 and TSBF 531 are the most effective strains and can be candidate strains for further testing to select inoculant strains for improved yields of promiscuous soyabeans. Further studies are suggested to determine the amount of nitrogen fixed by these strains under soil conditions.

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CHAPTER SIX

COMPETITIVENESS OF INDIGENOUS *BRADYRHIZOBIUM* STRAINS IN NODULES OF PROMISCUOUS AND SPECIFIC SOYABEAN VARIETIES IN KENYAN SOILS

Abstract

The ability of certain strains of *Rhizobium* and *Bradyrhizobium* to dominate nodulation of soyabean in a multi-strain environment is termed as competitiveness. Currently there is scant information on competitiveness for nodule occupancy of indigenous rhizobia on specific and promiscuously nodulating soyabean varieties in Kenya. The objective of the study was to determine the competitiveness of elite indigenous rhizobia strains in nodules of soyabean grown in soils derived from three agro-ecological zones. Varieties were grown in each of three previously autoclaved and non-autoclaved soils. PCR-RFLP fingerprinting profiles were used to identify the strains occupying nodules. In all the soils, Nyala variety got well nodulated with both indigenous strains isolated from promiscuous soyabean varieties (TSBF 531 and TSBF 442 but by comparison, the nodulation of promiscuous variety was more in the 3 soils irrespective of sterilization. In non-autoclaved soils, strain TSBF 531 was highly competitive (>57% occupancy) in nodules of all varieties and soils (except with the variety SB 19 in Bungoma soil). In both autoclaved and non-autoclaved soils, USDA 110 strain was not identified in any nodules analyzed. This suggests that a potential exists for the use of indigenous strains such as TSBF 531 and TSBF 442 as inoculants to replace strain USDA 110 currently recommended for the inoculation of soyabean production in Kenya.

6.1 Introduction

Soyabean is one of the most popular legumes in the world and its success stems from a number of factors related to its composition and productivity. Soyabean yields more than other common legumes, has relative few pest and disease problems and a good grain storage quality. Although 'traditionally-bred' soyabean varieties do not contribute much to the soil N status because most of the N fixed is removed by harvesting the grains, 'dual-purpose', promiscuous soyabean varieties were developed by the International Institute of Tropical Agriculture (IITA) between the mid-70s and early 90-s (Sanginga *et al.*, 2003). Their dual-purpose nature stems from the fact that these varieties produce a substantial amount of grain and leafy biomass, resulting in a relatively low N harvest index.

The promiscuous nature allows these varieties to nodulate freely with the indigenous *Bradyrhizobium* spp. population, precluding inoculation, a technique that has often failed in SSA (Mpepereki *et al.,* 2000). Promiscuous soyabeans were bred for compatibility with cowpea type soyabean rhizobia ubiquitous in tropical and sub-tropical soil (Rao *et al.,* 1981). Breeding for promiscuity was thought to be a better alternative to *Bradyrhizobium* inoculant use as developing countries were constrained in adequate facilities, personnel and effective production, storage and distribution mechanisms (Pulver *et al.,* 1982).

Since the release of these varieties, studies in many parts of Africa on the ability of promiscuous genotypes to establish effective symbiosis with indigenous *Bradyrhizobium* strains have yielded inconsistent results. While some studies (Choudhry, 1975; Pulver *et al.,* 1984) reported no yield responses of promiscuous soyabean cultivars after *Bradyrhizobium* inoculation, Sanginga *et al. (*1997) showed that inoculation increased total N and N_2 fixation and grain yield of early maturing promiscuous cultivars of soyabean. Okereke and Eaglesham (1993) reported an increase in nodulation and N_2 fixation of promiscuous soyabean cultivars using a commercial *Bradyrhizobium* inoculant, while Olufajo and Adu (1993) reported that US rhizobia strains failed to have significant yield responses when compared to local *Bradyrhizobium* strains on promiscuous soyabean varieties. These results show that there is still no scientific consensus on the ability of IITA bred promiscuous genotypes to be effectively nodulated with indigenous *Bradyrhizobium* strains. In addition, the competitiveness in nodulation of these indigenous rhizobia is not well elucidated. The competitive nodulation ability of a *Bradyrhizobium* strain is an important trait, as strains must compete with other rhizobia in the rhizosphere environment for nodulation sites on the host plant (Dowling and Broughton, 1986). For strains to succeed they must have the ability of to be competitive and be effective in symbiosis (Brockwell, 1980). Competitiveness is a

complex phenomenon which is influenced by interaction between soil factors and genetic traits of the host and *Rhizobium* symbiont (Thies *et al.,* 1992; Tripplet and Sadowsky, 1992). The establishment of effective nodulation can be enhanced by using effective nodulation and competitive strains of *Bradyrhizobium*. The objective of this study was to determine the competitiveness of elite indigenous rhizobia strains in nodules of soyabean varieties grown in soils derived from three agro-ecological zones.

6.2 Materials and Methods

6.2.1 Soil Sample Analysis

Composite soil surface samples collected from 0-15 cm were obtained from each selected site in Bungoma, Nakuru and Mitunguu. These soils were properly mixed, sieved and sub-sampled for chemical analysis as described by Anderson and Ingram (1993). Remaining soil was divided into two and one autoclaved at 121°C for 20 minutes, cooled to room temperature. Both samples were potted into sterile 4-kg polythene bags.

6.2.2 Estimation of Indigenous Population of Rhizobia in Soils

The most probable number of indigenous rhizobia in Nakuru, Bungoma and Mitunguu soils was estimated from the distribution of positive test plants in a plant infection test based on a five fold dilution series as described by (Brockwell *et al.,* 1975). Sand was sieved through 2 mm sieve and washed three times in double distilled water, dried and bagged into 4 kg plastic pots. Three varieties' (Nyala, SB 8 and SB 19) seeds were surface sterilized as described in Chapter five (Section 5.2.2) and pre-germinated in a growth chamber at 28°C. Varieties were inoculated with three soil samples each with four five fold dilutions (1:50; 1:250; 1:1250; 1:6250) (Brockwell *et al.,* 1975). Pots were laid out in a 3 x 3 x 4 factorial combination arranged in a randomized complete block design replicated four times. Dilutions were made by adding 10 grams of each soil to 90 ml of double distilled water and shaken for 10 mines. The 1:50 dilution was made by adding 1 ml of each soil to 4 ml of distilled water. Subsequent dilutions were made by adding 1 ml of previous dilutions to 4 ml of distilled water. Each five day old seedling was inoculated with 1 ml of the serially diluted soil suspensions. Seedlings were alternately watered with double distilled water and Broughton solution (Broughton and Dilworth, 1970). They were harvested after six weeks and roots were washed with tap water to remove sand taking care not to destroy the roots and nodules. The plants were scored for presence (+) or absence (-) of nodules. The presence of a single nodule on plants was considered a positive score. From the proportion of plants forming

nodules at each dilution level, the most probable number of rhizobia in the sample was calculated using a modified version of the Fisher and Yates as described by Brockwell *et al.*, (1975).

6.2.3 Soyabean Varieties

Seeds of promiscuous genotypes SB 8 and SB 19 and Nyala were obtained from TSBF-Maseno field station. SB 8 and SB 19 are genotypes bred at IITA to nodulate with cowpea type rhizobia ubiquitous in tropical soils. These two genotypes have been released to farmers in Kenya. Nyala, a specific nodulating genotype bred in Zimbabwe and released to farmers in Kenya, was used as a local control. Seed of each of these genotypes was surface sterilized as described in Chapter 5 (section 5.2.2). Germinated seedlings were transferred to sterilized polythene bags containing 4 kg each of autoclaved or non autoclaved soil.

6.2.4 Competitiveness of *Bradyrhizobium* strains

Elite strains were selected from a previous screening experiment in sand described in Chapter 5. Two strains were selected based on relative IGS group frequency in the isolated population (Table 4.1), superior biomass production abilities when inoculated to promiscuous soyabean genotypes as determined in Experiment 5 and their unique PCR fingerprint patterns (Figure 6.1). The two indigenous elite strains selected were TSBF 442 and TSBF 531. An introduced *Bradyrhizobium japonicum* strain USDA 110 obtained from Dr David Odee, Kenya Forestry Research Institute (KEFRI) was used as a control. Strains TSBF 442 and TSBF 531 were *Bradyrhizobium* selections isolated from Bungoma and Mitunguu environments, respectively.

Table 6.1: Selected strain treatments as described by IGS and phylogeny based on partial 16S rRNA gene sequence analysis

Trt	Treatment	Site*	Cluster**	Species affiliation	IGS group
1	TSBF-531	1	C	*Bradyrhizobium* spp.	I
	TSBF-442	2	A	*Bradyrhizobium elkanii*	III
	USDA 110	-	-	*Bradyrhizobium japonicum*	-
2	- ve control	-	-	-	-
3	+ ve control (70 $\mu g g^{-1}$)	-	-	-	-

*1= Mitunguu, 2 = Bungoma; ** = These were the major clusters of strains isolated from the Bungoma and Mitunguu sites in Chapter 4 (Figure 4.6)

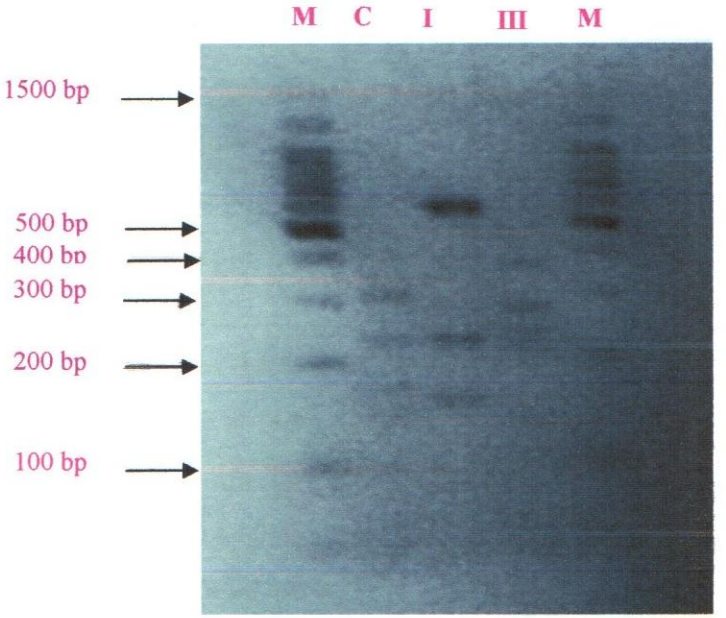

Figure 6.1: Fingerprint patterns of strains used in competitiveness trial in pot studies as revealed by *Msp* I enzyme. M-100 bp ladder, C-USDA 110, I-TSBF 531, III-TSBF 442

Pure cultures of these strains were each plated and pure colonies grown in broth liquid media. The broth was shaken on a rotatory shaker at 28°C operating at 250 revolutions per minute for 5 days until logarithmic phase as in previous experiment. These were mixed in a 1:1:1 cocktail and thoroughly shaken. Seedlings of each genotype (5 day old) were transferred into each 4-kg polythene bag filled with soils. A V shaped hole was made around soyabean plants grown in the polythene pots. Three milliliters (3 ml) of the broth cocktail mixture was applied at the base of the 5 day old soyabean plants and covered with soil. Negative and positive controls were included in the trial. Seedlings were grown at field capacity by alternate watering with Broughton solution (Broughton and Dilworth, 1970) and distilled water and grown in the greenhouse at approximately 12/12 light and 25 °C/32 °C.

6.2.5 Experimental Design

The experiment consisted of four treatments: three inoculation treatments (1:1:1 strain mix (USDA110:TSBF 531:TSBF 442), inoculated on three soyabean varieties (Nyala, SB 8, SB 19) in three soils (Nakuru, Bungoma, Mitunguu), each of which was either autoclaved or non autoclaved). The treatments were set up as 3 x 3 x 3 x 2 factorial combination arranged in a randomized complete block design replicated four times (Steel and Torrie, 1977). The three strain treatments were applied on three varieties, in three autoclaved and non autoclaved soils sampled from three agro-ecological zones. There was one cocktail inoculation treatment, a +Nitrogen control with no inoculation and a non-inoculated and a -N (-ve) control with no nitrogen. The plus nitrogen control received an equivalent of 98 kg ha^{-1} (800 mg per 4 kg pot of soil) N applied in the form of NH_4NO_3.

6.2.6 Harvesting and Data Recording

Plants were harvested at reproductive stage one (R1) Fehr *et al.,* 1971) for each of the genotypes by cutting at the first node level separating the shoot and the root system. Roots were washed free of soil and nodules separated, counted and recorded. Shoots and nodules were dried to constant weight 70°C for 48 hours and weights recorded. Plant shoot dry weight produced in this nitrogen free growth system was used as a proxy for nitrogen fixation. A sample of nodules was stored in sterilized glycerol at -80° C for DNA extraction.

6.2.7 Nodule Preparation for DNA Extraction

Nodules isolated from inoculated and uninoculated treatments were sampled, thoroughly cleaned to remove traces of soil, immersed in 95% ethanol and kept in glycerol at -30°C. Before analysis each nodule was surface sterilized with 96% ethanol for 30 seconds and rinsed with sterile water, then surface sterilized with 3.3% w/v $Ca(OCl)_2$ for 3 minutes, and three times rinsed with sterile distilled water as described by Krasova-Wade *et al.,* (2003). From this stage the nodules were manipulated aseptically.

6.2.8 Extraction of Nodule Bacteria DNA

Each nodule was crushed in 300 µl of sterile water with plastic micro pestles sterilized in 96% ethanol in a 1.5 ml Eppendorf tube. Total genomic DNA from a total of 125 nodules depending on the availability of nodules. Nodule analysis was carried out on 15, 14 and 12 nodules from varieties SB19, SB8 and Nyala respectively; 10, 14 and 9 nodules from SB19, SB 8 and Nyala respectively and 20, 18, 13 nodules from SB 19, SB 8 and Nyala

respectively grown on soils derived from Nakuru, Bungoma and Mitunguu, respectively was extracted by the method of Krasova-Wade *et al.*, (2003) in Chapter 4 (section 4.2.2).

The intergenic region between the 16S and 23S gene was amplified by PCR with the primers FGPS 1490-72; 5′ -TGCGGCTGGATCCCCTCCTT-3′ (Navarro *et al.*, 1992) and FGPL 132-38; 5′-CCGGGTTTCCCCATTCGG-3′ (Ponsonnet and Nesme, 1994). Polymerase chain reaction amplification was carried out in a 25 µl reaction volume containing 2µl of pure total DNA extract, freeze dried beads (Ready-to-Go PCR beads, Pharmacia Biotech) containing 2.5 U of Taq DNA polymerase, 200 µM Tris-HCL, (pH 9 at RT), 50 mM KCL, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1.0 µM of each primer as described in Chapter 4 (section 4.2.3).

6.2.9 Statistical Analysis

Statistical analysis was performed on nodule numbers, nodule dry weights and shoot weights using the following model.

$$\mathbf{Y_{ijklm} = \mu + E_i + A_j + EA_{ij} + R_{k(ij)} + EAR_{jl(i)} + S_k + ES_{ik} + EAS_{ijl} + V_m + EV_{im} + AV_{jm} + EAV_{ijm} + SV_{lm} + ASV_{jlm} + EASV_{ijlm} + \xi_{ijklm}}$$;

Where;

µ = is the general mean;

E_i = Effect due to i^{th} environment,

A_j = Effect due to j^{th} autoclaving treatment in the k^{th} replicate,

EA_{ij} = the interaction between i^{th} environment and j^{th} autoclaving treatment,

$R_{k(ij)}$ = The effect due to the j^{th} autoclaving treatment in the i^{th} environment in k^{th} replicate

EAR_{ijk} = Interaction between i^{th} environment and j^{th} autoclaving treatment and k^{th} replicate,

S_l = Effect due to l^{th} strain,

ES_{il} = Interaction between i^{th} environment and l^{th} strain,

EAS_{ijl} = Interaction between i^{th} environment, j^{th} autoclaving treatment and l^{th} strain,

V_m = Effect due to m^{th} variety

EV_{im} = Interaction between i^{th} environment, and m^{th} variety,

AV_{jm} = Interaction between l^{th} strain and m^{th} variety,

EAV_{ijm} = Interaction between i^{th} environment, j^{th} autoclaving and m^{th} variety,

SV_{lm} = Interaction between l^{th} strain, and m^{th} variety,

ASV_{jlm}= Interaction between m^{th} variety and the lth strain and j^{th} autoclaving treatment,

$EASV_{ijlm}$ Interaction between i^{th} environment and j^{th} autoclaving treatment, l^{th} strain and m th variety and

$\xi_{;jk\,m}$ is the random error.

Means were separated using LSD whenever they were significant at 95% confidence level.

6.3 Results

There was a highly significant ($p<0.01$) site, soil treatment, site x soil treatment effect on shoot biomass. Interactive effects of strain treatment x Site x Soil treatment x Variety on shoot biomass were also significant ($p<0.05$).

6.3.1 Chemical Characteristics of the Soils at the Sites

The soils from the different sites had variable chemical characteristics. Soil sampled from Bungoma had relatively lower extractable phosphorus than those from Mitunguu and Nakuru. The soil from Nakuru had relatively lower pH and C:N ratio than those from Mitunguu and Bungoma (Table 6.2).

Table 6.2: Soil chemical characteristics of the various environments from which promiscuous soyabean varieties were grown

Site	Depth	pH in water	Exch. Ca.	Exch. Mg	Exch. K	Extr. P	Total soil organic Carbon	Total soil Nitrogen
	cm		-------cmolckg $^{-1}$-----			mg Pkg^{-1}	(%)	(%)
Mitunguu	0-15	6.86	17.2	4.60	1.01	9.25	2.46	0.25
Bungoma	0-15	6.05	6.23	1.80	0.15	2.39	1.01	0.07
Nakuru	0-15	5.33	5.09	1.95	0.45	4.36	1.38	0.13

6.3.2 Indigenous Population of Rhizobia in Soils

Indigenous soil population of *Bradyrhizobium* strains nodulating varieties ranged from 11-49, 26-72 and 26-72 cells per gram of soil in Mitunguu, Nakuru and Bungoma respectively (Table 6.3). Varieties differed in their capacities to detect/trap rhizobia in different soils. When grown in Nakuru, Bungoma and Mitunguu soils, SB 8 estimated the indigenous

baseline population of rhizobia as being similar, while Nyala and SB 19 detected different amount of baseline population of rhizobia in the three soils.

6.3: Indigenous population[+] of rhizobia nodulating promiscuous and local soyabean varieties in soils from Nakuru, Bungoma and Mitunguu sites.

Soils	Rhizobia cells infecting varieties (bacteria cells gram^{-1} soil) Nyala	SB 8 (TGx. 1895-33F)	SB 19 (TGx. 1740-2F)
Mitunguu	2.6×10^1 - 4.9×10^1	2.6×10^1 - 4.9×10^1	1.1×10^1 - 3.0×10^1
Bungoma	4.6×10^1 - 7.2×10^1	2.6×10^1 - 4.9×10^1	2.6×10^1 - 4.9×10^1
Nakuru	2.6×10^1 - 4.0×10^1	2.6×10^1 - 4.9×10^1	4.6×10^1 - 7.2×10^1

[+] Estimated using the most probable number (MPN) method

6.3.3 Nodulation of Promiscuous Soyabean Varieties in Autoclaved and Non-Autoclaved Soils

All genotypes significantly responded to inoculation with cocktail (1:1:1) of TSBF 531:TSBF 442:USDA 110 0 in autoclaved and non-autoclaved soils for all the environments. There were significant nodulation responses to inoculation by genotypes in non-autoclaved soils than autoclaved soils. The highest number of nodules and nodule weights were observed on SB19 in both autoclaved and non-autoclaved soils while the least was on genotype Nyala. There was a significant interaction between genotypes and soil treatments on nodulation and nodule dry weights. The least nodulated genotypes in non-autoclaved and autoclaved soils were Nyala and SB 8 respectively. SB 19 was the most nodulated while Nyala was the least nodulated genotype in both soil treatments (non autoclaved and autoclaved) across all environments (Table 6.4 and Table 6.5).

6.3.4 Shoot Biomass Production for Soyabean Varieties in Autoclaved and Non-Autoclaved Soils

There was a significant ($p<0.05$) shoot biomass response to inoculation by elite multi strain mix for the promiscuous varieties in all sites in autoclaved soils. However, significant response to inoculation for variety Nyala was observed in all sites but Bungoma, where there was relatively lower P content (Table 6.6). The interaction between sites x soil treatment x varieties for biomass was only significant at $p=0.059$. In the non-autoclaved soils, there was a significant ($p<0.05$) shoot biomass response to inoculation by elite multi strain mix for the promiscuous varieties at all sites but Bungoma site. However, there was no significant inoculation response for variety Nyala at any of the sites.

Table 6.4: Nodulation of promiscuous soyabean varieties (TGx 1895-33F and TGx. 1740-2F) and the non promiscuous variety (Nyala) after inoculation by the multi-strain mixture in non-autoclaved soils isolated from Mitunguu, Nakuru and Bungoma Kenya

Soils	Nyala				SB 8 (TGx. 1895-33F)				SB 19 (TGx. 1740-2F)			
	Control +		Inoculated ++		Control		Inoculated		Control		Inoculated	
	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)
Mitunguu	2	6.7	75*	58.0*	7	33.0*	123*	117.6*	15	28.0	81*	57.5*
Bungoma	1	0	23	5.0	2	7.6	28*	5.0	1	3.0	49*	17.4
Nakuru	1	2.5	50*	33.0*	5	5.3	122*	95.0*	1	3.0	93*	80.0*

$LSD_{(0.05)}$ for Nodule number per plant =19.5

$LSD_{(0.05)}$) for nodule dry weight =23.3

+ Control: denotes no inoculation

++ Inoculated denotes: inoculation with indigenous *Bradyrhizobium* multi-strain mix consisting of TSBF 531, TSBF 442 and USDA 110 in 1:1:1 ratio

6.3.5 Nodule Occupancy of Indigenous Bradyrhizobia in Autoclaved and Non-Autoclaved Soils

In autoclaved soils, there was 100% nodule occupancy by TSBF 531 in all environments and genotypes (Table 6.7). In contrast, no nodule was occupied by strains USDA 110 and TSBF 442 in any environment or genotype indicating TSBF 531 as the most competitive strain. In non-autoclaved soil environment, strain TSBF 531 had higher (>57.1%) nodule occupancy than in nodules of each of the three genotypes grown in soils from all three environments except on SB19 at Bungoma. In the latter site, TSBF 442 had higher (70%) nodule occupancy than TSBF 531 (30%). There was a significant interaction among genotypes, environment and soil treatment (autoclaved and non autoclaved) on nodule occupancy. In autoclaved soils, all nodules on all genotypes grown in soil from the three environments had 100% nodule occupancy by strain TSBF 531. However, in non-autoclaved soils, nodule occupancy was influenced by strain, genotype and environment. Nodule occupancy by strain TSBF 531 ranged from 66.7% at Nakuru to 100% at Mitunguu and Bungoma on Nyala; 57.1% at Bungoma to 100% at Nakuru and Mitunguu on SB 8 and 30% at Bungoma to 77.8% and 91.7% at Nakuru and Mitunguu respectively on genotype SB 19 (). Genotypes had variable nodule occupancy (0%-75%) by background *Bradyrhizobium* strains belonging to IGS groups (XIII, IX, XI XIV and H) as previously reported by Wasike *et al,* (2009). However none of the background *Bradyrhizobium* strains were competitive in nodules of any genotype at any environment. Strain TSBF 531 was more competitive in nodulation than TSBF 442 and USDA 110.

Table 6.5: Nodulation of promiscuous soyabean varieties (TGx 1895-33F and TGx 1740-2F) and the non promiscuous variety (Nyala) after inoculation by a multi-strain mixture in autoclaved soils isolated from Mitunguu and Bungoma sites in Kenya

Sites	Nyala				SB 8 (TGx 1895-33F)				SB 19 (TGx. 1740-2F)			
	Control [+]		Inoculated [++]		Control		Inoculated		Control		Inoculated	
	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)	Nodule number per plant	Nodule dry wt per plant (mg)
Mitunguu	0	0	15	7.5	0	0	9	3.0	0	0	25*	5.0
Bungoma	0	0	17	10.0	0	0	7	2.5	0	0	22*	7.5
Nakuru	0	0	20*	17.5	0	0	10	5.0	0	0	30*	17.5

*Denotes significant nodulation response to inoculation by multi strain mix; $LSD_{(0.05)}$ for Nodule number = 19.5; $LSD_{(0.05)}$ for nodule dry weight =23.3;
[+]Control denotes no inoculation; [++] Inoculated denotes inoculation with indigenous *Bradyrhizobium* multi-strain mix consisting of TSBF 531, TSBF 442 and USDA 110 in 1:1:1 ratio

Table 6.6: Shoot dry weight (g) of promiscuous (TGx 1895-33F and TGx 1740-2F) and specific (Nyala) soyabean varieties after inoculation by multi-strain mixture in autoclaved and non-autoclaved soils from Mitunguu, Nakuru and Bungoma in Kenya

	Varieties	Nyala		SB 8 (TGx 1895-33F)		SB 19 (TGx 1740-2F)	
		Control[+]	Inoculated[++]	Control	Inoculated	Control	Inoculated
Sites		Shoot dry weight per plant (g)	Shoot dry weight per plant (g)	Shoot dry weight per plant (g)	Shoot dry weight per plant (g)	Shoot dry weight per plant (g)	Shoot dry weight per plant (g)
Mitunguu	Autoclaved	1.48 (0.11)	2.24 (0.39)*	1.21 (0.15)	2.26 (0.25)*	1.27 (0.27)	1.88 (0.24)*
	Non-Autoclaved	2.79 (0.40)	2.91 (0.15)	2.52 (0.25)	3.18 (0.12)*	1.59 (0.13)	2.27 (0.15)*
Bungoma	Autoclaved	2.46 (0.27)	2.63 (0.26)	2.07 (0.20)	2.84 (0.30)*	1.86 (0.34)	2.68 (0.31)*
	Non- Autoclaved	2.56 (0.24)	2.82 (0.36)	2.68 (0.40)	3.03 (0.18)	2.70 (0.26)	2.78 (0.15)
Nakuru	Autoclaved	2.86 (0.10)	3.48 (0.26)*	2.65 (0.18)	2.92 (0.21)*	2.75 (0.28)	3.84 (0.19)*
	Non-Autoclaved	4.35 (0.27)	4.84 (0.62)	4.12 (0.31)	4.76 (0.30)*	3.62 (0.27)	4.25 (0.59)*

* Indicates significant response to multi-strain inoculation; Values are means of 4 while those in brackets are standard errors (SE) of the individual means; Control[+]: denotes no inoculation; [++]Inoculated: denotes inoculation with indigenous *Bradyrhizobium* multi-strain strain consisting of TSBF 531 and TSBF 442 in 1:1 ratio.

Table 6.7: Nodule occupancy (%) of strains inoculated equally (TSBF 442: TSBF 531:USDA 110=1:1:1) to two promiscuous soyabean varieties cultivated in autoclaved soils from different sites in Kenya

Sites	Nyala (n=19)				SB8 (TGx 1895-33F) (n=24)				SB19 (TGx 1740-2F) (n=41)			
	TSBF 531	TSBF 442	USDA 110	Other	TSBF 531	TSBF 442	USDA 110	Other	TSBF 531	TSBF 442	USDA 110	Other
Mitunguu	100	0	0	0	100	0	0	0	100	0	0	0
Bungoma	100	0	0	0	100	0	0	0	100	0	0	0
Nakuru	100	0	0	0	100	0	0	0	100	0	0	0

+ Nodule occupancy calculated as a percentage of analyzed nodules containing strains with characteristic IGS groups inoculated on soyabean varieties grown in autoclaved soils sampled from different sites.

Table 6.8: Nodule occupancy (%) of strains inoculated equally (TSBF 442: TSBF 531:USDA110=1:1:1) to two promiscuous soyabean varieties cultivated in non-autoclaved soils from different sites in Kenya

		Nyala						SB 8 (TGx. 1895-33F)						SB 19 (TGx. 1740-2F)						
		Uninoculated (n=4)			Inoculated (n=15)			Uninoculated (n=4)			Inoculated (n=20)			Uninoculated (n=20)				Inoculated (n=31)		
Sites	Strain	TSBF 531	TSBF 442	IGS XIII	TSBF 531	TSBF 442	USDA 110	TSBF 442	IGS IX[+]	IGS H[+]	TSBF 531	TSBF 442	USDA 110	TSBF 531	TSBF 442	IGS XI[+]	IGS XIV	TSBF 531	TSBF 442	USDA 110
Mitunguu		0	50	50	100	0.0	0	50	25	25	100.0	0..0	0	33.3	33.3	33.3	0	91.7	8.3.	0
Bungoma		0	0	0	100	0.0	0	0	0	0	57.1	42.9	0	0.0	0.0	0.0	0	30.0	70.0	0
Nakuru		100	0	0	66.7	33.7	0	0	0	0	100.0	0.0	0	75.0	0.0	0.0	25	77.8	22.2	0

[+] **Nodule occupancy calculated as a percentage of analyzed nodules containing strains with characteristic IGS groups inoculated on soyabean varieties grown in non-autoclaved soils sampled from different sites.**

6.4 Discussion

Indigenous strain TSBF 531 was the most competitive strain in soyabean nodules of all varieties and soils except on SB 19 in soils at Bungoma. Competitiveness of *Bradyrhizobium* strains is influenced by interaction of the symbiont strains and host genotypes which differentially affect any or all the stages of symbiotic establishment including root hair curling, infection thread formation and penetration and nodule development (Lynch and Smith, 1993; Zhang *et al.*, 1995). Some of the factors implicated in influencing competitiveness include traits associated with individual strain, the host genotype and the environment. Characteristics associated with individual strains include their motility and chemotaxis, antibiotic production, selective substrate utilization, faster growth and colonization of the rhizosphere, rate of infection, cell surface determinants and nodule forming efficiency (Vlassak and Vanderleyden, 1997; Sessitsch *et al.*, 2002). The characteristics of the host genotype include the rate, amount, concentration of chemical signals (flavonoids, flavones and isoflavones) secreted by the host plant which induce synthesis of Nod factors by the strain (Peters and Verma, 1990) and the environment (the number of infective cells applied, the presence of competing indigenous (native) rhizobia (Thies *et al.*, 1991), deficiencies of essential elements (O'Hara, 2001). The competitive superiority of TSBF 531 over TSBF 442 and USDA 110 could be attributed to the secretion of antibiotic compounds which confer competitive advantage of this strain over the others.

Previous reports indicate that the production of peptide antibiotic trifolitoxin (TFX) by certain strains of rhizobia results in increased nodule occupancy values in non-sterile soils (Robleto *et al.*, 1998). Strain USDA 110 was least competitive in nodules of all soyabean genotypes in both autoclaved and non-autoclaved soils from all environments. Studies have previously reported that *Bradyrhizobium* spp in general do not nodulate specific soyabean genotypes (Abaidoo *et al.*, 2000). In this study, however, Nyala, a genotype conferred with specific genetic background, had significant nodulation response to inoculation by indigenous strains (TSBF 531 and TSBF 442). This confirms findings that the capacity of specific varieties of soyabean to nodulate with indigenous isolates in African soils is greater than is generally assumed (Musiyiwa *et al.*, 2005). However, this study also indicates that the nodulation of Nyala by competitive indigenous *Bradyrhizobium* strains such TSBF 531, does not necessarily result in any significant shoot biomass production. Shoot biomass production is often used as reliable proxy for nitrogen fixation (Abaidoo *et al.*, 2000). The significant nodulation and shoot biomass response to inoculation by strains at Mitunguu and Nakuru

relative to Bungoma could be attributable to a relatively high soil P status and competitiveness (nodule occupancy) by TSBF 531 at these sites. Phosphorus has previously been reported to improve nodulation and nitrogen fixation in soyabeans (Gunawardena *et al.*, 1993).

6.5 Conclusion

This study has shown that indigenous strain TSBF 531 is the most competitive of all strains tested in most soils. Strain 531 has been shown to be effective and competitive and can be selected for use in inoculants in order to optimize biological nitrogen fixation and thus increase soyabean yields at low cost in Kenya. However, further studies are suggested to determine the mechanism of competitiveness of TSBF 531 and find out why this strain is not competitive in some varieties and soils such as in SB 19 in Bungoma.

6.6 References

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CHAPTER SEVEN

RESIDUAL NITROGEN CREDIT TO MAIZE *(Zea mays, L.)* GROWN IN SEQUENCE WITH PROMISCUOUS SOYABEAN (*GLYCINE MAX L.*, MERR.) INOCULATED WITH INDIGENOUS *BRADYRHIZOBIUM* STRAINS IN CENTRAL RIFT OF KENYA

Abstract

Residual nitrogen benefits resulting from growing cereals after legumes have been reported world wide. In Kenya, higher fertilizer costs, especially N and declining soil fertility are among the key factors contributing to low crop yields. The contribution of promiscuous soyabean inoculated with indigenous rhizobia to soil N status and performance of succeeding maize (*Zea mays*, L.) was studied in a field experiment at Egerton-Njoro (LH_2) and Rongai (LH_4) in Rift Valley of Kenya from 2009-2010. Th experiment was laid out as a split plot. Treatments included growing promiscuous and non promiscuous soyabean during the short rains season (September-December) followed by maize in the long rains (March-July) season. During the short rains (precursor season), main plots received a blanket application of 60 kg ha^{-1} P but no applied nitrogen. Crop residues and vegetation of the weedy fallow were incorporated in the soil during seed bed preparation for the long rain season. Sub-plots were grown under 5 treatments: weedy fallow, maize (variety Duma), TSBF 531 inoculated to SB19, TSBF 531+TSBF 442 (1:1) inoculated to SB 19 and uninoculated local soyabean variety Nyala. In the long rains (test season), three levels N (0 $kgha^{-1}$, 30 kg ha^{-1} and 60 kg ha^{-1}) were applied to main plots and maize (variety Duma) was planted in all sub-plots. The trial was established in split plot design replicated four times at each site. A blanket application of 60 kg ha^{-1} P was applied across all plots in test season. Maize grain yield following promiscuous soyabean inoculated with TSBF 531 increased by 38.3% over that following maize, 20.6% following local soyabean, 18.5% following fallow and by 12.9 % following strain 1:1 mixture of TSBF 531 and TSBF 442. The study demonstrated that use of promiscouous soyabean variety inoculated with elite indigenous *Bradyrhizobium* strains in rotation with maize is a viable and preferable option to weedy fallows and maize- maize cropping sequences.

7.1 Introduction

Nutrient deficiencies especially those of N and P are among the major constraints to crop production in Kenya. Although this problem could be addressed by use of fertilizers, these are often inaccessible to most resource constrained farmers because of their high cost and inadequate supplies. Cultivation of leguminous crops in rotation or mixture with other food crops has been recognized as one of the cost effective ways by which farmers can maintain soil fertility. The legumes meet some of their N requirements through N fixation thus sparing some of the soil N for subsequent crops (Giller *et al.,* 1994). In addition to residual N that accrues due to nodule senescence and fallen leaves (Ledgard and Giller, 1995). Tropical grains legumes such as soyabean (*Glycine max* (L.) Merr.) are efficient in translocating the bulk of the fixed N to grains and even when the residues are returned to the soil, there may be a net removal of N from the field (Peoples and Crasswel, 1992; Giller *et. al.,* 1994). This however depends on the cultivar and the field history (Peoples *et. al.,* 1995). Some promiscuous soyabean varieties have indeterminate growth habit, relatively lower grain and N harvest indices hence great potential to add N to the soil than specifically nodulating commonly grown varieties (Mpepereki *et al.,* 1996). In Kenya, the soyabean crop is increasingly becoming one of the important crops both in terms of nutrition and soil fertility improvement. Previous studies conducted in moist savanna of Nigeria on soyabean-maize rotation have shown that a net N contribution of the soyabean crop to the soil increases with soyabean growth duration. This contribution ranges between -8 and 47 kg ha $^{-1}$, provided the soyabean stover is not harvested from the plots (Sanginga *et al.,* 1997, Sanginga *et al.,* 2001, Singh *et al.,* 2000). The usual practice of farmers in Kenya however is to harvest the crop without returning the stover in the field. Carsky *et al.* (1997) observed that even when the stover is exported, maize grain yield increase following soyabean that was given a basal application of 20 kg N ha^{-1} and was similar to that from 40 kg ha^{-1} applied to maize preceded by maize. The benefit of N fixed by the legume to subsequent cereal is usually reported as N fertilizer replacement value (NFRV) or the N fertilizer equivalent. This is defined as the quantity of fertilizer N required to achieve the same yield with continuous non legume as the yield obtained following a legume crop (Carsky *et al.,* 1997, Giller, 2001). For many small-scale farmers in Kenya, and with the recent fertilizer price escalation (Ksh 2500 for a 50 kg bag, 2010) access to fertilizer that will allow for even a basal application is limited. The objective of this experiment was to evaluate the residual soyabean N credit from a previous

best-bet promiscuous soyabean x elite indigenous bradyrhizobial strain on the yield of a subsequent maize crop.

7.2 Materials and Methods

7.2.1 *Bradyrhizobium* Strains

Having tested nine indigenous bradyrhizobia, a control (USDA 110), a positive and negative control in sand, two strains TSBF 531 (*Bradyrhizobium elkanii),* and TSBF 442 (*Bradyrhizobium* spp) were found to yield significantly more shoot biomass than the rest on inoculation to soyabean varieties SB 19, SB 9 and SB 8 (Chapter 5). Further, the two strains were tested for competitiveness in nodules and found to be more competitive than USDA 110 (Chapter 6). In this trial, TSBF 531, and a multi-strain mixture of TSBF 531 and TSBF 442 (1:1) were selected. The multi-strain mixture was selected based on prior confirmation in the field of its superior biomass production performance on inoculation to promiscuous varieties. These strains were hence selected for evaluation for their N replacement value in the experiment.

7.2.2 Site Selection and Characteristics

Field experiments were conducted at Egerton University (LH_2) near Njoro (0° 23' S and 35° 35' E) and on a farmer's field at Mang'u (LH_4) in Rongai (0° 10' S and 36° 01' E) both within Nakuru district (Cheruiyot *et al.,* 2001). Egerton University has an altitude of 2250 m above sea level and received annual precipitation of about 1000 mm and has a mean annual temperature range of 14-16 ^{0} C. Soils are well drained dark reddish clays classified as *Mollic Andosols*. Rongai site has an altitude of 1945 m above sea level and receives annual rainfall of about 900 mm with a mean annual temperature of 16-18^{0}C. The soils at this site are well drained clay loams classified as *Vitric Andosols (*Cheruiyot *et al.,* 2001). These sites were selected based on potential for soyabean to act as a sequential crop with maize, a staple crop. Most farmers in these areas have maize based diets, are resource poor and therefore require inexpensive sources of protein and an affordable source of N to boost their maize yields. Prior to planting each site in the first year, soil samples were collected from the top 30 cm. The samples were dried and sieved to pass through a 2 mm sieve before analyzing for physical and chemical properties (soil texture, pH, organic carbon, extractable P and exchange bases using method described by Anderson and Ingram, (1993). The experiment was laid out as a split plot; with N rate as main plot and fallow management options as sub-plots.

7.2.3 Precursor Season Treatments

During the first year, first season (precursor season), main plots received a blanket application of 60 kg ha^{-1} P but no applied Nitrogen. Sub-plots were grown under 5 fallow management options: weedy fallow, maize (Duma), TSBF 531 inoculated on SB 19, TSBF 531+TSBF 442 (1:1) inoculated on SB 19 and non promiscuous local soyabean variety Nyala. The crop residues and vegetation of the precursor season treatments were incorporated into the soil during seed bed preparation for the test season.

7.2.4 Test Season Treatments

In the following test season, three levels N (0, 30 and 60 kg ha^{-1}) were applied to main plots while maize (variety Duma) was planted in all sub-plots in order to test maize-maize, weedy fallow-maize, *Bradyrhizobium* + promiscuous soyabean variety-maize and non promiscuous soyabean-maize sequences under the respective three levels of N. Main, sub-plots and replications were independently randomized. A blanket application of 60 kg ha^{-1} P was applied across all plots in test season.

7.2.5 Experimental Layout

Main plots sizes had dimensions of 15.0 by 6.0 m while sub-plots consisted of plots measuring 3.0 m by 6.0 m with a net pot size of 2.0 m by 3.75 m. Two seeds were planted per hole at 75 cm by 30 cm for maize and 0.45 m by 0.10 m for soyabean and thinned to 1 plant per hole after germination to achieve a population of 53,333 plants per hectare and 222,222 plants per hectare for soyabean respectively at each of the sites. These were laid out in a randomized complete block design replicated three times at every site. The experimental plots were kept weed free by hand weeding during the growing season and pest management done using standard agronomic procedures.

7.2.6 Data Recording and Analysis

At physiological maturity, plants in each sub-plot net plot were counted and harvested. The harvested plants were air dried and weighed to get total dry matter. Maize cobs were dehusked and shelled. Sub-samples of the maize and maize stover were weighed and dried at 70° C for 24 hr before re-weighing to adjust total dry matter and grain yields for moisture content. Data was taken on variables: maize grain yields, maize stover, initial soil levels of N and P, soil pH and organic carbon. Leaf temperature, instant photosynthesis,

stomatal conductance and leaf chlorophyll were recorded using SPAD and the infrared gas analyzer (IRGA) as described by Coombs *et al.* (1982). Nitrogen and Phosphorus contents were determined following procedures described by Anderson and Ingram (1993). All data on maize growth, grain and stover yields were subjected to statistical analysis.

Analysis of variance was conducted on all the data using the following model.

$$\mathbf{Y_{ijklm} = \mu + E_i + N_j + EN_{ij} + R_{k(ij)} + NR_{jl(i)} + T_k + ET_{ik} + NT_{jk} + \xi_{ijkl}}$$

Where E_i = Effect due to i^{th} environment,

$R_{k(ij)}$ = Effect due to j^{th} replicate in the i^{th} environment.

T_k = Effect due to cropping sequence in the j^{th} replicate,

ET_{ik} = the interaction between i^{th} environment and k^{th} cropping sequence,

N_l = Effect due to l^{th} nitrogen rate in the j^{th} replicate,

NE_{il} = Effect due to interaction between i^{th} environment and l^{th} nitrogen rate,

$NR_{jl(i)}$ = The interaction effect between l^{th} nitrogen rate and j^{th} replicate,

NT_{kl} = the interaction effect of k^{th} cropping sequence, and l^{th} nitrogen rate,

NTE ikl = the interaction effect between i^{th} environment, k^{th} cropping sequence and l^{th} nitrogen rate while

ξ_{ijkl} is the random error.

Statistical analysis was conducted using ANOVA procedure of SAS (1996) and means separated using least significant difference (LSD) whenever treatment effects were significant at 95% confidence level (Steel and Torrie, 1977).

7.3 Results

7.3.1 Effect of Fallow Management on Grain Yield, Stover Yield, Chlorophyll Content and Carbon Exchange Rates

There were significant effects due to cropping sequences on grain yield ($p<0.05$) stover weight ($p<0.05$) and chlorophyll content ($p< 0.01$). The highest maize grain yield was realized when maize was planted after promiscuous soyabean inoculated with TSBF 531, while the least grain yield was realized when maize followed maize (Table 7.2). Across sites, maize grown after SB 19 inoculated with TSBF 531 was significantly different from the rest of the cropping sequences for grain and stover yield. On average, maize grain yield following SB 19 inoculated with TSBF 531 increased by 38.3%, 20.6%, 18.5% and 12.9% compared

with maize following maize, following fallow, following local variety Nyala and following SB 19 inoculated with TSBF mix respectively. Stover yield increased by 34.4%, 17.6%, 21.1% and 18.3% following maize, fallow, Nyala and SB 19 inoculated with TSBF 531 respectively while chlorophyll content increased by 8.0%, 8.5%, 13.4% over maize following maize, fallow and Nyala respectively (Table 7.2). For chlorophyll content, all but yields resulting from maize grown after fallow period were similar. Effect of fallow management was not significant ($p<0.05$) for mean leaf temperatures, stomatal conductance or instant photosynthesis. Their respective correlations of variations were very high.

7.3.2 Effect of Nitrogen on Grain, Stover Yield and Chlorophyll Content

There was no response to N application in terms of maize grain yield, stover yield and chlorophyll content at Njoro. However, application of 60 kg ha^{-1} nitrogen increased grain yield, stover yield and chlorophyll content by 31.0%, 26.5% and 16.9% respectively over the control at Rongai. Across sites, application of 60 kg ha^{-1} N resulted in a 16.1%, 18.4% and 8.5% increment in grain yield, stover yield and chlorophyll yield respectively across sites although these were not statistically different (Table 7.3).

7.3.3 Effect of Site on Grain, Stover Yield and Chlorophyll Content

There was a significant ($p<0.05$) site effect on maize grain, stover yield and chlorophyll content. Maize grain yield and chlorophyll content were significantly ($p<0.05$) higher at Njoro compared to Rongai by 11.5 % and 21.3% respectively. Stover yield however was significantly ($p<0.05$) higher at Rongai compared to Njoro by 43.1% (Table 7.4).

7.3.4 Effects of Rotation benefits of TSBF 531 inoculated SB 19, TSBF mix inoculated SB 19, Nyala and Fallow on maize grain

The rotation effect was separated from fixed N effects using a method described by Baldock *et al.,* (1981) and adopted by Karpstein-Machan and Stuelpnagel (2000). In this method, the total N effect was calculated as a yield of maize following a legume minus the yield following a non-legume both without added N while the rotational effect was calculated as the difference between rotations at the highest fertilizer N rate. The total N effects on maize grain yield due to SB19 inoculated with TSBF 531, SB19 inoculated with TSBF mix, Nyala and fallow management were 1674.3 kg ha^{-1}, 904.1 kg ha^{-1}, 561.8 kgha^{-1}, and 1286.4 kg ha^{-1} respectively (Table 7.5).

Table 7.1: Soil chemical characteristics for Njoro and Rongai sites in 2009

Site	pH+	Exch. K	Exch Ca	Exch. Mg	Extract P	Organic Carbon	Sand	Silt	Clay
		----------cmolc kg^{-1}----------			Mg P kg^{-1}	%	%	%	(%)
Njoro	5.55	1.19	10.51	1.84	4.16	3.47	20.3	61.0	18.7
Rongai	6.16	1.88	12.51	2.42	9.25	2.99	24.6	53.3	22.1

+ pH was measured in a 1:1 soil water ratio

Table 7.2: Effect of cropping sequences on maize grain, stover yield and chlorophyll content at Njoro and Rongai sites

Treatment	Grain yield (Kg ha^{-1})			Stover (Kg ha^{-1})			Chlorophyll content (SPAD)		
	Njoro	Rongai	Mean	Njoro	Rongai	Mean	Njoro	Rongai	Mean
SB 19 inoc with TSBF 531-Maize	5422.3 a	4438.0 a	4930.4 a	9794.1a	11290.7a	10542.4a	42.88ab	34.58abc	38.729 a
SB 19 inoc with TSBF Mix-Maize	4689.1 b	4221.0 ab	4364.4 b	7024.3b	11178.8 a	8912.1 b	43.22a	35.83ab	39.525 a
Nyala-Maize	4246.7 bc	4039.8 ab	4088.0 b	6994.9b	10979.1 a	8706.8 b	39.28c	37.16a	38.217 ab
Fallow-Maize	4102.9 bc	3929.4 b	4161.9 b	6948.0b	10799.9 a	8963.6 b	39.58bc	31.81 bc	35.696 b
Maize-Maize	3796.2 c	3332.4 c	3564.3 c	6235.2b	8692.1 b	7843.5 b	41.21 bc	30.50c	35.854 a
LSD $_{(0.05)}$	639.25	413.83	374.26	1843.4	1843.4	1238.8	3.35	4.775	2.27

+ Means followed by the same letter in a column are not significantly different from each other using the least significant difference test ($p<0.05$).

Table 7.3: Effect of nitrogen on maize grain, stover yield and chlorophyll content at Njoro and Rongai sites

N rate	Grain yield (Kg ha^{-1})		Stover (Kg ha^{-1})		Chlorophyll content (SPAD)	
	Njoro	Rongai	Njoro	Rongai	Njoro	Rongai
0	4440.1 a	3473.8 c	6997.5 a	9451.5 b	41.455 a	31.120 b
30	4277.9 a	3950.6 b	7683.6 a	10358.3 b	39.865 a	34.44 ab
60	4636.1 a	4552.4 a	7516.7 a	11954.4 a	42.375 a	36.370 a
LSD $_{(0.05)}$	NS	320.57	NS	1331.5	NS	3.699

+ Means followed by the same letter in a column are not significantly different from each other using the least significant difference test ($p<0.05$). NS-Not significant

Table 7.4: Effect of site on maize grain, stover yield and chlorophyll content at Njoro and Rongai sites

	Grain yield (Kg ha^{-1})	Stover yield (Kg ha^{-1})	Chlorophyll content (SPAD)
Njoro	4451.4 a	7399.3 b	41.231 a
Rongai	3992.3 b	10588.0 a	33.977 b
LSD $_{(0.05)}$	236.7	783.46	1.813

+ Means followed by the same letter in a column are not significantly different from each other using the least significant difference test ($p<0.05$).

Table 7.5: Effects of Total N benefits of SB 19 inoculated TSBF 531, TSBF mix, Nyala and fallow management on subsequent maize grain yield (kg ha^{-1})

N effect	SB 19 + TSBF 531	SB 19 + TSBF Mix	Nyala	Fallow
Total N benefits	1674.3	904.1	561.8	1286.35
Rotation benefit	823.0	768.9	607.7	0
N benefit*	851.3	135.2	-45.9	1286.35

* N benefit is the rotational effect attributable to N from previous crop treatment.

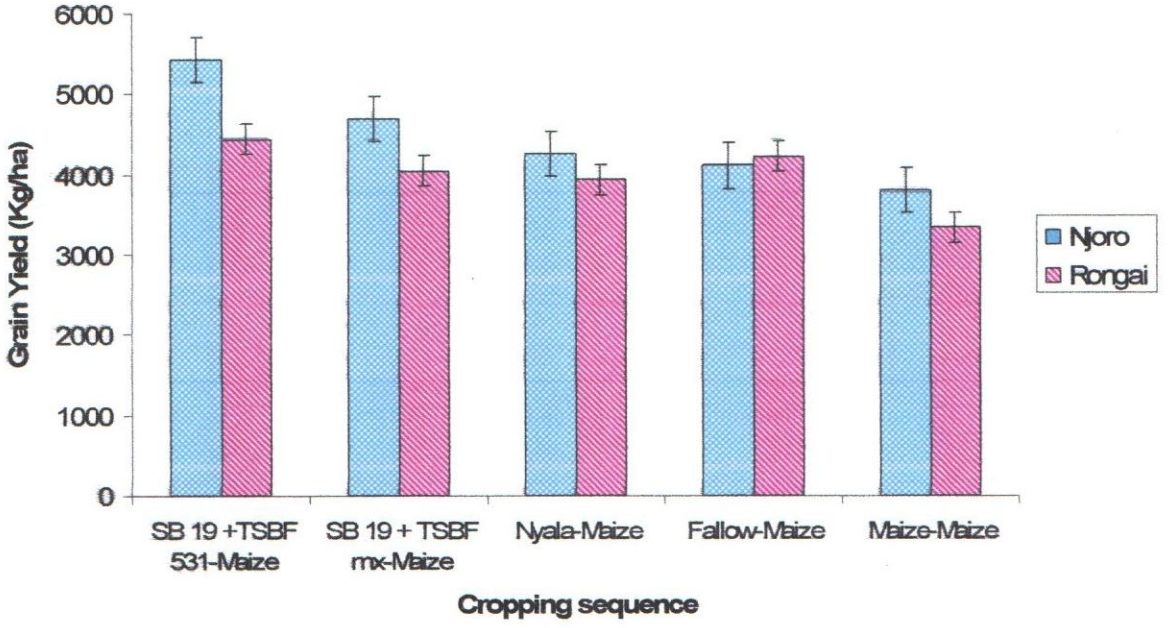

Figure7.1. Interaction between cropping sequence and site on maize grain yield in Central Rift of Kenya.

SB 19 + TSBF mix denotes SB19 inoculated with a 1:1 mixture of indigenous rhizobia strains TSBF 531 and TSBF 442; Nyala - uninoculated local non-promiscuous soyabean variety; SB 19 + TSBF 531 denotes SB 19 inoculated with single strain indigenous rhizobia TSBF 531. Bars represent standard errors of the individual means.

7.3.4 Effects of Applied N to Grain and Stover Yields

In the 2010 rotation, TSBF 531 inoculated SB 19 increased maize grain yield by 1674.3 kg ha^{-1} compared to 904.1 kg ha^{-1} and 561.8 kg ha^{-1} for TSBF mix and Nyala respectively. TSBF 531 had 85% yield increase over TSBF mix and 198% over Nyala. Contribution due to rotation was least for Nyala (607 kg ha^{-1}) but was nearly the same for TSBF 531 and TSBF mix inoculated promiscuous soyabeans variety. Fixed nitrogen benefit accounted for about 50.8% of the total yield increase of TSBF 531 inoculated SB 19 compared to 14.95% of TSBF mix inoculated SB 19 and 45.9% for Nyala (Table 7.6).

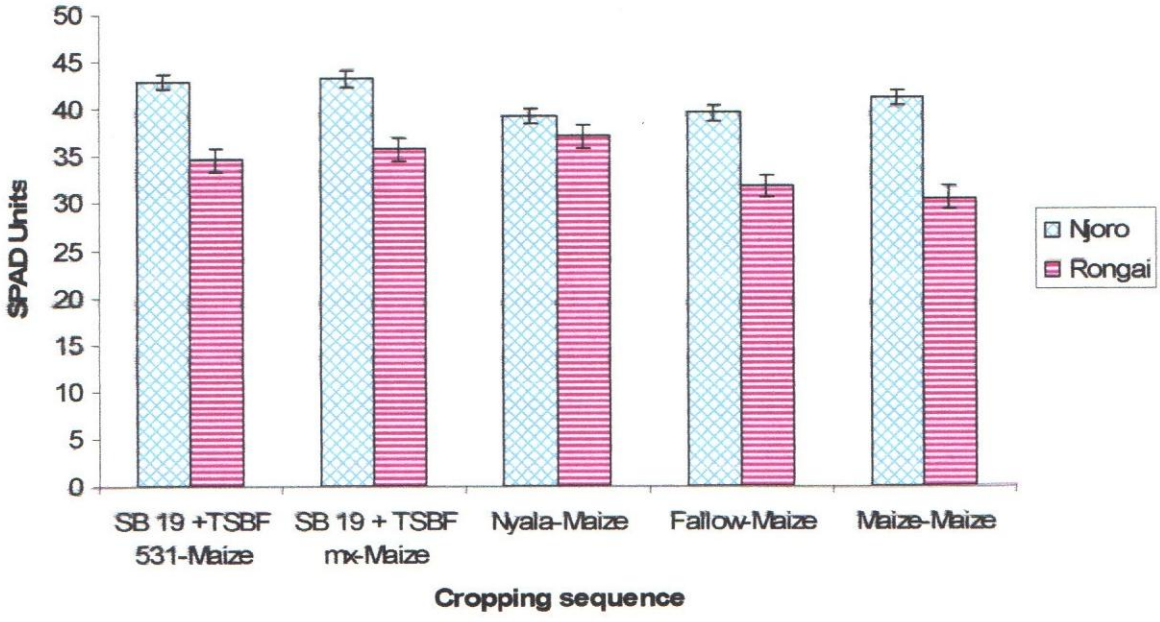

Figure 7.2: Interaction between cropping sequence and site on maize leaf chlorophyll in Central Rift of Kenya

SB 19+TSBF mix denotes SB19 inoculated with a 1:1 mixture of indigenous rhizobia strains TSBF 531 and TSBF 442; Nyala-uninoculated local non-promiscuous soyabean variety; SB 19 + TSBF 531 denotes SB 19 inoculated with single strain indigenous rhizobia TSBF 531. Bars represent standard errors for individual means.

7.4 Discussion

7.4.1 Effect of Previous Crop on Subsequent Maize Grain and Stover Yields, Chlorophyll and Carbon Exchange Rates (CER)

The hypothesis in this study was that maize grown after soyabean inoculated with elite rhizobia yields more than maize grown after non-nodulated soyabean (Nyala), fallow and maize grown after maize. Managing fallows with legume crops generally resulted in increasing maize yield by about 4460.9 kg ha^{-1} for legumes compared to 3863.1 kg ha^{-1} for weedy fallow and maize. This translates to 15.5% advantage in yields associated with improved fallow management practice. These results are comparable to others reported before (Carsky *et al.*, 1997; Kasasa *et al.*, 1999). Despite the large amount of residue in weedy fallow and continuous maize plots in the precursor season, the impact of these fallow management options on maize grain yield, biomass production and chlorophyll content on the subsequent season was negligible. This could be due in part to the fact that the critical enhancing feature of fallow species seems to be residue quality as defined by levels of tissue N and C: N ratio (Cheruiyot *et al.*, 2001). The degree to which residue quality affects crop

performance in tropical environments has been reported before (Tian *et al.,* 1994). While the benefits of improved fallow are largely attributable to improved N supply and low C:N ratios of the legume (Ojiem *et. al.,* 1998), other factors may have played a part. These include soil physical and chemical properties such as bulk density, water holding capacity and improved cation exchange capacity (McVay *et al.,* 1989). Maize could also be self incompatible since maize residue often retards growth of seedlings maize crop due to presence of phytotoxins (Yakie and Cruise, 1984). The enhanced yield increment could also be due to nitrogen credit resulting from N fixation. The difference in yield between maize following maize and response to fertilizer N and maize following nodulated soyabean was used to estimate N credit using the fertilizer replacement value method (Varvel and Wilheims, 2003). These calculations gave an estimate of 561.8 kg ha $^{-1}$,703.4 kg ha $^{-1}$ and 1574.8 kg ha $^{-1}$ hectare for maize following non-nodulating Nyala, SB 19 nodulated with TSBF mix and SB 19 nodulated with TSBF 531 respectively. These figures are considerably higher (45 kg ha^{-1}) than is commonly reported in American mid-west for fertilizer N recommendations (Kurtz *et. al.* 1984). This reduction indicates that N fixation played an important part of N credit. The fact that non nodulated soyabean also increased maize yield indicated that other non N factors also played a role in soyabean N credit. These non N factors have previously been reported (Varvel and Wilheim, 2003) and include improvement of soil tilth, breaking of disease and pest cycles and N sparing effect.

7.4.2 Effects of Applied N on Grain and Stover Yields

In the 2010 rotation, TSBF 531 inoculated SB 19 increased maize grain yield by 1674.3 kg ha^{-1} compared to 904.1 kg ha^{-1} and 561.8 kg ha^{-1} for TSBF mix and Nyala respectively. TSBF 531 had 85% yield increase over TSBF mix and 198 % over Nyala. Contribution due to rotation was least for Nyala (607 kg ha^{-1}) but was nearly the same for TSBF 531 and TSBF mix inoculated promiscuous soyabeans variety. This is expected since the TSBF mix contained a 1:1 strain mixture of a competitive strain TSBF 531 and TSBF 442. Fixed nitrogen benefit accounted for about 50.8% of the total yield increase of TSBF 531 inoculated SB 19 compared to 14.95% of TSBF mix inoculated SB 19. Nyala did not record any fixed nitrogen but had a rotation and total effect. This could be as a result of the mineralization of the biomass as this variety had no nodules in the precursor season. The increased yield due to inoculation with TSBF 531 could be due to the fact that this strain is effective (Chapter 5) and competitive in nodules (Chapter 6) and hence fixes a substantial amount of nitrogen that is left for utilization by a subsequent maize crop. Maize grain yield in

Njoro was significantly higher than in Rongai. This could be related to higher rainfall, organic carbon and exchangeable bases in Njoro compared to Rongai (Mungai *et al.*, 2009).

7.5 Conclusions

This study indicates that farmers can derive benefit from rotating maize with promiscuous soyabean especially when inoculated with indigenous competitive strains such as TSBF 531. It also indicates a potential for positive N benefit consequences on reduction of cost of maize production often cited as an impediment to crop production by small scale farmers in Kenya. However, further studies should be conducted to determine the actual amounts of N fixed by TSBF 531 inoculated SB 19.

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CHAPTER EIGHT

Conclusions and Recommendations

This study evaluated the suitability and performance of dual-purpose soyabean and indigenous soyabean rhizobia nodulating promiscuous soyabean varieties in selected agro-ecological conditions of Kenya. It set out to specifically assess the natural nodulation of introduced promiscuous soyabean varieties bred in West Africa under selected Kenyan agro-ecological zones, determine the genetic diversity of indigenous *Bradyrhizobium* strains nodulating seven promiscuous soyabean varieties in the different agro-ecological zones in Kenya, determine the effectiveness of potential best-bet promiscuous soyabean variety-indigenous *Bradyrhizobium* strain combination (s) for nodulation and biomass production as proxy for nitrogen fixation. Further, the study determined the competitiveness of selected elite indigenous *Bradyrhizobium* in promiscuous and specific soyabean nodules. Finally, the study determined the N credit to maize from promiscuous soyabean inoculated with elite indigenous *Bradyrhizobium* strains in two sites in Central Rift in Kenya.

This study has shown that varieties bred under West African conditions in fact nodulate and fix nitrogen with indigenous *Bradyrhizobium* strains but more so in the presence of P. Promiscuous varieties showed sensitivity to P. While a majority of varieties showed increased nodulation and biomass production with the addition of P others did not. Promiscuous varieties grown in sites with relatively high pH and phosphorus produced more nodules and shoot biomass. This indicates that initiatives to promote soyabean should promote the use of P to optimize biological nitrogen fixation for soyabean production. Studies are needed to determine the amounts of P required to produce optimum grain yields for P sensitive and P insensitive varieties in different agro ecological zones.

Genetic diversity and phylogeny of indigenous *Bradyrhizobium* strains nodulating seven introduced promiscuous soyabean varieties grown in two different regions (two in the highlands and three sites in the lowlands) in Kenya was assayed using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the 16S-23S rDNA intergenic spacer region and 16S rRNA gene sequencing. PCR-RFLP analysis applied on nodules using *Msp* I distinguished 18 intergenic spacer (I-XVIII) groups in the highlands and 8 (A, B, C, D, E, F, G, H) IGS groups in the lowlands. This study has revealed considerable diversity among *Bradyrhizobium* strains nodulating promiscuous soyabean varieties grown in

Kenya. The diversity identified in *Bradyrhizobium* populations in the five sites represents a valuable genetic resource that has potential utility for the selection of more competitive and effective strains to improve biological nitrogen fixation and increase soyabean yields at low cost. However, further studies are recommended to confirm the preponderance of *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* strains in the relatively acidic sites and whether this is related to strain acid tolerance or other soil characteristics. More sites need to be sampled in order to get a better estimate of the diversity of indigenous *Bradyrhizobium* strains nodulating promiscuous soyabean varieties in Kenyan soils.

Ten indigenous strains were selected from non-homologous IGS groups isolated from varieties grown in highland sites. These strains and an inoculant strain USDA 110 control were inoculated on three promiscuous (SB 8, SB 9 and SB 19) grown in sand media for 45 days. Dry shoot biomass was used to estimate effectiveness. All indigenous strains were significantly ($P<0.05$) more effective than USDA 110. However, inoculation of varieties with TSBF 442, TSBF 101 and TSBF 531 resulted in more shoot biomass and nodulation than USDA 110. These elite strains (TSBF 442 and TSBF 531) belonged to predominant IGS groups and were selected and tested for competitiveness in nodules.

Further screening of elite indigenous *Bradyrhizobium* strains for competitiveness in autoclaved and non autoclaved soils indicated that indigenous strains isolated from Mitunguu (TSBF 531) and Bungoma (TSBF 442) were more competitive than USDA 110 in both specific (Nyala) and promiscuous (SB 8 and SB 19) soyabean varieties tested in the three soils. TSBF 531 was more competitive than TSBF 442 in all varieties and soils except in SB 9 nodules in Bungoma. Preponderance of TSBF 531 strains in nodules of promiscuous varieties under the background of relatively improved P conditions resulted in significant increment in nitrogen fixation (using shoot biomass as reliable proxy) in Mitunguu and Nakuru compared to relatively low P conditions in Bungoma. This indicates that P and competitive *Bradyrhizobium* strains are necessary for optimum soyabean production. However, studies are suggested to decipher the biological mechanisms which confer competitive superiority of TSBF 531 over TSBF 442 and USDA 110. This competitive superiority also needs to be confirmed in field experiments in order to more reliably inform the deployment of TSBF 531 as potential inoculant strain for soyabean production in Kenya. The amounts of N fixed by elite strains (TSBF 531 and TSBF 442) also need to be determined.

Nitrogen credit to maize following managed fallows was estimated. The treatments included maize-maize, maize-fallow, maize-specific soyabean and maize-inoculated cropping

sequences. The study showed maize grain yield increase following inoculated soyabean (single or mixed strain). In the 2010 rotation, TSBF 531 inoculated SB 19 increased maize grain yield by 1674.3 kg ha^{-1} compared to 904.1 kg ha^{-1} and 561.8 kg ha^{-1} for TSBF mix and Nyala respectively. TSBF 531 had 85% yield increase over TSBF mix and 198% over Nyala. Contribution due to rotation was least for Nyala (607 kg ha^{-1}) but was nearly the same for TSBF 531 and TSBF mix inoculated promiscuous soyabeans variety. Fixed nitrogen benefit accounted for about 50.8% of the total yield increase of TSBF 531 inoculated SB 19 compared to 14.95% of TSBF mix inoculated SB 19 and 45.90% for Nyala.

This supports the view that soyabean inoculated with indigenous rhizobia is important in nitrogen credit. It also suggests that non N benefits accrue from growing maize as previous crop. The study also concludes that inoculation of promiscuous soyabean provides a sustainable option to reduce fertilizer N requirements of maize grown after inoculated soyabean. However, it is recommended that further studies are needed to determine:

(i) The benefit: cost relationship of using the indigenous strain TSBF 531 as inoculants in a promiscuous soyabean-maize sequence.

(ii) The quantity of nitrogen fixed by TSBF 531 inoculated soyabean in different sites.

(iii) The soyabean N credit to maize of elite strain inoculated promiscuous soyabean varieties with varying maturity periods in different agro-ecological zones in Kenya.

ANNEX

Annex 1.1: Mean squares of shoot dry weight, nodule number and nodule fresh weight of three varieties inoculated with 12 strains of indigenous *Bradyrhizobium* strains under greenhouse at ICIPE, 2008

Source of variation	df	Dry Shoot biomass	Number of nodules	Nodule dry weight
Varieties	3	0.035	0.0955	0.00038
Replication	4	0.036	1.556	0.00075
Varieties × Replication	8	0.023	1.402	0.000037
Strain	11	0.379**	68.50*	0.00338*
Varieties × Strain	22	0.029	2.870	0.00119*
Error	132	0.022	1.8069	0.025761
CV (%)		11.86	29.3	2.45

Values with ** are significant at $p< 0.01$, while values with * are significant at $p< 0.05$.

Annex 1.2: Mean squares of treatments, sites, soil treatment and variety of three promiscuous varieties inoculated with a 1:1:1 strain mix of TSBF 531, TSBF 442 and USDA 110

Source of variation	df	Dry shoot biomass	Number of nodules	Nodule dry weight
Strain treatment	2	1.157 NS	557.1**	0.0042**
Site	2	41.97 **	17.12**	0.0012**
Strain treatment x site	4	0.355 NS	10.31*	0.0006**
Soil treatment	1	50.60 **	139.23**	0.0049**
Strain treatment xsSoil treatment	1	1.05 *	80.51**	0.0022**
Site x Soil treament	2	4.60 **	14.43**	0.0009**
Strain treatment x Site x Soil treatment	4	0.254 NS	7.56**	0.0050**
Variety	2	2.02 *	6.65*	0.0004*
Strain treatment x Variety	4	0.59 NS	4.83 NS	0.00011NS
Site x Variety	4	0.11 NS	0.791*	0.00011NS
Strain treatment x Site x Variety	8	0.30 NS	1.358 *	0.00009**
Soil treatment x Variety	2	1.607**	8.42 NS	0.00069*
Strain treatment x Soil treatment x Variety	4	0.108 NS	4.69 NS	0.0021 NS
Site x Soil treatment x Variety	4	0.557 NS	0.71 NS	0.0014 NS
Strain trtxSite xSoil treatmentxVariety	8	0.016 *	1.37* NS	0.00011 NS
Error	71	0.2702	1.022	0.000063

Values with ** are significant at $p< 0.01$, while values with * are significant at $p< 0.05$.

Annex 1.3: Mean sum of squares for Site, Nitrogen and Cropping sequences in combined (Rongai and Njoro) sites

Source of variation	df	Grain yield (kg ha^{-1})	Stover (kg ha^{-1})	Chlorophyll content (SPAD units)$^{+}$	Chlorophyll content (µgcm^{-2})$^{++}$	Leaf Energy (W m^{-2})	Photosynthesis (µmol m^{-2}sec^{-1})	Leaf Temperature (^{o}C)	Stomatal conductance (mm m^{-2}sec^{-1})
Site	1	6324429.0	305045159.2*	1579.05*	139922.8*	2.840*	6.66	98.1	0.016
Nitrogen	2	4408808.0	22854458.0	101.29	58763.4	0.030	29.15	42.5	0.007
Site × Nitrogen	2	2077613.0	11819798.1	71.98	10396.2	0.070	30.07	11.77	0.005
Cropping Sequence(CS)	4	5857733.0*	22868943.7**	72.21*	11032.6	0.005	50.5	7.10	0.008
Site × CS	4	996936.0	13830391.1 *	59.53	13598.3	0.003	47.1	0.57	0.009
Nitrogen × CS	8	832155.0	7114168.8	30.16	12939.9	0.012	45.7	1.13	0.011
Site × Nitrogen × CS	8	590979.0	2133810.7	11.22	26554.5	0.015	45.	1.08	0.011
Error	72								
CV		15.40	23.93	13.25	30.6	68.4	-5719	12.4	768.5

* denotes significant at $p<0.05$ while ** denotes significant at $p<0.001$. $^{++}$ Data taken using infra red gas analyzer while $^{+}$ denotes data taken using SPAD.

Annex 1.4: Mean sum of squares for cropping sequences and nitrogen at Njoro

Source of variation	df	Grain yield	Stover	Chlorophyll ntent (SPAD) units
Nitrogen	2	643422	2560575	32.25
Cropping sequences	4	2685415**	22794728**	39.56
Nitrogen × cropping quences	8	699973	6244390	17.55
CV (%)		17.34	30.1	9.81

** indicates significant at p<0.001

Annex: 1.5: Mean sum of squares for cropping sequences and nitrogen at Rongai site

Source of variation	df	Grain yield	Stover	Chlorophyll content (SPAD)
Nitrogen	2	5842998.9	32113680	1.00
Cropping sequences	4	2079355.3 **	13904605.9*	2.77*
Nitrogen x cropping sequences	8	723161*	300388.6	0.72
CV (%)		19.5	19.6	16.97

** indicates significant at P< 0.001; while * indicates significant at P< 0.05

Annex 2: Publications

Annex 2.1: Plant and Soil Journal 2009.

Plant Soil (2009) 322:151–163
DOI 10.1007/s11104-009-9902-7

REGULAR ARTICLE

Genetic diversity of indigenous *Bradyrhizobium* nodulating promiscuous soybean [*Glycine max* (L) Merr.] varieties in Kenya: Impact of phosphorus and lime fertilization in two contrasting sites

V. W. Wasike · D. Lesueur · F. N. Wachira · N. W. Mungai · L. M. Mumera · N. Sanginga · H. N. Mburu · D. Mugadi · P. Wango · B. Vanlauwe

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Abstract While soybean is an exotic crop introduced in Kenya early last century, promiscuous (TGx) varieties which nodulate with indigenous rhizobia have only recently been introduced. Since farmers in Kenya generally cannot afford or access fertilizer or inoculants, the identification of effective indigenous *Bradyrhizobium* strains which nodulate promiscuous soybean could be useful in the development of inoculant strains. Genetic diversity and phylogeny of indigenous *Bradyrhizobium* strains nodulating seven introduced promiscuous soybean varieties grown in two different sites in Kenya was assayed using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the 16S-23S rDNA intergenic spacer region and 16S rRNA gene sequencing. PCR-RFLP analysis directly applied on 289 nodules using *Msp* I distinguished 18 intergenic spacer groups (IGS) I–XVIII. Predominant IGS groups were I, III, II, IV and VI which constituted 43.9%, 24.6%, 8.3% 7.6% and 6.9% respectively of all the analyzed nodules from the two sites while IGS group VII, IX, X, XI, XII, XIV, XVI, XVII, XVIII each constituted 1% or less. The IGS groups were specific to sites and treatments but not varieties. Phylogenetic analysis of the 16S rRNA gene sequences showed that all indigenous strains belong to the genus *Bradyrhizobium*. *Bradyrhizobium elkanii*, *Bradyrhizobium* spp *and Bradyrhizobium japonicum* related strains were the most predominant and accounted for 37.9%, 34.5%, and 20.7% respectively while *B. yuanmingense* related accounted for 6.9% of all strains identified in the two combined sites. The diversity identified in *Bradyrhizobium* populations in

Responsible Editor: Euan K. James.

V. W. Wasike
Kenya Agricultural Research Institute (KARI),
P.O. Box 57811-00200, Nairobi, Kenya

V. W. Wasike · N. Sanginga · H. N. Mburu · D. Mugadi · P. Wango · B. Vanlauwe
Tropical Soil Biology and Fertility Institute of the International Centre for Tropical Agriculture (TSBF-CIAT),
United Nations Avenue, P.O. Box 30677, Nairobi, Kenya

V. W. Wasike · N. W. Mungai · L. M. Mumera
Department of Crops Horticulture and Soils,
Egerton University,
P.O. Box 536, Njoro, Kenya

F. N. Wachira
Department of Biochemistry and Molecular Biology,
Egerton University,
P.O. Box 536, Egerton, Kenya

D. Lesueur (✉)
CIRAD, PERSYST Department,
UPR Ecosystems of Plantations, Tropical Soil Biology and Fertility Institute of the International Centre for Tropical Agriculture (TSBF-CIAT),
United Nations Avenue, P.O. Box 30677, Nairobi, Kenya
e-mail: d.lesueur@cgiar.org
e-mail: didier.lesueur@cirad.fr

Annex 2.2: East African Agriculture and Forestry Journal, 2009:

Environmental Influence on the Genetic Diversity of Indigenous Bradyrhizobia Nodulating Promiscuous Soyabean in Kenya.

V.W. Wasike, H.N. Mburu, B. Vanlauwe, L.A. Wasilwa, N. Sanginga, N.W. Mungai, L.M. Mumera, D. Lesueur, F.N. Wachira, J.O. Owuoche

Abstract

Kenya continues to face a large and growing deficit in soyabean [*Glycine max* (L.) Merr] with annual production of 5000 metric tones against a demand of over 100,000 metric tones. Among the reasons advanced for low production is low soil fertility. Promiscuous soyabean varieties known as tropical glycine cross (TGx) have recently been introduced in Kenya. These varieties are crosses between non-promiscuous North American and promiscuous Asian soyabean genotypes bred by the International Institute of Tropical Agriculture IITA to nodulate and fix nitrogen with a population of indigenous strains of bradyrhizobia resident in African soils. These strains are diverse and exhibit this diversity in competitiveness and effectiveness within and between hosts. While the diversity of indigenous rhizobia nodulating other leguminous plants in Kenya has been reported, the genetic diversity of indigenous rhizobia nodulating promiscuous soyabean has not been reported. The objective of this study was to assess the genetic diversity of indigenous *Bradyrhizobium* strains isolated from six promiscuous soyabean varieties grown in five sites differing in altitude, rainfall and soil chemical characteristics in Kenya. Genetic diversity was assayed by amplifying the 16S-23S rDNA intergenic spacer (IGS) region. The differences in fingerprints were used to group strains into IGS groups. Sequencing of the 16S rDNA gene was used to determine strain phylogeny. Strains nodulating varieties in lowland sites were grouped into 8 while those in highland sites were grouped into 18 different IGS groups respectively. Predominant groups were A, B and D and I, III and II in the lowland and highland sites respectively. Sequencing of 16S rDNA gene showed that 37.5 % of the strains nodulating soyabean in all sites were related to *Bradyrhizobium elkanii,* 30.0 % to *Bradyrhizobium japonicum*, 25.0 % to *Bradyrhizobium* spp and 7.5% to *Bradyrhizobium yuanmingense.* The polymorphism in *Bradyrhizobium* populations from these sites represents a valuable genetic resource that has potential variability for the selection of more effective and competitive strains for use as inoculants to facilitate soyabean production at low cost.

Annex 2.3: Kenya Science Technical and Innovation Journal

The competitiveness of *Bradyrhizobium* spp strains on promiscuous and specific soyabean varieties in Kenyan soils

Victor Wasike [a, b, c]*, Nancy Mungai [c], Laettittia Herrmann [b], Francis Wachira [d], Louis Mumera [c], Nteranya Sanginga [b], Marine Wagner [b], Harrison Mburu [b], Phillip Malala [b], Lusike A. Wasilwa [a].

[a] Kenya Agricultural Research Institute (KARI) P.O. Box 57811-00200, Nairobi, Kenya

[b] Tropical Soil Biology and Fertility Institute of the International Centre for Tropical Agriculture (TSBF-CIAT), United Nations Avenue, P.O. Box 30677, Nairobi, Kenya

[c] Department of Crops Horticulture and Soils, Egerton University, P.O. Box 536, Njoro, Kenya.

[d] Department of Biochemistry and Molecular Biology, Egerton University, P.O. Box 536, Egerton, Kenya.

Corresponding author: Victor W. Wasike

Kenya Agricultural Research Institute (KARI) P.O. Box 57811-00200, Nairobi, Kenya

Tel: + 0722.850805

Email: vwwasike@yahoo.com

Abstract

The ability of certain strains of Rhizobium and Bradyrhizobium to dominate nodulation in a multi-strain environment is termed as competitiveness. Currently there is scant information on competitiveness for nodule occupancy of indigenous rhizobia on specific and promiscuously nodulating soyabean varieties in Kenya. The objective of the study was to determine infectiveness and competitiveness of two selected rhizobia isolated from eastern and western Kenya to nodulate two promiscuous and one specific nodulating soyabean local variety, Nyala, grown in soils derived from three agro-ecological zones with no known previous history of soyabean cultivation. Varieties were grown in each of three previously autoclaved and non-autoclaved soils. PCR-RFLP fingerprinting profiles were used to identify the strains occupying nodules. In all the soils, variety Nyala was well nodulated with both indigenous strains (TSBF 531 and TSBF 442) isolated from promiscuous soyabean varieties. In non-autoclaved soils, strain TSBF 531 was in most cases competitive in nodules of all varieties and soils. This suggests that a potential exists for the use of indigenous strains such as TSBF 531 and TSBF 442 as inoculants for soyabean production in Kenya.

Annex 3: Dunnets test for pair wise comparison of *Bradyrhizobium* strains

Strain IDs.	Strains
1	TSBF 404
2	TSBF 101
3	TSBF 131
4	TSBF 531
5	TSBF 534
6	TSBF 331
7	TSBF 442
8	TSBF 344
9	TSBF 336A
10	USDA 110
11	Negative control
12	Positive control

t Tests (LSD) for Biomass Dry Weight

Alpha	**0.05**
Error Degrees of Freedom	**131**
Error Mean Square	**0.16915**
Critical Value of t	**1.97824**

Comparisons significant at the 0.05 level are indicated by *.**

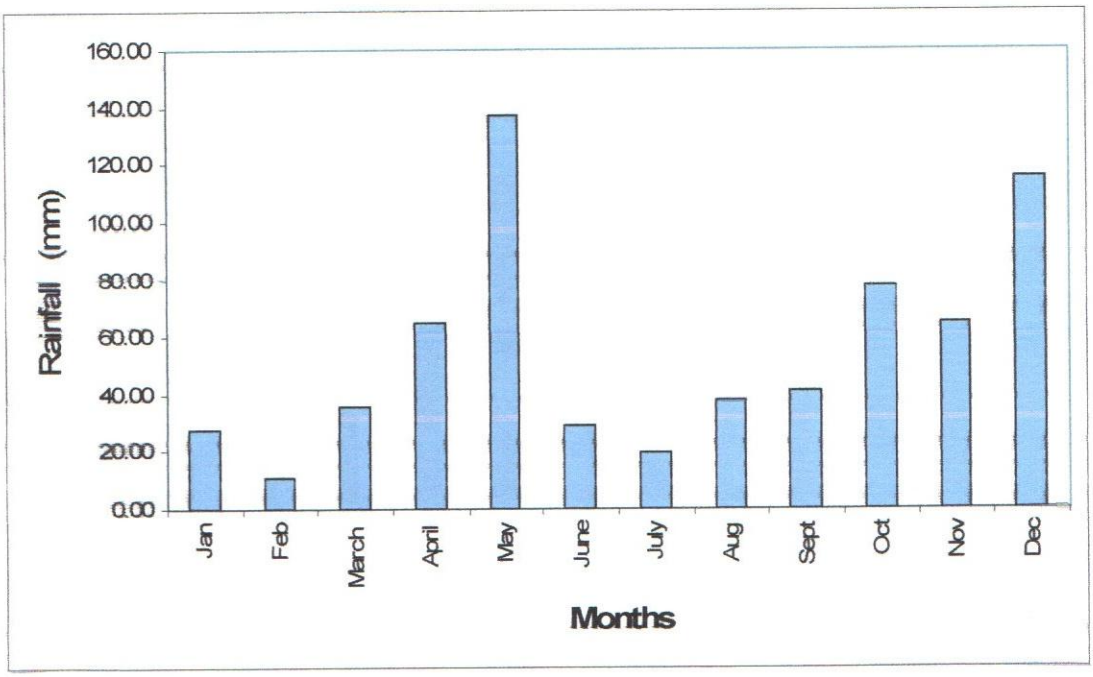
Strain Comparison	Difference Between Means	95% Confidence Limits		
12 - 7	1.2877	0.9853	1.5900	***
12 - 2	1.3393	1.0422	1.6364	***
12 - 4	1.3547	1.0576	1.6518	***
12 - 8	1.4412	1.1441	1.7383	***
12 - 5	1.4440	1.1469	1.7411	***
12 - 9	1.4680	1.1709	1.7651	***
12 - 6	1.4860	1.1889	1.7831	***
12 - 1	1.5600	1.2629	1.8571	***
12 - 11	1.6147	1.3176	1.9118	***
12 - 10	1.7660	1.4689	2.0631	***
12 - 3	1.7980	1.5009	2.0951	***
7 - 12	-1.2877	-1.5900	-0.9853	***
7 - 2	0.0517	-0.2507	0.3540	
7 - 4	0.0670	-0.2353	0.3693	
7 - 8	0.1535	-0.1488	0.4559	
7 - 5	0.1563	-0.1460	0.4587	

7	- 9	0.1803	-0.1220	0.4827	
7	- 6	0.1983	-0.1040	0.5007	
7	- 1	0.2723	-0.0300	0.5747	
7	- 11	0.3270	0.0247	0.6293	***
7	- 10	0.4783	0.1760	0.7807	***
7	- 3	0.5103	0.2080	0.8127	***
2	- 12	-1.3393	-1.6364	-1.0422	***
2	- 7	-0.0517	-0.3540	0.2507	
2	- 4	0.0153	-0.2818	0.3124	
2	- 8	0.1018	-0.1952	0.3989	
2	- 5	0.1047	-0.1924	0.4018	
2	- 9	0.1287	-0.1684	0.4258	
2	- 6	0.1467	-0.1504	0.4438	
2	- 1	0.2207	-0.0764	0.5178	
2	- 11	0.2753	-0.0218	0.5724	
2	- 10	0.4267	0.1296	0.7238	***
2	- 3	0.4587	0.1616	0.7558	***
4	- 12	-1.3547	-1.6518		
4	- 7	-0.0670	-0.3693	0.2353	
4	- 2	-0.0153	-0.3124	0.2818	
4	- 8	0.0865	-0.2106	0.3836	
4	- 5	0.0893	-0.2078	0.3864	
4	- 9	0.1133	-0.1838	0.4104	
4	- 6	0.1313	-0.1658	0.4284	
4	- 1	0.2053	-0.0918	0.5024	
4	- 11	0.2600	-0.0371	0.5571	
4	- 10	0.4113	0.1142	0.7084	***
4	- 3	0.4433	0.1462	0.7404	***
8	- 12	-1.4412	-1.7383	-1.1441	***
8	- 7	-0.1535	-0.4559	0.1488	
8	- 2	-0.1018	-0.3989	0.1952	
8	- 4	-0.0865	-0.3836	0.2106	
8	- 5	0.0028	-0.2943	0.2999	
8	- 9	0.0268	-0.2703	0.3239	
8	- 6	0.0448	-0.2523	0.3419	
8	- 1	0.1188	-0.1783	0.4159	
8	- 11	0.1735	-0.1236	0.4706	
8	- 10	0.3248	0.0277	0.6219	***
8	- 3	0.3568	0.0597	0.6539	***
5	- 12	-1.4440	-1.7411	-1.1469	***
5	- 7	-0.1563	-0.4587	0.1460	

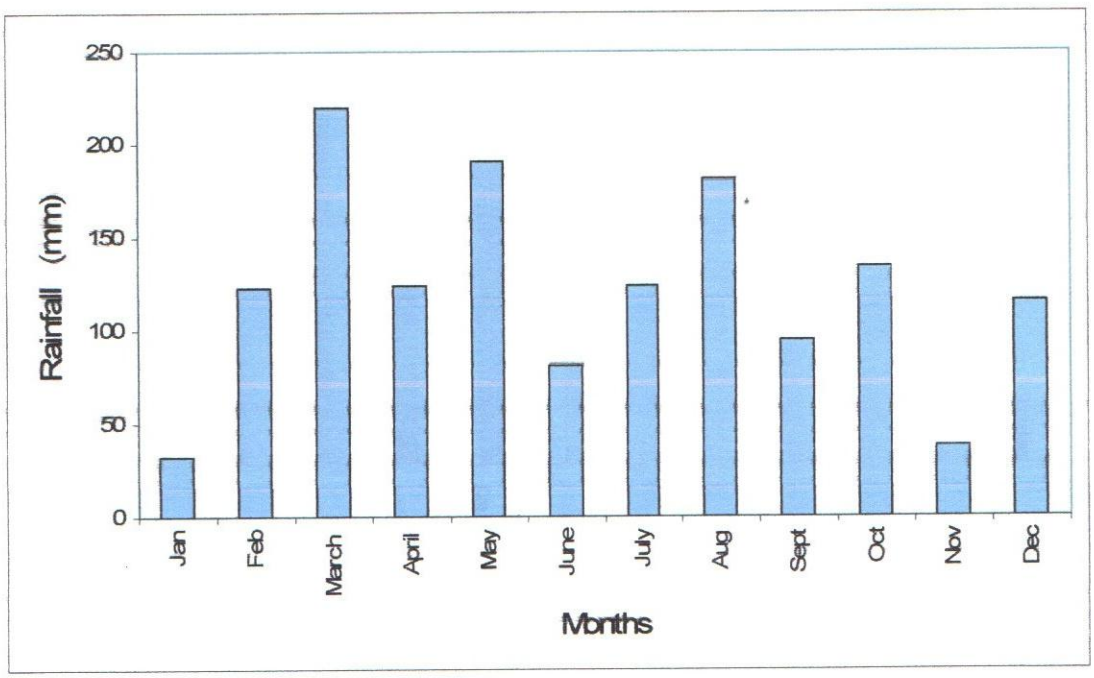
5	- 2	-0.1047	-0.4018	0.192	
5	- 4	-0.0893	-0.3864	0.2078	
5	- 8	-0.0028	-0.2999	0.2943	
5	- 9	0.0240	-0.2731	0.3211	
5	- 6	0.0420	-0.2551	0.3391	
5	- 1	0.1160	-0.1811	0.4131	
5	- 11	0.1707	-0.1264	0.4678	
5	- 10	0.3220	0.0249	0.6191	***
5	- 3	0.3540	0.0569	0.6511	**
9	- 12	-1.4680	-1.7651	-1.1709	***
9	- 7	-0.1803	-0.4827	0.1220	
9	- 2	-0.1287	-0.4258	0.1684	
9	- 4	-0.1133	-0.4104	0.1838	
9	- 8	-0.0268	-0.3239	0.2703	
9	- 5	-0.0240	-0.3211	0.2731	
9	- 6	0.0180	-0.2791	0.3151	
9	- 1	0.0920	-0.2051	0.3891	
9	- 11	0.1467	-0.1504	0.4438	
9	- 10	0.2980	0.0009	0.5951	***
9	- 3	0.3300	0.0329	0.6271	***
6	- 12	-1.4860	-1.7831	-1.1889	***
6	- 7	-0.1983	-0.5007	0.1040	
6	- 2	-0.1467	-0.4438	0.1504	
6	- 4	-0.1313	-0.4284	0.1658	
6	- 8	-0.0448	-0.3419	0.2523	
6	- 5	-0.0420	-0.3391	0.2551	
6	- 9	-0.0180	-0.3151	0.2791	
6	- 1	0.0740	-0.2231	0.3711	
6	- 11	0.1287	-0.1684	0.4258	
6	- 10	0.2800	-0.0171	0.5771	
6	- 3	0.3120	0.0149	0.6091	***
1	- 12	-1.5600	-1.8571	-1.2629	***
1	- 7	-0.2723	-0.5747	0.0300	
1	- 2	-0.2207	-0.5178	0.0764	
1	- 4	-0.2053	-0.5024	0.09	
1	- 8	-0.1188	-0.4159	0.1783	
1	- 5	-0.1160	-0.4131	0.1811	
1	- 9	-0.0920	-0.3891	0.2051	
1	- 6	-0.0740	-0.3711	0.2231	
1	- 11	0.0547	-0.2424	0.3518	
1	- 10	0.2060	-0.0911	0.5031	
1	- 3	0.2380	-0.0591	0.5351	
11	- 12	-1.6147	-1.9118	-1.3176	***
11	- 7	-0.3270	-0.6293	-0.0247	***

11 - 2	-0.2753	-0.5724	0.0218	
11 - 4	-0.2600	-0.5571	0.0371	
11 - 8	-0.1735	-0.4706	0.1236	
11 - 5	-0.1707	-0.4678	0.1264	
11 - 9	-0.1467	-0.4438	0.1504	
11 - 6	-0.1287	-0.4258	0.1684	
11 - 1	-0.0547	-0.3518	0.2424	
11 - 10	0.1513	-0.1458	0.4484	
11 - 3	0.1833	-0.1138	0.4804	
10 - 12	-1.7660	-2.0631	-1.4689	***
10 - 7	-0.4783	-0.7807	-0.1760	***
10 - 2	-0.4267	-0.7238	-0.1296	***
10 - 4	-0.4113	-0.7084	-0.1142	***
10 - 8	-0.3248	-0.6219	-0.0277	***
10 - 5	-0.3220	-0.6191	-0.0249	***
10 - 9	-0.2980	-0.5951	-0.0009	***
10 - 6	-0.2800	-0.5771	0.0171	
10 - 1	-0.2060	-0.5031	0.0911	
10 - 11	-0.1513	-0.4484	0.1458	
10 - 3	0.0320	-0.2651	0.3291	
3 - 12	-1.7980	-2.0951	-1.5009	***
3 - 7	-0.5103	-0.8127	-0.2080	***
3 - 2	-0.4587	-0.7558	-0.1616	***
3 - 4	-0.4433	-0.7404	-0.1462	***
3 - 8	-0.3568	-0.6539	-0.0597	***
3 - 5	-0.3540	-0.6511	-0.0569	***
3 - 9	-0.3300	-0.6271	-0.0329	***
3 - 6	-0.3120	-0.6091	-0.0149	***
3 - 1	-0.2380	-0.5351	0.0591	
3 - 11	-0.1833	-0.4804	0.1138	
3 - 10	-0.0320	-0.3291	0.2651	

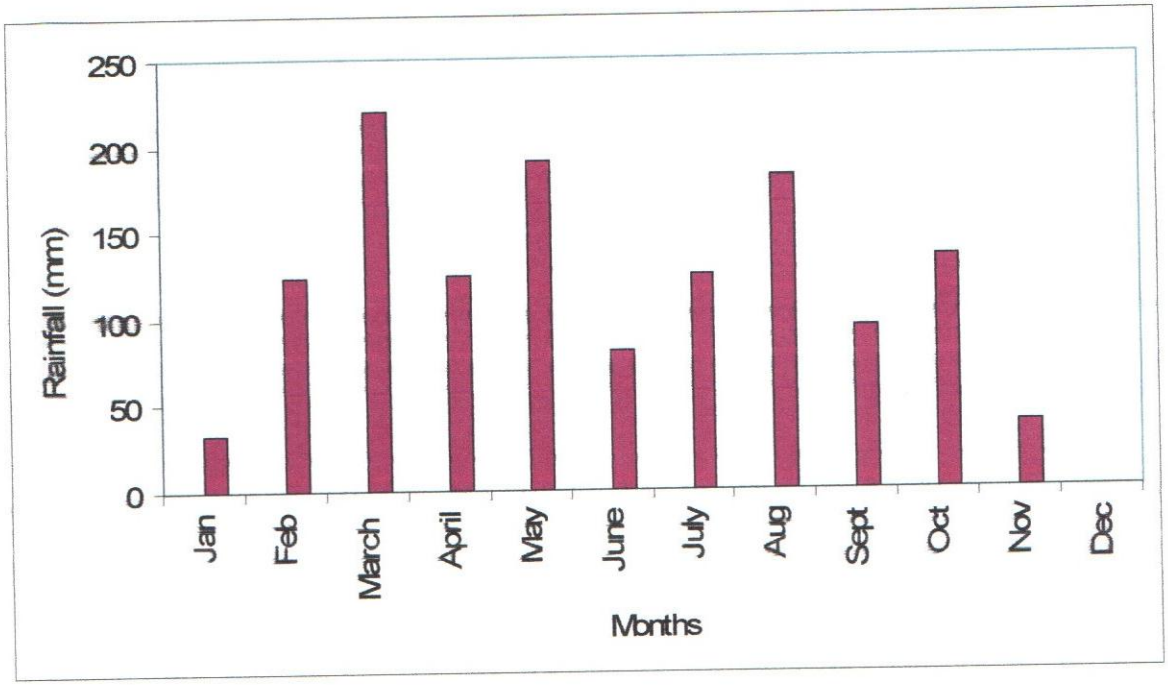
Annex 4: Rainfall Data



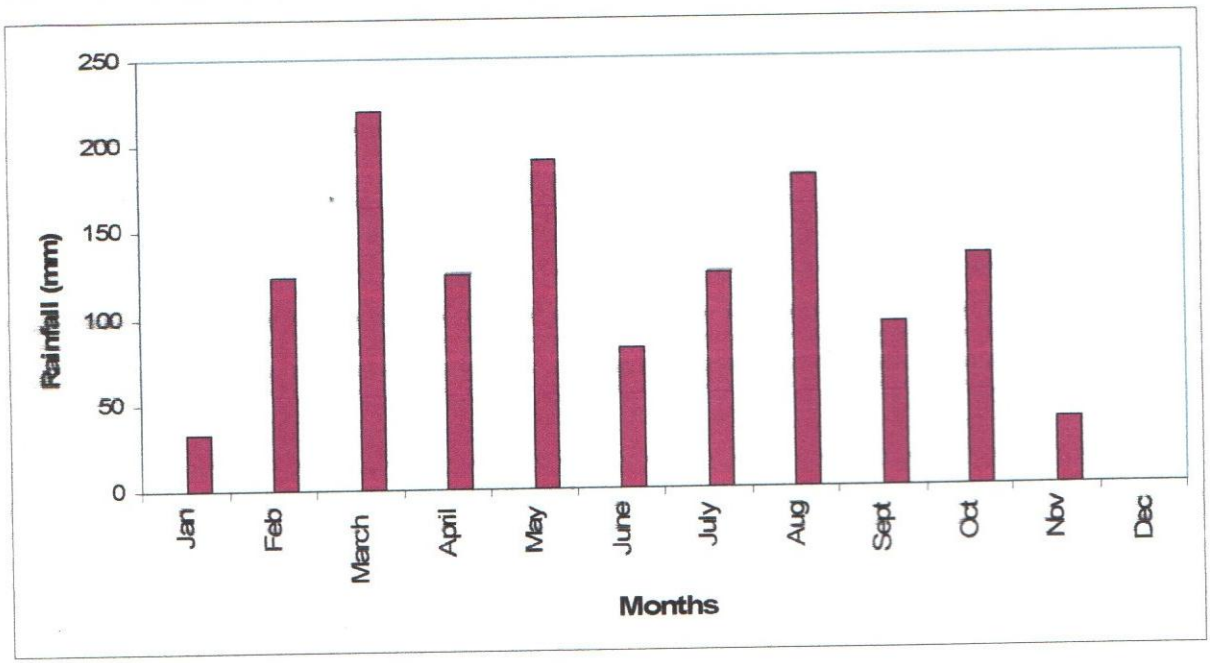
Annex 4.1: Distribution of rainfall during the 2009 precursor season in Rongai.



Annex 4.2: Distribution of rainfall during the maize test season in Rongai in 2010.



Annex 4.3: Distribution of rainfall during the pre-cursor season in Njoro in 2009



Annex 4.4: Distribution of rainfall during the maize test season in Njoro in 2010

Annex 5: Preparation of DNA Extraction Buffers

Preparation of 0.2M Tris HCL, pH 8 (Sigma@ T3069): Add 200 ml of 1M Tris HCL in 100ml double distilled water.
Adjust pH to 8.0 with addition of NaoH

Preparation of 0.04 EDTA, Sigma@ P6755 pH 8: Dissolve 372.2 g into 1 litre x 0.04= 14.888 g/litre

Or: Dissolve 3.722 g in 250 of double distilled

Preparation of 2.8 M NaCl (Sigma@ S3014): Dissolve 58.44 g/litre x 2.8

13.32 g into 1 litre of double distilled water

Or: 40.908 g in 250ml of double distilled water

Preparation of CTAB 4% (w/v) (Sigma@ H6269): Dissolve 4 g of CTAB in 100ml of double distilled water
Or: Dissolve 10 g CTAB in 250ml of double dstilled water

Preparation of polyvinyl polypyrolidone (Sigma@ P6755)(PVPP 2 % (w/v):
Dissolve 2 g of PVPP in 100ml of double distilled water
Or: Dissolve 20 g PVPP in 250ml of double dstilled water