BIOLOGICAL NITROGEN FIXATION IN DROUGHT RESISTANT TEPARY BEAN (*Phaseolus acutifolius* A.Gray var. latifolius) IN THE SEMI- ARID MAKUENI DISTRICT OF KENYA.

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B.Ed

A Thesis submitted in partial fulfilment for the award of the degree of Master of Science in Botany of Kenyatta University

BOTANY DEPARTMENT

MAY 2001

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award

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To the memory of my late father, Timothy Ong'are Ochieng ;
..... *The dead are well(Eccl).*

ACKNOWLEDGEMENT

I would like to express my most sincere appreciation to my supervisors, namely Dr. Mwaniki, J.W., Dr. Shisanya, C.A., and Dr. Gitonga, N.M. without whose patience and critical guidance this work would not have been realized.

A special mention goes to both teaching and non-teaching staff of Kenyatta University for their support and advice. Of particular note are Dr. C.A. Ogol, Zoology Department, Dr. Geoffrey Muluvi, Biochemistry Department and Mr. John Nzau of Botany Department.

I am also greatly indebted to Dr. David Odee, KEFRI, Muguga for allowing me use of their facilities and for facilitating the conveyance of some of my samples to Dundee, Scotland for N analysis.

Special thanks also go to Messrs. Mutinda, Nobert and Juma at KARI/ ICRISAT field station, Kiboko, for their immeasurable support during my field trials.

Lastly I would like to acknowledge the immense support and encouragement I received from my loving wife Victoria; in more ways than I care to mention she bore the brunt of this grueling undertaking.

ABSTRACT

Laboratory trials were carried out to assess infectivity of tepary bean (TB) by the various selected rhizobium strains. Strains 446 and 3254 were obtained from the Microbiological Resources Centre (MIRCEN), University of Nairobi; while 578 and 579 were from Iserlohn, Germany. A local isolate, KTB1 was obtained from Kiboko soils. All treatments except the nitrogen treatment GUNF and the control nodulated under greenhouse conditions, though there were variations in the proportions of nodulated plants. The highest nodulation proportion (100%) was observed with strain 3254 followed by 446 (75%), then 578 and 579 (both at 25%). The lowest nodulation proportion was observed with strain KTB1 (20%).

Field trials were conducted at KARI/ ICRISAT station, Kiboko to compare the dry matter yields of tepary bean inoculated with various rhizobial strains. Plants were sampled at three stages during growth and separated into several fractions viz. root, shoot and nodules. There were no significant differences in the dry matter yield for the first harvest and second harvest. In the final harvest, total plant dry weight and mean pod dry weight from treatment 3254 were significantly higher ($p<0.05$) than for all the other treatments. Sample diffusive resistance readings were high in TB (about 0.17) compared with indigenous crops such as maize (0.150). This was indicative of good water economy by the plant as exemplified by the corresponding low relative transpiration rates.

DNA samples from the various treatments did not give good amplification due to the scarcity of specific primers. However, one primer produced good amplification revealing a close phylogenetic relationship between rhizobial strains 578 and 579 due to the similarities of their bands while strains 3254, 446 and KTB1 had quite dissimilar bands.
N analysis was carried out at the division of environmental and applied biology, University of Dundee; both the % N (2.55) and mean N per plot (19.26g) for treatment 3254 were significantly higher than for all other treatments. All data processing was carried out using the Microsoft Excel and means separated using Tukey's procedure.

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LIST OF ABBREVIATIONS

ATP..... Adenosine Triphosphate

ASALs.....Arid and semi-arid lands

BTB .....Bromothymothymol blue

C .....Carbon

$CaCl_2$.....Calcium chloride

DNA .....Deoxyribonucleic acid

Fe Citrate .....Ferrous Citrate

H_3BO_3 .....Boric acid

HCl .....Hydrochloric acid

ICRISAT.....International Centre for Research on Arid and Semi-Arid Tropics

K.....Potassium

KARI.....Kenya Agricultural Research Institute

KEFRI.....Kenya Forestry Research Institute

KH_2PO_4.....Potassium dihydrogen Phosphate

K2SO4..... Potassium sulphate

MIRCEN.....Microbiological Resource Centre

Mo-Fe protein .....Molybdenum- Iron protein

MPN..... Most probable Number

N .....Nitrogen

NaOH..... Sodium Hydroxide

P ..... Phosphorus

CHAPTER 1

1. INTRODUCTION

Today Africa faces a myriad of crises; the food crisis, the energy crisis, the debt crisis and the crisis of economic mis-management. We may also add to this list the climatic and ecological crises resulting in the growing desertification of the continent, persistent droughts and consequent crop failure, hunger and famine (Shisanya, 1996). The significance and magnitude of the African food problem i.e. declining per capita food production over nearly three decades, rising levels of malnutrition, and increased food insecurity, is matched only by how little is understood about the problem, its causes and possible solutions. The agricultural frontier has closed in the higher potential, high population density areas of Africa. Shifting agriculture and trans-human systems have either disappeared or come under real stress, while land-use has either intensified or been degraded to the point of abandonment. There has been a consistent search by farmers as well as research and extension workers for sources of productivity growth. Productivity in African agriculture needs to be stepped up to match the demands of the growing population for development and to reverse trends of the post-colonial period (Lynam, 1978).

Kenya's situation is not much different from that in other African countries. Kenya's marginal areas, which comprise both the arid and semi-arid lands (ASALs) cover approximately 506,000 km^2 or 88% of the country's total area (Braun and Mungai, 1981; Darkoh, 1990). These ASALs have typical characteristics of tropical African drylands

i.e. low and variable rainfall, high evaporation rates, sparse vegetation cover, shallow soils and widely spaced rivers which flow intermittently (Bernard, 1985). Kenya's problem is compounded by the high population growth rate which was estimated at 3.34% in 1993 with a population of 24 million people (Kenya, 1993) and a negative economic growth rate (Kenya, 2001). Kenya's population currently stands at 30 million people (Kenya, 2001), giving a national average density of more than 60 people km^{-2}. Figures of population density, however, do not take into account the carrying capacity of land. Thus, because of differentiated agroecological conditions in Kenya, 80% of the population occupy 20% of the country's total land area (Hammes and Bauer, 1985). For this reason there is need to harness the vast potential of Kenya's ASALs so as to step up agricultural productivity and break the vicious cycle of famine and malnutrition endemic to these zones. This would also help to ease pressure on present farmlands and to some extent curb the rural- urban migration trend. The National Development Plan of Kenya (Kenya, 1993), stresses that ASALs will receive increasing attention albeit at a higher cost in recognition of the important contribution they can make to the national development.

In light of the aforementioned challenges facing the country, more research needs to be geared towards optimising land use patterns in ASALs. One possible approach is by use, or introduction, of drought tolerant crop varieties that can survive the harsh environmental conditions prevalent in the ASALs. It would be of added advantage if such crop plants could help in enrichment of soil nutrients. Tepary bean, *Phaseolus acutifolius* A. Gray var *Latifolius* holds much potential in this regard. This research is focussed on

the ecophysiological performance of tepary bean (TB), in terms of drought tolerance and nitrogen fixation in symbiotic association with various rhizobial strains. This would help identify the best symbiotic pairing for promotion in the semi- arid Makueni District of Kenya. In order to accomplish the objectives of this study infectivity of the various rhizobial strains on TB was assessed under greenhouse conditions in the Botany Departmental laboratories, Kenyatta University. A local isolate and several commercial rhizobial strains were used in this trial. Field investigations were carried out at the Kenya Agricultural Research Institute (KARI), experimental station, Kiboko, Makueni District. These studies set out to investigate the performance of TB under semi- arid conditions and the comparative dry matter yield of TB with different rhizobial treatments.

1.1 Research hypotheses

1. There are significant differences in the amount of nitrogen fixed by tepary bean inoculated with different rhizobial strains.

2. There are significant differences in biomass production in Tepary beans inoculated with various strains of rhizobia in Makueni district.

3. The rhizobial strains used in this study are genetically different.

1.2 Research objectives

Using tepary bean in symbiotic association with various rhizobial strains the objectives of this study were as follows:

Overall objective

To assess nitrogen fixation and drought tolerance in tepary bean.

Specific objectives

1. To screen the Kiboko soils for indigenous strains of rhizobia capable of nodulating and fixing nitrogen with tepary bean.

2. To quantify the degree of nodulation and amount of nitrogen fixed in tepary bean inoculated with various strains of rhizobia.

3. To quantify the biomass of the root, shoot and leaves of plants inoculated with various strains of rhizobia.

4. Perform DNA analysis of the rhizobial strains used to establish their phylogenetic relationships.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 The problem of soil fertility and nitrogen deficiency

Many shifting cultivation systems in East and Central Africa are characterised by alternate fallow and arable phases. The fallow phase, however, must be long enough to allow adequate restoration of fertility. In a study in Uganda, Jones (1972) reported that a three-year resting phase was needed to restore the soil organic C, N, P, K, and Mg that were depleted in a three-year growth phase. In many systems the arable phase is extended beyond the period needed to restore soil fertility, hence the observed decline in fertility. The basis of future yield growth in African agriculture will depend first on improved nutrient supply to crops and forages, as much of food production on the continent over the past couple of decades has been based on the mining of inherent soil fertility. Unless the significant rates of nutrient depletion are reversed, the negative trends in food production are likely to get worse rather than better (Jones, 1972).

Stoorvogel *et al.*, (1993), calculated N, P, and K balances for 38 African countries and found that most East and Southern African countries experience nutrient depletion rates as a result of:

(i) high population density and continuous cultivation,

(ii) hilly and mountainous terrain, rendering the land susceptible to erosion and

(iii) soils being relatively fertile hence having a lot of nutrients to lose.

Studies done 40 years ago showed that the savanna soils of West and East Africa contained amounts of N ranging from as low as 0.02% to 0.07%, and forest soils from

0.17% to 0.30% (Nye and Greenland, 1960). Similarly, low amounts of organic matter were recorded, 1.80 - 2.90 % C, in forest soils and 0.23%-1.30% for savanna (Nye and Greenland, 1960). With the increased intensity of cultivation since then, the content of soil N and organic matter has further declined, and this accounts partly for the poor farm yields obtained today. Recovery to 95% of the pre-agricultural levels is predicted to require 180 years for nitrogen and 230 years for carbon (Johannes, 2000). The extreme environments and low species diversity of arid ecosystems make them especially susceptible to changes caused by land use change, especially grazing (Evans, 1999).

N nutrition is one of the major determinants of crop yield. In contrast to many ecosystems experiencing large increases in N input, many arid ecosystems are experiencing loss or redistribution of nutrients due to land use change (Evans, 1999). Over a broad range of plant species, the rate of photosynthesis is directly correlated to leaf nitrogen content when both are expressed on a mass basis (Reich *et al.*, 1995). Nitrogen and carbon assimilatory processes in crop plants are interdependent. If the activity of either assimilatory system is disrupted, adjustments occur in the other (Rufty *et al.*, 1992). Nitrogen stress also alters partitioning of fixed carbon between starch and sucrose in leaves and utilisation of sucrose in sink tissue (Rufty *et al.*, 1992). Because most land plants grow under conditions of nitrogen deficiency we might expect adjustments in nutrient use to maximise photosynthesis (Schlesinger, 1997).

Increased use of N from chemical fertilizers has resulted in significant increases in food production world-wide, yet Africa's consumption of mineral fertilizers per hectare is the lowest in the world ranging from 2.2%-3.9% of the global expenditure compared to

3.6%-7.3% for South America, 30.4%-43.1% for Asia and 31.4- 42.4 % for Europe from 1982 (FAO, 1986). The low use of chemical fertilizers, particularly in Africa is attributed to their high cost, unavailability, poor infrastructure for marketing and their detrimental effects on the environment (e.g. eutrophication). These factors have collectively limited N fertiliser use by subsistence and small-scale farmers throughout Africa (FAO, 1986).

2.2. The Leguminosae

Leguminosae is one of the three largest families of flowering plants with some 690 genera and about 18,000 species of herbs, shrubs, trees and climbers; the other large families being Compositae and Orchidaceae (Purseglove, 1989). Despite the great variety of forms, Leguminosae constitutes a very natural and easily recognisable family, which resolves itself, according to the structure of the flower, into three very natural subfamilies. According to the taxonomic framework of Polhill and Raven (1981), the family is divided into 3 subfamilies, the Caesalpinioideae, Mimosoideae and Papilionoideae. Some people prefer to give these subfamilies the rank of family, in which case they are known as Caesalpiniaceae, Mimosaceae and Papilionaceae. In the subfamily Caesalpinioideae, nodulation is less common than in the other subfamilies. It is largely restricted to the tribe Caesalpinieae and the genus Chamaecrista from the tribe Cassieae. In the Mimosoideae, nodulation is general except for four groups within the tribe Mimoseae and very few species of *Acacia.* The only tribe from the Papilionoidea which appears not to nodulate is the Dipterygeae. Nodules vary greatly in size and appearance (Sprent and Sprent, 1990). Mimosoideae are mainly tropical and are of little importance agriculturally (Purseglove, 1989). Papilionoideae is the

largest of the subfamilies with about 12,000 species with the more primitive woody genera mostly in the tropics and the more advanced herbaceous genera common in the temperate regions. It is widely distributed in both tropical and temperate regions and provides a large number of crop plants. Although the value of legumes in improving and sustaining soil fertility has been known since ancient time, it was only towards the end of the 19th century that the discovery was made that legumes added N to the soil and the mechanism of this addition elucidated (Purseglove, 1989). The advantage of using legumes is that they are able to use both nitrate and ammonium in their metabolism. They are also able to synthesise ureides. This property is highly correlated with N fixation (Sprent, 1990); plants assimilating combined nitrogen from the soil have low levels of ureides. The latter may also confer herbivore, especially insect resistance on N fixing plants.

The genus *Phaseolus,* in the subfamily Papilionoideae, contains about 150 species of annuals and perennials throughout the warm regions of both hemispheres. They are usually twining herbs, rarely woody at the base. Their leaves are pinnately trifoliate and very rarely unifoliate with stipules persistent and usually entire leaflets. The flowers are few to numerous and attached on axillary peduncles (Purseglove, 1989). Germination in this genus is either hypogeal or epigeal. Morphological and other features which distinguish the domesticated from the wild species are: increase in seed size, reduction in fleshiness of root system and loss of perennialism; reduction of parchment layers of pods and violent seed dispersal; reduction in the amount of glucoside phaseolunatin in seeds which is hydrolysed by enzyme action to produce hydrocyanic acid and decrease in impermiability of seeds to water intake.

Pulses obtained from the *Phaseolus* species are one of the most important sources of protein in the diet of many tropical people and supplement the carbohydrate staple foods of rice, maize, and other cereals. The immature pods and leaves of most beans are eaten as vegetables. A number of species are grown as green manure and cover crops and also for fodder (Purseglove, 1989).

2.2.1 Legume- rhizobial symbioses

The rate of carbon accumulation in soils is controlled by the rate of nitrogen accumulation, which is in turn dependent on atmospheric nitrogen deposition and symbiotic nitrogen fixation by legumes (Johannes, 2000). All nodules on legumes are formed by symbioses between host plants and rhizobia. The legume root nodule represents a symbiotic association between a colony of bacteria and the plant (deBruijn and Downie, 1991). Rhizobia, currently comprising the genera *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium,* are widely distributed and abundant soil bacteria belonging to the alpha subclass of the Proteobacteria (Vinuesa *et al.,* 1998). Isolates of the same serotype can significantly differ in the N_2- fixing efficiencies and in their abilities to occupy nodules in competition with other closely-related strains (Streit *et al.*, 1992; Triplett and Sadowsky, 1992). Despite differences between the rhizobia, some legume species nodulate effectively with more than one bacterial genus (Sprent and Sprent, 1990). Although nodule size and tissue distribution may vary with the bacterial genus, the morphology and anatomy of the nodule is largely host controlled in nature, though this may not be so for engineered systems (Sprent and Sprent, 1990).

The infection and nodulation processes involve development steps that have been comprehensively treated by Date (1977). Infection in roots may be (1) by hairs, (2) by wounds or cracks or (3) between cells of intact epidermises (Sprent and Sprent, 1990). In all cases the cell walls must be amenable to bacterial penetration. Other microorganisms can modify both root and root hair growth in ways that may inhibit or stimulate nodulation. In soybean and some other legumes (Bhuvaneswari *et al.*, 1980; Calvert *et al.*, 1984) infections are most common in root hairs that emerge shortly after inoculation, suggesting that rhizobia may influence development in a way that facilitates infection.

In many leguminous species nodule development commences with rhizobial infection of a root hair (Date, 1977; Bauer, 1981). Different nodule structures are formed on infection of different plants, varying from cylindrical to spherical and from annular to irregular. Two broad classes are recognised as determinate and indeterminate nodules (Sprent, 1980). Within a nodule several proteins are produced. Two of these are nitrogenase and leghaemoglobin. The latter act to maintain a high flux of oxygen at low concentration (Appleby, 1984), while nitrogenase is responsible for the reduction of nitrogen to ammonia (Equation 1). Nitrogenase has been purified from all types of nitrogen-fixing organisms with the exception of archaebacteria (Haaker and Klugkist, 1987). Most nitrogenase complexes contain a Mo-Fe protein (nitrogenase) and a Fe protein (nitrogenase reductase). To make the active enzyme an iron-molybdenum co-factor (FeMoco) is inserted.

A minimum of 16 ATP are needed per molecule of nitrogen reduced, two for each of the 8 electrons transferred (Equation 1). In terms of overall cellular energy, the reducing power is equivalent to at least a further 9 ATP per nitrogen molecule assuming that, if not used for N_2, the electrons would be available for oxidative phosphorylation (Sprent and Sprent, 1990).

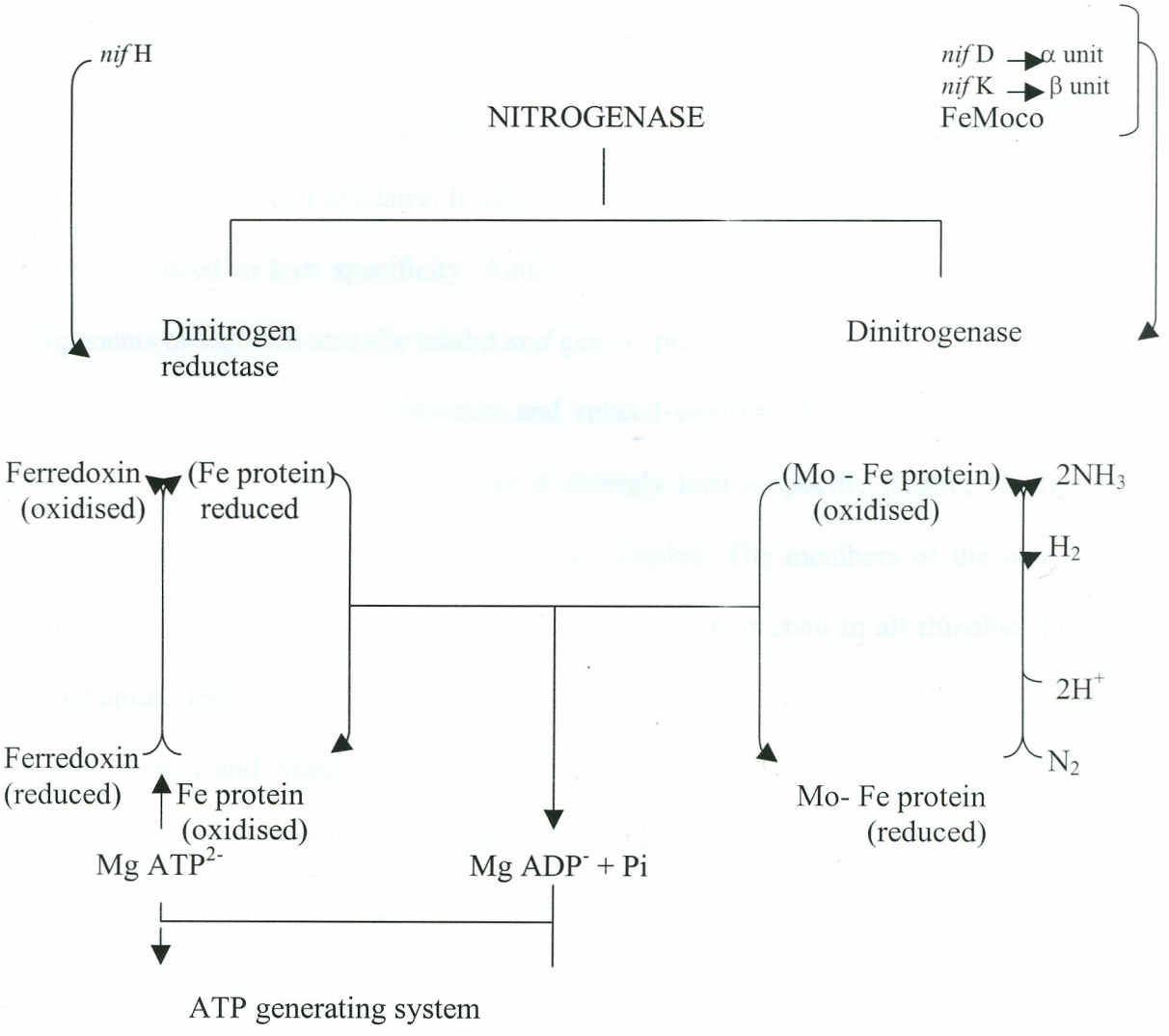


Fig. 2.1 Nitrogenase enzyme complex (Sprent and Sprent, 1990)

$$8H^+ + N_2 + 8e^- \longrightarrow 2NH_3 + H_2$$

Equation 1: Reduction of nitrogen to ammonia.

2.2.2 Rhizobium – host specificity

Nutrients needed by rhizobia and other soil micoorganisms in order to multiply are likely to be supplied by root exudates and sloughed off cells. There is evidence that in some cases rhizobia are preferentially nourished (Sprent and Sprent, 1990). During this period of multiplication the rhizobia genes for nodulation (the *nod* genes) are switched on by components of plant cell exudates. It had been thought that the switching on of *nod* genes would be related to host specificity. Additionally, some flavones that are characteristic components of legumes actually inhibit *nod* gene expression (Sprent and Sprent, 1990). There are many flavones, isoflavones and related compounds that vary in ability to induce or inhibit *nod* genes. The range is strongly host – specific (Rolfe, 1988). The structure and operation of the *nod* genes are complex. The members of the *nod* ABC groups are called the common *nod* genes as they may be present in all rhizobia (Gallon and Chaplin, 1987). They appear to be involved in induction of nodules on legumes (Marvel, Torrey and Ausubel, 1987). In particular *nod* C was found to code for a protein which becomes incorporated into the bacterial surface membrane so that cysteine – rich clusters of amino acids protrude.

Another gene, *nod* D, appears to be directly under the influence of flavones or isoflavones and to regulate other genes of the *nod* complex including sets of host specific nodulation (hsn, also known as *nod* E, F) genes (Fig.2.2). Thus *nod* D genes appear to be at the centre of the paradox of promiscuity coupled with specificity. Gyorgypal, *et al.*,

(1988) suggest that *nod* genes have a dual function: a common one to activate *nod gene* expression and the diverse one to recognize different flavonoids. Rhizobia with a relatively restricted host range may have only a single copy of *nod* D. Some rhizobia have three copies of *nod* D which may vary in their flavonoid recognition and, in this way, may vary the host range or factors such as the numbers of nodules formed, or the time between inoculation and nodule formation.

Attachment to roots and root hairs proceeds in stages with increasing levels of specificity and decreasing levels of reversibility, the later stages involving recognition. Two types of substances appear to be involved in both recognition and attachment; these are lectins on the plant side, which may recognize particular residues in rhizobia in a species-specific way and the particular carbohydrate residues i.e. extracellular polysaccharides (EPS). In addition to EPS, there are three other types of surface polysaccharides which may be involved in infection (Table 2.1).

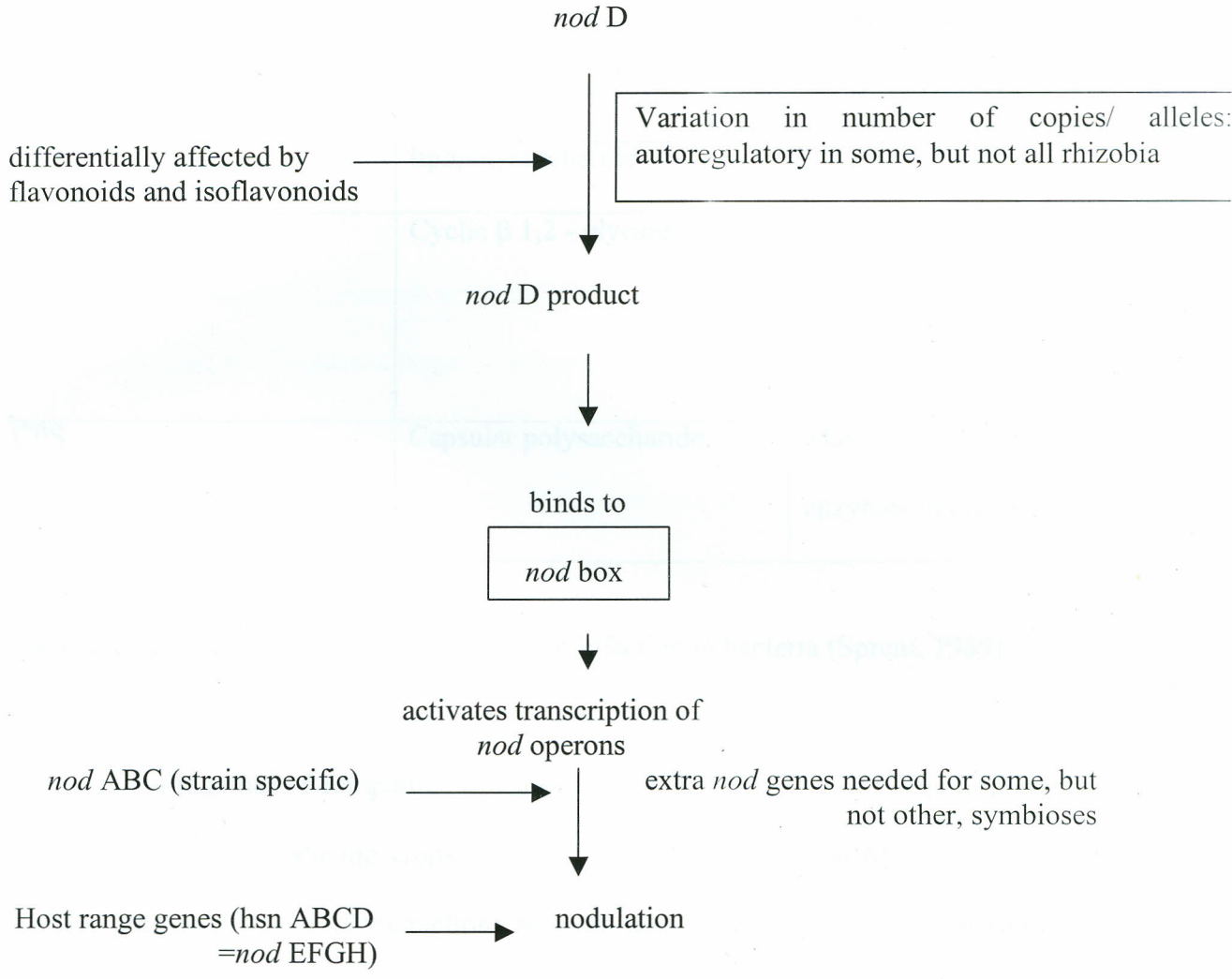


Fig. 2.2 An outline of ways in which *nod* genes in rhizobia may act, Young and Johnston (1989)

Abbreviation	Full name	comment
EPS	Extracellular polysaccharide	Usually acidic. Complex heteropolymer; may react with lectins
LPS	lipopolysaccharide	As above
*	Cyclic β 1,2 - glycans	Homopolymers probably involved in many pathogenic and symbiotic systems
CPS	Capsular polysaccharide	May be affected by host enzymes to effect attachment

* No standard abbreviation

Table 2.1 Surface polysaccharides involved in infection in bacteria (Sprent, 1989)

The bringing together of compatible rhizobia and host strain is of paramount importance, particularly when introducing crops into new areas. Fernandez and Miller (1987) have pointed out that because of interactions between host genotype and rhizobial strain for nitrogen-fixing potential, it is inappropriate to select genotypes simply for high nitrogen-fixing potential per se, because nitrogen- fixing efficiency is dependent on the rhizobial strain used.

2.3 Biological nitrogen fixation: potentials and limitations

There has been a tremendous interest in biological nitrogen fixation as an alternative to mineral fertilizers in agricultural systems (Kenya, 1989). The Kenya government has encouraged complementation of inorganic fertilizers through alternatives such as biofertilizers (using N_2 - fixing agents) as stated in the National Development Plan (Kenya, 1989). Biological nitrogen fixation (BNF) contributes to productivity both directly, where the fixed N_2 is harvested in grains or other food for human and animal consumption or indirectly through enhancement of soil fertility (Giller and Cadish, 1995). Biological nitrogen fixation represents a particularly renewable source of N for many farming systems (Peoples and Jensen, 1999). Global estimates of BNF to soil nitrogen vary from 135-175 million tonnes annually (Burns and Hardy, 1975; Paul, 1988). The calculated annual amounts fixed range from 30 - 160 kg N ha^{-1} yr^{-1} shoot N for annual pasture species, 37 – 128 kg N ha^{-1} yr^{-1} for lucerne, and 14 – 160 kg N ha^{-1} yr^{-1} by pulses (Peoples *et al.,* 2001). Other sources of nitrogen accumulation in the soil include decomposition of organic matter, action of lightning and use of N fertilisers among others.

Legumes of all categories contribute to N fertility in various cropping practises including fallow, agro-forestry and rotational systems. Low input legume- based agriculture exists in a continuum between subsistence farming and intensive arable and pastoral systems. Pastoral systems reliant solely on fixed N are capable of moderately high production with

modest N losses (Ledgard, 2001). The principal factors regulating BNF can be summarised in terms of environmental or management constraints to legume growth (basic agronomy, nutrition, water supply, diseases and pests) or result from local practises that impact on % N fixation (Peoples and Jensen, 1999). The low moisture contents in soils of Sahelian Africa can reduce nodule functioning in symbiotic legumes through drought-induced collapse of lenticels (Pankhurst and Sprent, 1975), decreased nitrogenase activity, reduced respiratory capacity of bacteroids and decline in leghaemoglobin content of nodules (Guerin *et al.,* 1990). In many parts of the world reduced water supply limits fixation in the field. In the semi-arid tropics, crops often suffer water stress for various periods during the growth cycle. Drought can also affect longevity of introduced rhizobia and a decline occurs with low moisture and soil desiccation. Consequently nodulation fails to occur through loss of infection sites due to changes in the morphology of infectible root hairs (Eaglesham and Ayanaba, 1984). In the tropics legumes have the ability to produce nodules on more acid soils and soils deficient in P, Ca, and other nutrients than in temperate countries (Purseglove, 1989). In the temperate regions nodulation is poor in seasons characterised by low light intensity. It is generally considered that at the high temperatures in the tropics significant excretion of nitrogen by the roots does not take place.

A study in Australia found that N fixation was primarily regulated by biomass production and that both pasture and crop legumes fixed between 20 and 25 kg shoot N for every tonne of shoot dry matter (DM) produced (Peoples *et al.,* 2001). Although pulses often fixed more N than pastures, legume – dominant pastures provided greater net inputs of

fixed N, since a much larger fraction of the total plant N was removed when pulses were harvested for grain than was estimated to be removed or lost from grazed pastures. The conclusions about the relative size of the contributions of fixed N to the N- economies of the different farming systems depended upon the inclusion or omission of an estimate of fixed N associated with the nodulated roots (Peoples *et al.,* 2001). The net amounts of fixed N remaining after each year of either legume – based pasture or pulse crop were calculated to be sufficient to balance the N removed by at least one subsequent non – legume crop only when below – ground N components were included. This has important implications for the interpretation of the results of previous N fixation studies undertaken all over the world, which have either ignored or underestimated the nodulated root when evaluating the contributions of fixed N to rotations.

So far, few studies have assessed the direct effects of drought on legume symbiotic performances in Africa. A study (Schulze *et al.,* 1991) in the desert and savanna areas of Namibia has shown that symbiotic *Acacias* spend more water per unit carbon assimilated than non-legumes. The water spent on carbon assimilation probably represents the cost of supplying extra carbohydrate for N_2 fixation (Danso *et al.,* 1992). There is a trade- off in photosynthesis; when plant stomata are open, allowing CO_2 to diffuse inward, O_2 and H_2O diffuse outward to the atmosphere (Schlesinger, 1997). The loss of water relative to photosynthesis is often expressed as water use efficiency (WUE), viz:

$$WUE = \frac{\text{mmoles of } CO_2 \text{ fixed}}{\text{Moles of } H_2O \text{ lost}} \qquad \text{equation 2}$$

Or

$$WUE = \frac{\text{mol } CO_2 \text{ fixed}}{\text{mol } H_2O \text{ transpired}} \qquad \text{equation 3}$$

For most plants, WUE (Equation 2, 3) typically ranges from 0.86 to 1.50 mmol/mole depending upon environmental conditions (Osmond *et al.*, 1982). High water loss per CO_2 fixed is generally not a problem so long as plenty of water is available for transpiration (Nobel, 1991). WUE (Equation 2,3) is higher at lower stomatal conductance (Bazzaz, 1990; Ceulemans and Mousseau, 1994). Low stomatal conductance is a feature for drought tolerance.

Symbiotic response to physiological stress is a highly variable parameter in which tolerance or susceptibility seems to result from heritable factors in both the host and the rhizobium (Bitangi, 1985). In soil, a particular bacterial strain has to survive fluctuations in temperature, moisture and ion content, and be able to compete successfully for nutrients with a wide variety of microorganisms. It also has to survive diseases such as bacteriophages to which it might be prone, to say nothing of the effects of natural and artificial herbicides and other assorted chemicals.

Although a few legumes are able to tolerate rising temperatures (Thomas and Sprent, 1984), the thermal effects of high temperature in Sahelian Africa in particular are likely to adversely affect legume symbiotic activity. Species adapted to this zone are able to achieve significant rates of fixation with high soil temperatures as exemplified by native *Acacia* species in Sudan and other Sahelian countries (Habish, 1970). Soil acts as an important component in the energy balance of plants (Nobel, 1991) since it has a

relatively high heat capacity but a low conductivity coefficient. Considerable energy can be stored by it. Plants that grow in arid regions and their microsymbionts must have mechanisms for temperature tolerance (Habish, 1970). Weber and Miller (1972) working with *Rhizobium japonicum* J. found that soil temperature may influence which symbiotic pairings are selected from soil populations.

2.4 The Kenyan situation and the tepary bean (*Phaseolus acutifolius* A. Gray var. *latifolius*) alternative

2.4.1 The tepary bean: an overview

Tepary bean, which belongs to the genus *Phaseolus* and subfamily Papilionoideae of the family Leguminosae, originates from the semi-deserts of NW-Mexico and SW-USA. It is native to the Sonoran Desert, one of the most diverse ecosystems in North America. It has been grown in these areas for about 5000 years, predominantly by means of 'floodwater- farming' agriculture (Brucher, 1977; Nabhan and Felger, 1978). The first scientific papers on tepary beans appeared at the beginning of the 20Th C (Freeman 1913; Hendry, 1919), the emphasis being placed on the botanical and ethno-historical aspects. The period 1915-1930 could be referred to as the 'Tepary boom' (Nabhan and Felger, 1978) because it was the first time that the crop was incorporated into the Dryland Farming Project through the Arizona Agricultural Experimental Station. It has also been tried as a hay and cover crop. Freeman (1918) isolated and grew 47 cultivars in Arizona differing in colour, shape, and size of seeds,

From 1960 there were increased research activities on tepary beans, particularly the biochemical and biophysiological characteristics of the crop (Coyne and Serrano, 1963;

Sullivan and Kinbacher, 1967;). The drought, heat, and disease resistance properties of the crop were exploited and used in cross-breeding programmes with other less environmentally-hardened *Phaseolus vulgaris* L. varieties during the 1970s (Mok *et al.*, 1978). Tepary bean seems to possess capabilities for drought-resistance and gives good yield in arid regions that are too dry for other beans. The results of biochemical analysis (Coyne and Serrano, 1963), showed that the plant produces high amounts of soluble solids such as glucose and sucrose, in times of good as well as poor water supply. These may act as osmotica during desiccation (Hornetz, 1988).

Tepary bean is a sub erect annual, approximately 25cm high, spreading or twining. The first pair of leaves are simple, about 5.5 x 3.5 cm. The mature leaves are trifoliate, lanceolate and contain stipules. The pods are compressed and the seeds absorb water very easily. The testa wrinkles within 5 minutes in moist soil and 3 minutes in pure water. Germination of the plant is hypogeal. The method of pollination is not known with certainty, but it is presumably self – pollinated. The dried bean has the following approximate composition: water 9.5 %; protein 22.2 %; fat 1.4 %; carbohydrate 59.3 %; fibre 3.4 %; and ash 4.2 % as compared to the common bean whose average composition of dried pulse is approximately: water 11.0%; protein 22%; fat 1.6%; carbohydrates 57.8 %; fibre 4.0%; ash 3.6%.

2.4.2 State of the art in tepary bean research in Kenya

The tepary bean is a relatively new crop in the region having been introduced in Kenya through the research activities of Hornetz (1988). Systematic research on the

ecophysiological demands, drought resistance and yield potentials of tepary bean in potential cultivation areas in Kenya started during the late 1980s and is continuing to date (Hornetz, 1988; 1990; 1991; Shisanya, 1996). In the semi-arid regions of S.E. Kenya the percentage loss of plants from germination to harvest time ranges from 44%-65% (Shisanya, 1996). Cutworms contribute a lot to this loss. Of the bean plants grown in this region Mwezi moja and Rose coco varieties appear to be more susceptible to cutworms than tepary bean (Shisanya, 1996). Studies by Hornetz (1990; 1991) show that tepary bean takes a shorter time to flower (32 days) than either Mwezi moja (39 days) or Rose coco (45 days). The total number of days taken to reach physiological maturity in tepary bean (60 days) is also shorter than for either Mwezi moja (65 days) or Rose coco (70 days) (Shisanya, 1996). Hornetz (1990) found the water requirement of Mwezi moja beans to be about 30% higher than that of tepary bean.

The harvest index (the proportion of the total crop that is harvested) for tepary bean is 28% (Shisanya, 1996). In cereal crops the harvested portion is grain and a harvest index of 40% or more is usually sought. However, in developing countries, where livestock depend on straw or strover for feed, a lower harvest index may be acceptable by the farmer if this substantially increases the livestock feed supply. Thus, the harvest index of 28% is reasonable for a leguminous crop given that short cycle crop varieties are known to produce relatively large amounts of seed, which contribute minimally to the dry weight (National Academy of Science, 1979).

2.4.3 Justification for selecting Tepary bean

Tepary bean (TB) is a new crop in the region whose potential for survival in Kenyan ASALs has not been fully studied. The research aims at supplementing land use patterns in Makueni district with a leguminous crop that has displayed a measure of drought tolerance. No research has been carried out to quantify the degree of nitrogen fixation in this crop. If the introduction of tepary bean together with a compatible rhizobial strain is successful, it will complement other protein foods in the semi arid areas, reduce the risk of crop failure, while at the same time restoring soil conditions through biological nitrogen fixation. The multifunctional tepary bean (Shisanya., 1996) was stigmatized as poor man's crop in the past. For instance it improves soil properties, enriches soil N, leaves used as vegetable, strover used as cattle feed while the beans are a rich source of protein. It seems to be well adapted to hot, arid sites of Kenya (Hornetz, 1990).

CHAPTER 3

3 MATERIALS AND METHODS

This chapter details all the materials and methods used in carrying out laboratory, greenhouse and field experiments.

3.1 Laboratory and Greenhouse Experiments

This section describes the materials and methods used to:

(i) assess nodulation in tepary bean (TB) treated with five rhizobium strains, viz; R446, R3254, R578, R579, and the Kiboko isolate, KTB1(Code being used for the first time and expected to be maintained).

(ii) compare the chlorophyll content in TB and other closely related legumes.

(iii) quantify the degree of N-fixation in TB with various rhizobial treatments

(iv) perform DNA analysis of the various rhizobia to ascertain that they are indeed different strains

3.2 Plant growth media

Plant nutrient solution was prepared as described by Somasegaran *et al.,* (1985). Five different stock solutions were prepared as follows;

Stock 1- $CaCl_2.2H_20$; 294.1 g/L

Stock 2-KH_2P0_4 ;136.1 g/L

Stock 3-$MgS0_4.7H_20$; 123.3 g, K_2S0_4 ;87.0 g, $MnS0_4.H_20$; 0.3338 g: /L

Stock 4- H_3B0_3 ;0.27 g, $ZnS0_4.7H_20$;0.288 g, $NaMo0_2.2H_20$; 0.048 g, $CoS0_4.7H_20$; 0.056 g: /L

Stock 5-Fe Citrate; 5.4g/L

For each litre of full-strength solution, 0.5ml was added from each of the five stock solutions. The pH of the solution was adjusted to 6.8 using NaOH (1.0 M) and HCl (1.0M). All solutions were sterilized by autoclaving at 121^{0}C for 30 minutes.

3.2.1 Yeast extract mannitol agar (YEMA) and yeast extract mannitol broth (YEMB)

Yeast extract mannitol agar and Yeast extract mannitol broth were used in routine culturing of root nodule bacteria. The YEMA medium contained: -

Mannitol 10.0g, $MgS0_4 .7H_20$ 0.1g, K_2HP0_4 0.5g, NaCl 0.2g, yeast extract 0.5g and agar 15g (Beck *et al.,* 1993).

All ingredients were dissolved in distilled water and the volume adjusted to 1 litre. The pH was adjusted to 6.8 using 1.0 M NaOH and determined by use of a pH meter (Chemtrix 40, Chemtrix Inc., USA). The combined medium mixture was sterilized by autoclaving at 121^{0}C for 20 minutes. Composition of YEMB was the same but minus the agar.

3.2.2 Yeast extract mannitol agar (YEMA) plates

These were prepared by pouring sterilized YEMA into petri dishes. To prevent excessive water condensation on the plates, the YEMA medium was cooled to about 45^{0}C before dispensing in petri dishes at the rate of 20ml per plate. Pouring of medium into plates was carried out in a laminar flow chamber (Klenzaids, MIDC, Bombay, India) to avoid contamination.

3.2.3Yeast extract mannitol agar slants

Slants were used for the storage of rhizobia. These slants were prepared by pouring YEMA into McCartney bottles. Approximately half of the volume of the McCartney bottles was filled with YEMA media and the caps loosely adjusted. After autoclaving, screw caps were tightened and the bottles left to cool slanted at 45^0.

3.2.4 Congo red indicator medium

One-ml aliquot of sterile Congo red solution (prepared by adding 2.5g of Congo red powder to 100ml of sterile water) was added to 1 litre of sterilized YEMA at 45-50^0C. The two were mixed by shaking the flask and the medium was then poured into sterile petri dishes. This was used for the initial screening of rhizobia.

3.2.5 Litmus milk

Litmus milk was prepared by rehydrating litmus milk by addition of 100g of the powder to a litre of distilled water, which had been pre- heated to 50^0C. Thorough mixing was done to obtain a uniform suspension. The latter was dispensed in petri dishes and sterilized at 121^0C for 20 minutes. This was used in the differentiation of bacterial strains.

3.2.6 Bromothymol blue indicator medium

Five ml of bromothymol blue solution were added to 1 litre of sterilized YEMA. The medium was sterilized by autoclaving for 20 minutes at 121^0C. The sterile medium was cooled to between 45-50^0C and then poured into sterile petri dishes. This was also used for the initial screening of rhizobia. This indicator changes colour depending on the pH of the added medium for instance it is yellow at pH 6.0, and green between pH 6.1 and 7.6.

3.3 Cultures

Rhizobium strains R446 and R3254 were obtained from Microbiological Resources Centre (MIRCEN), University of Nairobi. Strains R578 and R579 were obtained from Iserlohn, Germany, and a local isolate, KTB1 obtained from the Kiboko soils according to Beck *et al* (1993).

3.3.1 Culture maintenance and preservation

The cultures were first streaked onto YEMA plates and pure colonies selected for inoculation on YEMA slants. The slants were incubated for 4-7 days. Stock cultures of all the strains were stored on slants in screw cap McCartney bottles at 4^0C in a refrigerator. Subculturing was carried out after every 3 months to avoid loss of strains through death .

3.3.2 Broth cultures

All cultures used in these experiments were routinely grown in 50ml of YEMB contained in 250ml flasks. Incubation was carried out at room temperature on an orbital shaker (Gallenkamp SG97/07/375, U.K) at 120 revolutions per minute. The cultures were first sub-cultured from stock cultures in McCartney bottles to YEMA plates before transferring them to the YEMB medium. All transfers were carried out under laminar

flow conditions using a sterile inoculating loop, and next to a spirit lamp flame to reduce contamination.

3.4 Gram staining

Bacterial smears from 3-4 day old colonies were prepared on clean microscope slides. The smears were air-dried, heat fixed and then Gram stained as described by Beck *et al* (1993). The slides were observed under oil immersion using a compound microscope.

3.5 Sterilization and pre-germination of seeds

Clean, undamaged seeds of uniform size and colour were dipped momentarily in 95 % ethanol and then immersed in 0.1 % $HgCl_2$ solution for 2 minutes. The seeds were then washed thoroughly in ten changes of distilled water to remove all traces of $HgCl_2$,leaving them in the final rinse for 1 hour to imbibe water. Twenty seeds were then transferred aseptically with forceps to the surface of a 2 % water agar in a petri dish, where they were spread to give sufficient room for germination. They were then placed in an incubator at 25-26^{0}C for 24 hours. Seedlings whose radicles attained a length of 1-2 cm after incubation were considered ready for transfer into the tubes.

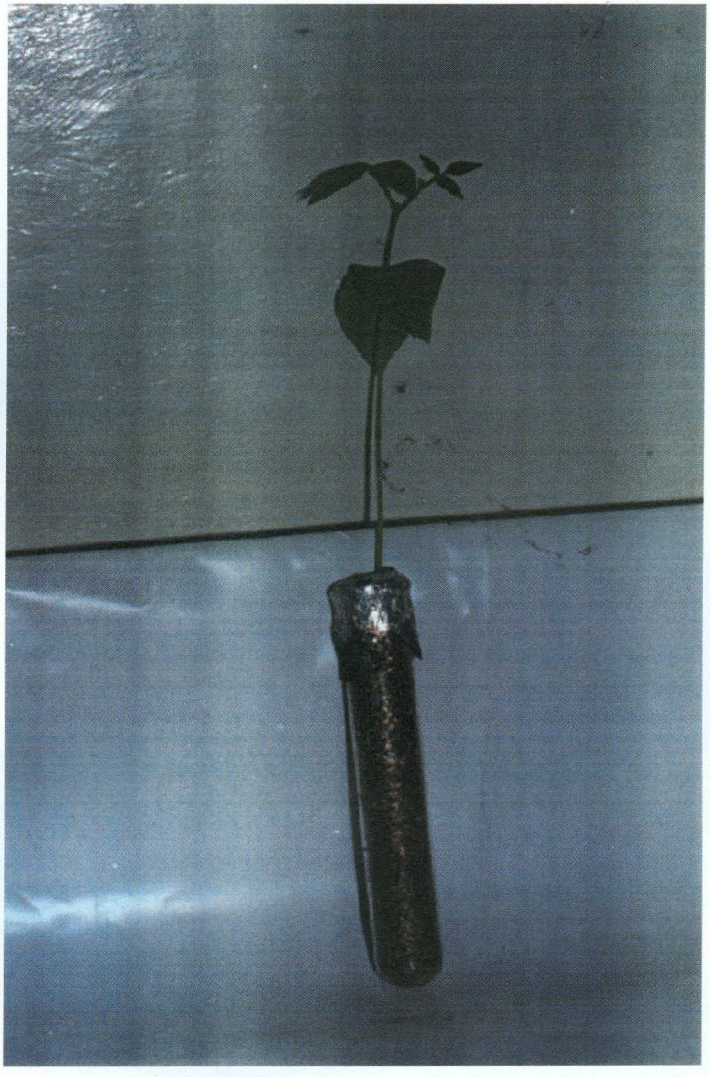


Plate 3.1
The test– tube system.

Plate 3.2
Performance of various rhizobial treatments.

3.5.1 Test tube system.

The test tube system (plates 3.1and 3.2) was chosen as it allows monitoring of nodulation through the transparent glass of the tube. Inoculation and watering is done using syringes to reduce chances of contamination. This is in contrast to the Leonard jar assembly where direct monitoring of nodulation is not possible. The laborious set up in the latter system and the watering process also increase chances of contamination. The vermiculite was thoroughly washed for 3 continuous days by changing the water at least twice a day and stirring frequently. The final rinse was with distilled water. The pH was adjusted to 6.8 using NaOH (1.0 M) and HCl (1.0 M). Test tubes were filled with the rooting medium (vermiculite) and moistened lightly with N-free nutrient solution. Test tube tops were sealed using aluminium foil, placed on racks and autoclaved for 15 minutes at 121^{0}C. After autoclaving and cooling the tubes were prepared for planting seedlings by making a hole in the aluminium foil using a sterile needle.

3.5.2 Planting, treatment, and care of plants in tubes.

A sharpened, sterile probe was used to make a hole in the edge of the foil. Seedlings of uniform appearance and approximately 15mm in radicle length were held by the radicle just below the cotyledon using sterile forceps and the radicle carefully inserted into the hole in the foil. The tubes with sown seedlings were covered in moistened paper tissues to stimulate growth. Plants were transferred to the greenhouse after 4 days. A sterile probe was also used to make a hole in the foil cap diagonally opposite the seedling. This hole served as a watering and inoculation port. Inoculation was carried out by pipetting 1ml of 4-5 day old rhizobia broth onto the radicle base. Each treatment was replicated 4

times. The material control was treated with sterile distilled water. Watering, with N-free medium was carried out 3 times a week by use of a syringe, through the watering hole.

3.5.3 Harvesting of plants

The plants were harvested after 28 days. At harvest, the stems were cut at the level of the growth medium. Roots and the adhering rooting medium were removed and put into a coarse sieve to trap nodules. The rooting medium was washed off using a gentle stream of water to avoid nodule detachment. Nodulation was then assessed according to Vincent (1970).

3.6 Estimation of the number of rhizobia in the Kiboko soils specific to Tepary beans.

The number of rhizobia in Kiboko soils specific to tepary beans was determined using the Most Probable Number (MPN) plant infection technique (Vincent, 1970; Beck *et al.*, 1993). Cylindrical soil subsamples, 3-5cm in diameter and from the 5-20cm-depth zone were collected from Kiboko. In the laboratory, the soil subsamples were thoroughly mixed together to obtain a homogeneous composite sample. Test tube systems were prepared (see section 3.2.2). Pre-germinated seedlings (see section 3.2.3) were planted in the tubes. A hundred grams of the composite soil sample was diluted in 900ml of sterile distilled water. A 10 fold dilution series was made from 10^{-1} to 10^{-10} and used for inoculating the plants. The plants were grown for 28 days and at harvest the nodulation status was observed and recorded. Uninoculated controls were used to check for sterile

conditions. The MPN was calculated from the MPN tables according to the formula: - (Equation 4) (Beck *et al.*, 1993)

$$\text{MPN} = \frac{m \times d}{v} \qquad \text{Equation 4}$$

where m is the most likely number, d, the lowest dilution in the series and v, the lowest aliquot used for inoculation.

3.7 Nodule assessment

At harvest nodule assessment was carried out according to Vincent (1970) to check for:

(i) Presence or absence of nodules.

(ii) Proportion of plants nodulated.

(iii) Distribution of nodules on the roots

(iv) Nodule colour based on the presence of leghaemoglobin.

3.7.1 Isolation of rhizobia from nodules

Ten nodules from each of the treatments were collected at random from the roots as described in section 3.5.3 The nodules were surface sterilized by dipping them momentarily in 95% $CaOCl_2$ for 3 minutes. Immediately after sterilization, the nodules

were rinsed in 7 changes of sterile distilled water, leaving them in the final rinse for 1 hour to get rid of all traces of $CaOCl_2$. The nodules were then crushed using a pair of sterilized forceps in a large drop of distilled water in a petri dish. A sterile inoculation loop full of the resultant suspension was streaked on YEMA plates incorporating Congo red. The inoculated petri dishes were incubated at 28^0C for colonies to appear. Colonies that were typical of rhizobia were streaked onto YEMA. Pure isolates were transferred to McCartney bottles to form stock cultures and stored at 4^0C in a refrigerator. The authenticity of the isolates as pure cultures of rhizobia was confirmed by plant nodulation test under aseptic conditions in tube systems.

3.8 Chlorophyll content determination

Chlorophyll content was determined for tepary bean, cowpea and the common bean. All experiments were carried out in the dark to prevent deterioration of chlorophyll. It was done on 15-day-old plants by collecting leaves from the 3rd node. One gram of leaf sample was homogenized using a mortar and pestle with addition of small amounts of 80 % acetone. The process was repeated until the washings and filtrates were colourless. A final homogenization was done to make the total acetone volume $60cm^3$. The combined filtrates and washings were transferred to a 250ml volumetric flask. Twenty five ml of the extract was transferred to 50ml di-ethyl-ether in a separating funnel and mixed well. Water was added until the chlorophyll passed into the ether layer. The ether layer was washed 5 times with water. The ether phase was transferred to a 250ml volumetric flask. Two grams of Na_2S0_4 was added and allowed to stand with occasional shaking till a clear

solution was obtained The filtrates were taken in cuvettes and the optical densities measured at 652, 647 and 664 nm by use of a mass spectrophotometer (Spectronic 20 model, Bausch and Lomb, U.S.A). The readings at 664 and 647 are close to the absorpion peaks in 80% acetone of chlorophylls a and b respectively (Coombs et al., 1985).

3.9 DNA analysis

This was done by means of the randomly amplified DNA (RAPD) technique via the polymorphic chain reaction (PCR) machine (PCR Thermocycler, Techne Ltd, Duxford, Cambridge).

3.9.1 Sources of genomic DNA

Rhizobium DNA was isolated from the strains R446, R3254, R578, R579 and KTB1.

3.9.2 Isolation of bacterial DNA

A well-separated colony was resuspended in 5ml LB broth. 1ml of the cultured cells (37^0C overnight) was transferred into a micro-centrifuge tube and the cell pellets collected after centrifuging at 11,000 xg for 10 min. at room temperature. The pellets were resuspended in 1 ml TES- sucrose buffer and incubated at 25^0C for 5 min with 1mg/ml lysozyme. A hundred µl 10% SDS was added and the tube vortexed. The DNA was extracted with phenol chloroform and precipitated with 0.3-M sodium acetate and 1 volume isopropanol. The pellets were washed with 70% ethanol and centrifuged at 11,000-x g for 10 min at room temperature. The DNA pellets were dissolved in 20µl TE and digested with 1µg/ ml RNase A at 37^0C for 10 min. The phenol- chloroform extraction and isopropanol precipitation were repeated. The final DNA was dissolved in 200µl TE.

3.9.3 Amplification conditions

Amplification reactions were performed in volumes of 24µl containing 10mM Tris- Cl, pH8.3, 50mM KCl, 2mM $MgCl_2$, 0.001 % gelatin, 100µM each of dATP, dCTP, dGTP and TTP (Pharmacia), 0.2µM primer, 25ng of genomic DNA, and 0.5 unit of *Taq* DNA polymerase. Amplification was performed in a Perkin Elmer Cetus DNA Thermal cycler programmed for 45 cycles of 1 minute at 94^0C, 1 minute at 36^0C, 2 minutes at 72^0C, using the fastest available transitions between each temperature. Amplification products were analysed by electrophoresis in 1.4 % agarose gels and detected by staining with ethidium bromide.

3.9.4 Genetic analysis of amplified DNA polymorphisms

Amplified polymorphic DNA fragments and Restriction Fragment Length Polymorphisms (RFLPs) were mapped by scoring marker segregation in the respective bacteria used to create these maps. Multipoint maps and LOD scores were calculated using the Mapmaker programme.

3.9.5 Use of amplified segments as Restriction Fragment Length Polymorphism (RFLP) probes

Polymorphic DNA segments (amplified as described in 3.9.3) were resolved by electrophoresis in a 1.4 % agarose gel and excised from the gel. A 5µl slice of gel was added to a 100µl-reaction mixture and was amplified under the condition described in 2.3.2. The re- amplified DNA samples were labeled with ^{32}P (BRL Random Primers

DNA Labeling System, Life Technologies, Inc.) and used as hybridization probes to detect RFLPs.

3.10 Nitrogen content determination

(a) Due to cost prohibitions, only final harvest plant samples were analyzed for nitrogen. The plants were dried at 70^0C to constant weight for 72 hrs and the dry matter weights recorded. The root, shoot and seeds were ground into fine powder using a Retsch ball grinder (Glen Creston, West Germany). Nitrogen was analysed in Dundee University laboratories, Scotland using an automatic high sensitive Carlo Erba CHN Elemental analyzer (Model 1106) using an atropine calibration standard. Two standards were placed at the beginning of a run of samples, two every 10^{th} sample thereafter and two at the end. Background nitrogen and carbon values were obtained by running a number of empty tin capsules and the means subtracted from the sample values. Masses of 0.5 to 0.8 mg of each sample were weighed into a tin container using a CAHN/ Ventron microbalance. They were then loaded into the autosampler and injected into the combustion reactor with a purge of O_2. Combustion gases, carried by a flow of Helium, were oxidised to CO_2, H_2O, N_2 and N_xO_y. The mixture was passed into a second reactor (filled with copper) for the reduction of the nitrogen oxides and then into a chromatographic column for N_2, CO_2, and H_2O separation. Each component together with the carrier gas was passed over a thermal conductivity detector producing a signal which was sent to an LDC computing integrator Model 308/9. Areas of peaks were recorded on the integrator print out.

3.11 Field experiments

Field trials were carried out at KARI/NRRC/ICRISAT sub-centre, Kiboko in Makueni District. The experiments were carried out during the short rains October 1999- January 2000. This station lies in SE Kenya (2^{0} 10/ S, 37^{0} 40/ E) about 165 km from Nairobi. Rainfall is bimodally distributed with mean monthly maximum in April (147mm) and November (164mm). The soils are classified as Acri – rhodic Ferrasols (Touber, 1983); i.e. well drained, very deep, dark reddish brown to dark red, friable with high structural stability. There is high desilication of the soils i.e. chemical migration of silica out of the soil solum, a factor that is favoured by high temperature and extreme leaching. They have low water- holding capacity, low native fertility and very low active acidity (Touber, 1983).

3.12 Field experimental layout.

The plants were arranged in a 7 x 7 Latin square design (Fig. 3.1). This design was chosen as it eliminates row and column effects from the experimental error thus yielding increased power of ANOVA (Steel and Torrie, 1980).

FUNC	GNF	578	579	446	3254	KTB1
GNF	FUNC	579	578	3254	KTB1	446
578	579	446	3254	KTB1	GNF	FUNC
579	446	3254	KTB1	GNF	FUNC	578
446	3254	KTB1	GNF	FUNC	578	579
3254	KTB1	FUNC	446	578	579	GNF
KTB1	578	GNF	FUNC	579	446	3254

Fig. 3.1 Field experimental layout

There were 7 replications per treatment. The treatments were as follows;

GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from kiboko soils

3.13 Field planting and crop management

Land preparation was carried out by disc ploughing followed by disc harrowing. A basal dose triple superphosphate (TSP) fertilizer was applied at the rate of 40kg ha^{-1} on all the plots. The seeds were dry planted on 31st October 1999 towards the end of the dry season

and harvested on 17th January 2000. Before planting, inoculation with the respective rhizobia was carried out by adding gum arabica sticker material to the filter mud carrier (Kibunja, 1984). They were thoroughly mixed with TBs and a little water added. For each of the treatments 2 seeds were planted per hole. Thinning was carried out after 2 weeks leaving one plant per hole.

3.14 Plant sampling

Plants were sampled at 3 weeks, 6 weeks after germination and at full maturity. Two plants were randomly sampled from each plot and various parameters assessed. The leaf area index (LAI) was measured by use of the LAI-2000 plant canopy analyzer. The LAI-2000 meter uses 'fish eye' optics to project a hemispheric image of the canopy onto five silicon detectors that are arranged in concentric circles. The detectors measure light interception at five zenith angles in degrees (7, 23, 38 53 and 68 degrees) to compute light transmission and LAI in a crop. Because LAI is proportional to the logarithm of the gap fraction, their logarithms are averaged in order to relate to LAI. All related statistics are calculated with the help of an integrated micro- computer in the LAI – 2000. Two readings were taken by the sensor, i.e. A-readings (above canopy) and B-readings (below canopy). The diffusive resistance, transpiration rate, leaf temperature and the photosynthetic active radiation (PAR) were measured using the LI-1600 steady state porometer (International Business Machines, U.S.A). Water potential measurement for TBs was carried out by use of the dew point micro-voltmeter (model 33T, I.B.M, U.S.A). Dry weights of whole plants, roots, shoots and nodules were also determined for each

treatment. The number of whole plants, nodules and pods were also determined per treatment. Samples were oven-dried at 70^0C to constant dry weight.

3.15 Data analysis

Data from the field was analysed using descriptive statistical procedures. MS Excel was employed for ANOVA and graphics. Means were separated by Tukey's procedure.

CHAPTER 4

4 RESULTS

4.1 Laboratory and Green-house experiments

The aim of the laboratory experiments was to determine infectivity of tepary bean by the various rhizobial strains, the degree of nitrogen fixation by tepary bean in symbiotic associations with the various rhizobial strains and to characterize the genetic relationships between the rhizobial strains used in these studies. The experiments involved nodule assessment, MPN counts and authentication of rhizobial isolates.

4.1.1 Nodule assessment

All treatments nodulated (Table 4.1) except the control (this had neither rhizobial inoculation nor N enrichment) and treatment GUNF (this was enriched with nitrogen but had no rhizobial inoculation). Poor nodulation was observed in plants inoculated with strains 578 and 579 (25%); plants inoculated with strain 446 had good nodulation. The highest nodulation proportion was displayed by treatment 3254 (100%). All the nodules were pink in colour showing presence of leghaemoglobin. The control and treatment GNF, as expected, failed to nodulate.

Table 4.1 Proportion of nodulated plants per rhizobial treatment.

Treatment	Proportion nodulated (%)
578	25
579	25
446	75
3254	100
Control	0
GNF	0
KTB1	20

Notes
GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
100 plants were used per treatment

4.1.2 Presumptive tests.

Presumptive tests were carried out to establish the cultural characteristics of the rhizobial strains 446, 578, 579, 3254 and the isolate from Kiboko soils, KTB1. The tests carried out were Gram staining, growth on YEMA, growth on bromothymol blue (BTB), growth in Congo red and growth in litmus milk. All the isolates were Gram negative rods. None of the colonies exhibited absorption of Congo red. Growth in YEMA showed that all the isolates were fast growers (colonies appearing within 2 –3 days) except for 3254 which exhibited very fast growth, with colonies appearing within 24 hours. All the colonies were round and milky. The rhizobia produced diffusible substances that changed the deep green colour of BTB: Strains 446, 578, 579, and KTB1 gave results in conformity with alkali production, changing the colour of BTB to blue; 3254 was acidic and changed BTB to yellow. On litmus milk, strains 446, 578, 579, and KTB1 produced a blue colouration, while 3254 produced a pink colour. Strains 578, 579 and KTB1 exhibited peptonization

of the media. These bacteria cannot ferment lactose but do possess proteolytic enzymes capable of hydrolysing casein (proteolysis) resulting in the release of large amounts of ammonia whose pungent smell can be detected; 446 produced a rennet curd (due to clotting of casein) and 3254 displayed gas production (due to fermentation of lactose to acid resulting in production of gas bubbles) in the litmus milk media.

The morphological characteristics were based on the rate of growth of the rhizobia on litmus milk and their colony shapes. For the very fast growers (e.g. *Rhizobium fredii*) visible colonies appear within 24 hrs, fast growers within 3 days (e.g. *Rhizobium phaseoli*) while for slow growers (e.g. *Bradyrhizobium japonicum*) it takes up to 7 days for colonies to appear. In this instance 3254 strain exhibited very fast growth while the rest fell in the category of fast growers.

Table 4.2 Growth of the rhizobia in YEMA.

Rhizobium identity	Growth rate	Colony shape and type
446	Fast grower	Round, milky
578	Fast grower	Round, milky
579	Fast grower	Round, milky
3254	Very fast	Round, milky
KTB1	Fast grower	Round, milky

R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils

Table 4.3 Rhizobial reactions in litmus milk

Rhizobium	Colour in litmus	Inference	Reaction type
446	blue	Alkali producer	Rennet curd
578	blue	Alkali producer	peptonization
579	blue	Alkali producer	peptonization
3254	pink	Acid producer	Gas production
KTB1	blue	Alkali producer	peptonization

R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils

Table 4.4 Reactions of rhizobia in bromothymol blue.

Rhizobial strain	Colour on bromothymol blue
446	blue
3254	Yellow
578	blue
579	blue
KTB1	blue

R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils

4.1.3 Authentication of the isolates as rhizobia

The experiments to authenticate the isolates as rhizobia were carried out in the greenhouse and plants harvested 28 days after emergence. The isolates from all the treatments were confirmed as rhizobia using the plant infection technique. Nodulation was observed in all treatments except the control and GNF.

4.1.4 Estimation of the number of rhizobia in the Kiboko soils

Estimation of rhizobia in Kiboko soils was done by the MPN plant infection technique as outlined in section 2.2.6 using the test- tube system. Table 4.5 shows the nodulation status for the various treatments. The rhizobium populations in Kiboko soils were found

to be 100 cells g^{-1} of soil; these are adequate for satisfactory nodulation results (Nambiar *et al.,* 1988). However the trials showed that introduction of new rhizobial strains could greatly increase plant productivity and N fixation.

Table 4.5 Nodulated test tube units planted with tepary bean as MPN counts.

Dilution	Nodulation (+) or (-)	Total number of nodulated units
10^{-1}	+ + + +	4
10^{-2}	+ + + +	4
10^{-3}	+ + + -	3
10^{-4}	- - - -	0
10^{-5}	- - - -	0
10^{-6}	- - - -	0
10^{-7}	- - - -	0
10^{-8}	- - - -	0
10^{-9}	- - - -	0
10^{-10}	- - - -	0
Total		**11**

Notes :

Number of replications (n) = 4 Lowest dilution = 10^{-1} Dilution steps = 10 + units= 11
Number of rhizobia = 1.0 x 10^2 rhizobia /g (Factor, 95 % fiducial limits n = 4, 3.8. Calculated from table VIII Fisher and Yates, 1963.)

4.2 Chlorophyll content determination

The chlorophyll content was determined for tepary bean and two comparison legumes i.e. common bean and cowpeas (Table 4.6 a, b). The concentration of chlorophyll in the leaves of plants is an indication of their potential to harvest photosynthetic active radiation (PAR). Higher photosynthetic rates would result in more robust growth.

The optical density measurements were taken at 3 different wavelengths i.e. 652, 647 and 664 nm. This was in order to facilitate calculations for the various chlorophyll fractions and total chlorophyll. The optical density for tepary bean was 0.1, 0.11 and 0.07

at 652, 647 and 664 nm respectively. These values were higher than for either cowpea or common bean. This translated into higher absorbance and higher chlorophylls a, b and total chlorophyll content.

Table 4.6 a; Optical density/ absorbance of chlorophyll extracts from three crops *viz*. Tepary bean, cowpea and common bean.

	Light wavelength			
	652nm		647nm	664nm
Optical density	TB	0.1	0.11	0.07
	CP	0.053	0.061	0.042
	CB	0.075	0.08	0.059

Key;
CP = cow pea nm = nanometer
CB = common bean TB= tepary bean

Table 4.6 b; Chlorophyll a, b and total chlorophyll in TB, CP and CB

	Chl a	Chl b	Total Chl
TB	0.641b	2.063b	2.900b
CP	0.397a	1.127a	1.636a
CB	0.573b	1.458a	2.174a

Key;
CP = cow pea chl = chlorophyll
CB = common bean TB= tepary bean

Plate 4.1
Measurement of leaf area index using the LAI-2000 Meter.

4.3 Readings from the steady state leaf porometer.

A porometer model LI-1600 was used to take readings for diffusive resistance for 3 continuous days at flowering stage for maize, common bean and tepary bean. The porometer also measured the transpiration rate, leaf temperature and the quantum value.

Maize and common bean (Rose coco) were used for comparison as they are routinely cultivated in Makueni District. These measurements were meant to give an indication of their water retention properties in comparison with the tepary bean. All readings were taken at 9.00 a.m. before the photosynthetic peak was attained (Table 4.7a, b, c). The corresponding air temperature was also recorded (Table 4.7d). There was no significant difference in transpiration rates between common bean and tepary bean though the common bean had generally lower readings with a correspondingly higher diffusive resistance. Both of these beans had lower transpiration rates than maize and thus better water economy. Build up of clouds on day 2 of the measurements reduced the transpiration rate, leaf temperature and the quantum for all the crops. These values remained low even on day 3 following precipitation on day 2.

The air temperature remained at a relatively high mean value of 30^0C that would have favoured rapid transpiration. Rainfall was received on the second day of taking these readings. This resulted in lowered transpiration rates for all the plant types as a consequence of increased relative humidity. Leaf temperature readings for tepary bean were on average lower than for either common bean or maize. Factors such as air temperature, rainfall, and leaf temperature have a direct bearing on transpiration rates and the hydration status of plants

Table 4.7 a: Day 1 porometer readings.

Crop Porometer readings	TB	Maize	Common bean
Diffusive resistance (scm^{-1})	0.07	0.02	0.06
Transpiration rate ($\mu g cm^{-2} s^{-1}$)	76.69	93.70	79.12
Leaf temperature ^0C	23.20	23.50	23.90
Quantum (μ mol $s^{-1}m^{-2}$)	160.00	36.00	81.99

Table 4.7 b: Day 2 porometer readings

Crop	TB	Maize	Common bean
Diffusive resistance (scm^{-1})	0.17	0.15	0.29
Transpiration rate($\mu g cm^{-2}s^{-1}$)	32.22	37.69	31.89
Leaf temperature (^0C)	21.80	23.40	24.30
Quantum (μmol $s^{-1}m^{-2}$	154.00	34.00	79.34

Table 4.7 c: Day 3 porometer readings

Crop	TB	Maize	Common bean
Diffusive resistance (scm^{-1})	0.32	0.37	0.48
Transpiration rate ($\mu g cm\text{-}^{2}s^{-1}$)	29.67	31.34	26.20
Leaf temperature (^0C)	26.30	28.30	27.30
Quantum (μmol $s^{-1}m^{-2}$)	37.00	30.00	46.00

Table 4.7 d: The corresponding air temperature, max. (^{0}C) and rainfall (mm) for the three days for which porometer readings were taken.

Day	Air temperature (^{0}C)	Rainfall
1.	30.0	0.0
2.	31.0	20.5
3.	29.0	0.0

4.4 Leaf water potential

Measurements for the leaf water potential were carried out by use of a dew point micro-voltmeter. The mean leaf water potential for tepary bean, maize and common bean at flowering were -0.18, -1.6, - 0.25 Mpa respectively. Tepary bean displayed a higher value for leaf water potential than the two comparison crops, both of which are routinely cultivated in Makueni District. This points to a higher degree of tissue hydration in Tepary bean as compared to these two crops.

4.5 Leaf area index

Readings for leaf area index were taken using the LAI – 2000 plant canopy analyser (plate 4.2); the readings were taken for maize, tepary bean and common bean (Table 4.8).

Table 4.8 LAI- 2000 plant canopy analyzer readings for tepary bean, maize and common bean.

TEPARY BEAN	MAIZE	COMMON BEAN
2*(S+I) = 3.34	2* (S+I) = 0.98	2* (S+I) = 2.00
SMP = 18	SMP = 18	SMP = 18
SEM = 1	SEM = 2	SEM = 5
MTA = 40	MTA = 43	MTA = 44
SEL =0.19	SEL = 0.25	SEL =0.25
DIFN = 0.047	DIFN = 0.425	DIFN = 0.198
LAI = 3.53	LAI = 1.05	LAI = 2.17

Key
* Statisical computation function LAI: Leaf area index SMP: Pairs of above and below canopy readings
SEM: Standard error of mean LAI: Leaf area index SEL: Standard error of LAI
MTA: Mean tilt angle DIFN: Diffuse non- interceptance

The LAI value for Tepary beans (3.53) was higher than for either maize (1.05) or common bean (2.17) and is well within range for annual crops. This factor is supported by the former's low MTA value. The DIFN for TB, indicating the fraction of sky not blocked by foliage, was also quite low with respect to the comparison plants. The high LAI value means that teparies can trap more of the incident solar radiation for photosynthetic purposes.

4.6 Meteorological data for the KARI field station (Makueni) during the season October- December 1999.

Meteorological data was taken on a daily basis throughout the growing season (Table 4.9 and Appendix 1). Readings were taken for air temperature (maximum and minimum), rainfall and relative humidity. In addition the soil temperature was also taken at depths of 15 and 30 cm. No rainfall was recorded in the months of September and October. The highest mean rainfall per day was noted in the month of October (13.51mm). The study site had rather high mean air temperatures and little accompanying rainfall (Table 4.10). The month of October proved to be the hottest in terms of mean maximum air temperature (32.65^0C) while December had the lowest readings (29.05^0C). The minimum readings for temperature were generally recorded at night and the lowest data were collected in the month of September while the highest minimum temperature was noted in November (Appendix 1). During the growth period the mean soil temperatures remained at mean levels of 24.26^0C and 22.81^0C at depths of 15cm and 30cm respectively. On average the temperatures were higher near the soil surface than at lower depths.

Table 4.9 Mean air temperatures (maximum and minimum) and mean rainfall during the growing season.

Month	Mean maximum temperature (^{0}C)	Minimum temperature (^{0}C)	Mean rainfall (mm) day^{-1}
September	30.66	14.73	0.00
October	32.65	16.63	0.00
November	30.23	18.15	13.51
December	29.05	17.97	3.19

Table 4.10 Mean soil temperatures (^{0}C) during the growth period at depths of 15cm and 30cm taken thrice daily.

Time	Soil temperature (15 cm)	Soil temperature (30cm)
9:00 am	21.77^{0}C	22.58^{0}C
12:00 noon	24.27^{0}C	22.65^{0}C
3:00 pm	26.75^{0}C	23.21^{0}C

Plate 4.2
Performance of tepary beans in various symbiotic associations .Treatment in the foreground is GNF ,the one on the right ,3254 ;left is 446 and in the background is FUNC.

4.7 Dry matter yield for various plant fractions; first, second and final harvest.

The plants were sampled at three stages i.e. at 21 days after emergence (when nitrogen fixation starts), at flowering (peak of nitrogen fixation) and at full maturity. Whereas the dry mass kept on increasing during the growth period, the nodules regressed after the flowering phase with a subsequent decrease in nitrogen fixation. The nodule number in rhizobial treatment 3254 was significantly higher than for all the other rhizobial treatments during the first harvest (Table 4.11). However, there was no significant difference in nodule number between treatments 3254 and KTB1, both of which were significantly higher than the other treatments. The nodule dry weight in treatment 3254 was significantly higher than for the other treatments during the first harvest. During the second harvest treatments 3254, KTB1, GNF and 578 displayed significantly higher nodule dry masses than FUNC, 446, and 579 (Table 4.11). FUNC (which had neither rhizobial treatment nor N fertiliser had the lowest readings. For all treatments there was an increment, both in nodule number and dry mass between the first and second harvest.

There were no significant variations between treatments in terms of root dry mass in both the first and second harvest (Table 4.12). Although the shoot masses were different in both the first and second harvest, these differences were not statistically significant. The

shoot dry mass was highest in treatment 3254 (Fig. 4.1) followed by N fertiliser treatment (GNF). The lowest dry mass was recorded in treatment KTB1. There was a progressive increase in shoot dry mass between the first and second harvest for all treatments. This was accompanied by slight increment in root mass in all treatments. The pod dry weights were also not significantly different (Table 4.13).

Treatment 3254 was significantly superior to all the other treatments in terms of total plant dry weight, pod and seed dry weight at the final harvest. A fairly high dry matter yield and seed yield was also observed in GNF. The pod weights for all treatments were higher than recorded during the second harvest (Table 4.14).

The number of days from planting taken to attain 50% flowering for treatments GNF, FUNC, 578,446, 579, 3254, and KTB1 were 35, 35, 34, 34, 33, 33 and 34 respectively. As expected rhizobial treatment had no effect on the host plant's phenology.

Table 4.11 Mean nodule number and nodule dry weight (g) per treatment per plot for first (21 days after emergence) and second harvest (flowering stage).

Treatment	Mean nodule number		Mean nodule dry weight (g)	
	First harvest	Second harvest	First harvest	Second harvest
GNF	2.00a	10.00a	0.04a	0.39b
FUNC	0.00a	9.00a	0.01a	0.19a
578	2.00a	16.00a	0.03a	0.36b
446	0.00a	2.00a	0.00a	0.19a
579	5.00a	8.00a	0.05a	0.12a
3254	30.00b	47.00b	0.24b	0.32b
KTB1	4.00a	20.00ab	0.04a	0.45b

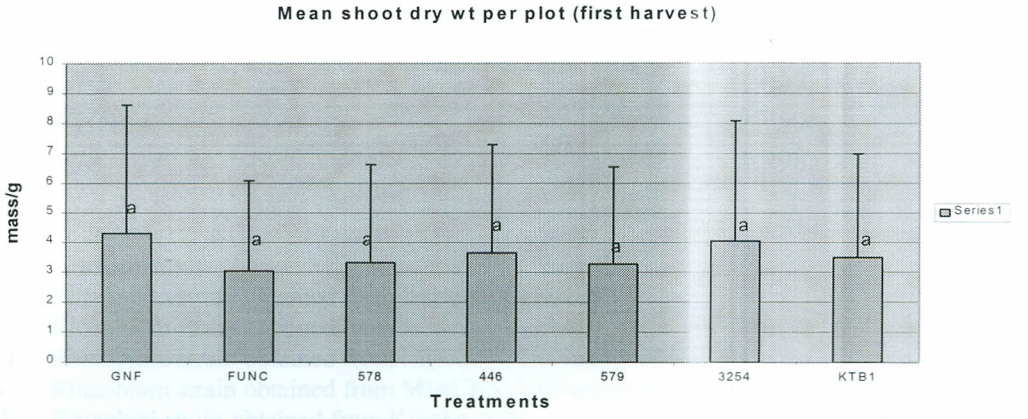
GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
Means followed by the same letter are not significantly different by Turkey's *w* procedure at 5% level.

Table 4.12 Mean root dry weight (g) per treatment per plot for the first (21 days after emergence) and second harvest (flowering).

Treatment	Mean root dry weight	
	First harvest	Second harvest
GNF	0.451a	0.763a
FUNC	0.381a	0.617a
578	0.405a	0.661a
446	0.443a	0.534a
579	0.412a	0.699a
3254	0.507a	0.688a
KTB1	0.423a	0.479a

GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
Means followed by the same letter are nor significantly different by Turkey's *w* procedure at 5% level.

Fig. 4.1 Mean shoot dry weights for tepary bean with different rhizobial treatments for the first harvest.



GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
Means followed by the same letter are not significantly different by Turkey's *w* procedure.

Table 4.13 Mean pod dry wt per treatment per plot; second harvest.

Treatment	Mean Pod dry wt. per plot (g)
GNF	3.336a
3254	4.433a
KTB1	2.834a
446	2.736a
579	3.097a
FUNC	2.759a
578	3.998a

GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
Means followed by the same letter are not significantly different by Turkey's *w* procedure at 5% level.

Table 4.14 Mean total dry mass (g) and yield per treatment per plot; final harvest.

Treatment	Whole plant dry weight per plot.	Pod dry weight per plot.	Seed dry weight per plot.
GNF	518.86[a]	1871.43[a]	1415.29[a]
FUNC	333.29[a]	1437.29[a]	1069.43[a]
578	329.14[a]	1569.00[a]	1167.71[a]
446	372.29[a]	1641.29[a]	1304.29[a]
579	410.00[a]	1730.00[a]	1316.00[a]
3254	755.29[b]	3978.00[b]	3126.29[b]
KTB1	333.71[a]	1512.57[a]	1133.86[a]

GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
Means followed by the same letter are not significantly different by Turkey's *w* procedure at 5% level.

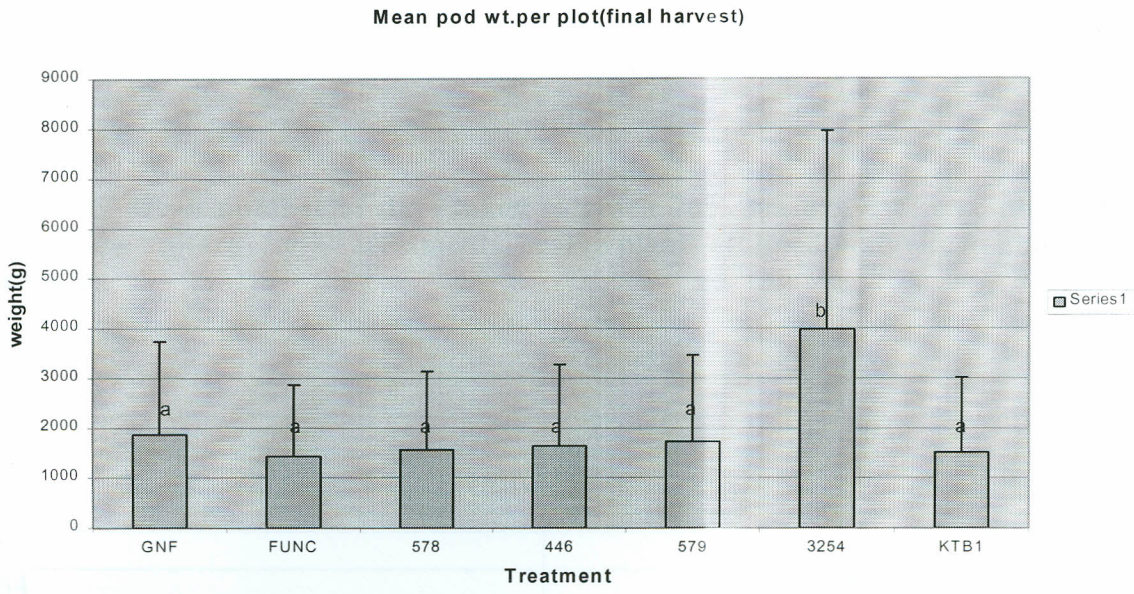


Fig. 4.5 mean pod dry weights (g) per treatment per plot, final harvest

GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
Means followed by the same letter are not significantly different by Turkey's *w* procedure at 5% level.

4.8 PCR analysis

This was done in order to establish phylogenetic relationships between the various rhizobia used in this study. The respective rhizobial DNA were isolated and amplified with the aid of primers. The 7 random primers tried failed to produce any amplification. The specific primers (sequence 5' to 3') TCC CGA CCT G and ACC CAT GCG G (Life Technologies, UK) also failed to produce amplification. However, one of the primers produced good amplification (plate 4.3) showing quite similar bands for strains 578 and

579; strains 446, 3254 and KTB1 had banding patterns quite distinct from each other and from strains 578 and 579.

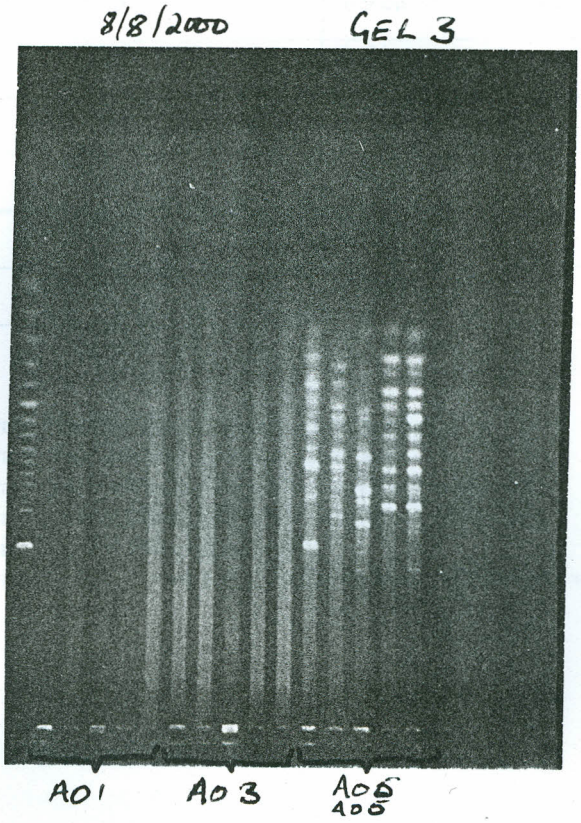
Table 4.17 Mean number of days taken to reach 70% maturity. The results were more or less uniform for all treatments, i.e. the plants attained maturity at about the same time.

Treatment	Days
GNF	68.14
FUNC	67.00
578	66.71
446	66.57
579	67.57
3254	69.71
KTB1	67.00

GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils

4.9 N analysis

This was done in order to find the percentage N in the samples and consequently the total N per plant for tepary bean in symbiotic association with the various rhizobial strains. These data would allow for the calculation of N per plot resulting from the activity of the various strains of rhizobia. The analysis was done using a Carlo Erba CHN elemental analyser that also computed values for % C and area C (Appendix 2). The total N for tepary bean plants inoculated with rhizobial treatment 3254 was significantly higher than for all the other rhizobial treatments at 5 % significance level (Table 4.18). The plant weight in tepary bean with treatment 3254 was significantly higher than the other treatments although the highest % N was observed in treatment KTB1. However, plants in treatment KTB1 were of comparatively low weights.



DNA amplification results. The band on the extreme left is for the maker. Primers A01 and A02 are showing poor amplification. For A06 the bands are for the following rhizobial DNA (Extreme R to L) 578, 579, 446, 3254 & KTBI respectively.

Table 4.18 Mean % N and Total N per treatment per plant

Rhizobial treatment	Mean plant weight (g)	Mean % N per plant	Mean total N per plant (g)
GNF	41.51ab	1.48a	0.61a
FUNC	26.66a	1.92a	0.51a
578	26.33a	1.91a	0.57a
446	29.78a	1.99a	0.59a
579	32.80a	1.83a	0.60a
3254	60.52b	1.60a	0.97b
KTB1	26.70a	2.55b	0.68a

GNF : N treatment.
R578 : Rhizobium strain obtained from Iserlohn, Germany
R579 : Rhizobium strain obtained from Iserlohn, Germany
R3254 : Rhizobium strain obtained from MIRCEN, University of Nairobi
R446 : Rhizobium strain obtained from MIRCEN, University of Nairobi
KTB1 : Rhizobial strain obtained from Kiboko soils
Means followed by the same letter are not significantly different by Turkey's *w* procedure at 5% level.

CHAPTER 5

5. DISCUSSION

The need for inoculation of plants to improve biological nitrogen fixation must be determined as a pre-requisite to Rhizobium strain selection programmes (Date, 1977). The need for inoculation will depend on the legume grown, the presence or absence of native rhizobia and their effectiveness in N_2 fixation with the introduced host, and on previous history of husbandry in the area (Date, 1977). The results obtained from gram staining and growth of the isolates in YEMA conformed with the standard cultural and morphological characteristics of rhizobia as described by Vincent (1970) and Somasegaran *et al.,* (1985). No isolate showed absorption of congo red i.e. the colonies were all milky to transluscent.

Litmus milk is a convenient medium for maintenance of lactic acid bacteria and for determining the action of bacteria on milk. It has been widely used for determining the action of bacteria on milk. Bacteria may coagulate or peptonize the milk. The litmus present indicates acid (pink) and alkaline (blue) changes, and also reduction, in which condition the litmus becomes colourless (Rohde, 1973). The roles of litmus dye in the litmus milk are as a pH indicator, and as a reducible molecule. In addition to these dye reactions, many other reactions can be determined by the manner in which different bacteria utilize the ingredients of the milk itself. Some bacteria ferment the lactose, some hydrolyze the casein, others produce a rennin – like enzyme, while still others

simultaneously ferment the sugar and hydrolyze the protein. The varied reactions of the rhizobia in the different media would point to the fact that we are in actual sense dealing with different rhizobia. This was borne out more clearly in treatment 3254 whose reactions were rather distinct from the other isolates suggesting it's distinct identity from the rest.

The test tube system proved to be quite convenient in terms of monitoring growth of plants, monitoring nodulation, and preventing contamination. The method is also economical in terms of space since the tubes occupy relatively little space. Watering of plants is easily carried out by means of syringes. In greenhouse experiments tepary bean produced effective nodules for all rhizobial treatments. All nodules were counted regardless of size. The principal factor that could have determined nodulation in the greenhouse trials was the infectivity of the various rhizobial strains used in these trials. Combined nitrogen may reduce nodulation and nodule activity (Becana and Sprent, 1987). In growing plants it may reduce haemoglobin synthesis leading to lower nodule activity even if nitrogenase is unaffected. The absence of nodulation in the control was expected since the treatment contained only sterile vermiculate and no rhizobia. The higher percentage nodulation seen in treatment 3254 is indicative of higher infectivity.

The pink colouration of the nodules, depicting presence of leghaemoglobin, was indicative of good N fixing potential. Strain 3254 produced more nodules per plant as compared to the other treatments (table 4.6). The higher number of nodules produced in treatment 3254 are indicative of a higher N fixing potential than the other rhizobial strains used in this survey. This was also proven by the higher plant weight and % N

content of teparies inoculated with this rhizobial strain. Nodule number is frequently used as a measure of infectiveness (Beck *et al.,* 1993).

The plant test is the only confirmatory test for rhizobia (Vincent, 1970). Rhizobia were successfully isolated from nodules and used to inoculate tepary bean. The latter nodulated confirming that we were dealing with rhizobia.

The enumeration of specific rhizobia in soils is required to predict the need for inoculation (Beck *et al.,* 1993), rate of nodulation and to study the fate of inoculum in soil. The MPN technique (Table 4.5) helps in enumeration and avoids the antagonistic effects of other microorganisms that hamper the counting of rhizobia in soils. The rhizobial populations in Kiboko soils (100 cells g^{-1} of soil) proved to be satisfactory for nodulation results. These are in line with the findings of Nambiar *et al.,* 1988, that most tropical soils have a rhizobial population of more than 100 rhizobia cells g^{-1} of soil capable of nodulating legumes grown on such soils. However, the performance of the indigenous rhizobia in symbiotic relationship with tepary bean was inferior to that of introduced strains, more specifically 3254, in terms of dry matter yield and % N content. This makes a case for the introduction of such a high performance rhizobial strain despite the presence of local rhizobial strains in the soil.

Soil temperature has direct effects upon both nodulation and nodule activity. Air temperature may have indirect effects via photosynthesis. Gibson (1969) found that more nitrogen was retained in root nodules at low temperatures, suggesting that export to the shoot may be retarded. High temperatures may interact with light. Frings (1976) found that in *Penissetum sativum* L, enhanced photosynthetic activity, either by extension of the

light period or by increasing irradiance, raised the probability of plants being nodulated at high temperature. Weber and Miller (1972), working with *B. japonicum* found that soil temperature might influence which symbiotic pairings are selected from soil populations. Temperature-sensitive strains fail to nodulate at extremes of temperature (Sprent and Sprent, 1990). During the growth period the mean soil temperatures remained at mean levels of 24.26^{0}C and 22.81^{0}C at depths of 15cm and 30cm respectively. Thus the high soil and air temperatures prevalent in Makueni district could impact negatively on the functioning of most plants in terms of photosynthetic function, general physiological processes and the functioning of introduced rhizobia. This makes a case for the introduction of the drought tolerant TB with a compatible rhizobial strain for improved yields. There was not much difference exhibited in diffusive resistance between TB and the common bean, though TB had a denser root mass that could aid in water uptake. Rainfall was received during the taking of diffusive resistance measurements. This might have affected the results since drought tolerant crops do not behave differently, in terms of water economy, from ordinary crops when water is available.

In spite of the high mean temperatures and little accompanying rainfall during the growth period tepary bean exhibited good growth. As growth continued, the superiority of treatment 3254 became quite distinct as evidenced by the greener plants and denser foliage (plate 4.2) and total dry matter yield (Table 4.14). This fact was evident at all sampling stages where the root, shoot and nodule dry weights were higher for this treatment than the other treatments. Normally in any legume, nodulation and N fixation are closely related to plant growth; the plant produces those nodules which it can support

(Sprent, 1989). No difference was seen, however, in the number of days needed to reach maturity with the different treatments as this is largely dependent on the host plant (the same in this case) rather than on the symbiotic association.

If we specifically wish to consider the productivity of crops or natural ecosystems, it is convenient to express their performance per unit land area. The leaf area ratio is therefore inappropriate and the leaf area index (LAI) is used; this is the leaf area per unit land area. LAI is the major factor determining the amount of light intercepted by the plant canopy. It's value for a closed canopy is related to the ability of the lower leaves to intercept sufficient light to maintain a positive carbon balance. Some coniferous canopies are able to support LAI> 15. For decidous forest, maxima between 6 and 8 are observed, while for annual crops maxima are between 2-4. All crop types react to stress by producing canopies with lower LAI. The high value for LAI (Table 4.8) for TB would be contradictory for drought-tolerant crops that combat water stress solely by reducing the evaporative surface. The relatively high leaf water potential for teparies, combined with their high LAI value would seem to point to a mechanism for maintenance of hydration by accumulation of certain solutes combined with tissue elasticity (Jones *et al.*, 1981; Nabhan and Felger, 1989). The higher LAI for TB would also point to a higher conversion efficiency. The conversion efficiency would not be at its maximum under ASAL conditions since crop plants are only able to photosynthesize at their maximum potential when other factors are not limiting (Beadle *et al*,1992). Jones (1981) has categorized desert plants as;

1. Drought escapers. These display either

(a) Rapid physiological development

(b) Developmental plasticity.

2. Drought tolerant with high tissue water potential. These attain this attribute by;

(a) Maintenance of water uptake through increased rooting or increased hydraulic conductance.

(b) Reduction of water loss by either reduction in epidermal conductance, reduction in absorbed radiation or reduction in the evaporative surface.

3. Drought tolerant with low tissue water potential. This is achieved through;

(a) Maintenance of turgor either by solute accumulation or increased elasticity.

(b) Desiccation tolerance through protoplasmic resistance.

High tissue (leaf) water potential was observed in tepary bean (section 4.4) though its diffusive resistance was not much different from that of the common bean (Table 4.7). Accumulation of glucose and sucrose in times of extreme water stress is a mechanism employed by this bean to assist in maintenance of cell turgor. Their relatively high LAI value would facilitate faster growth and setting of seed by trapping more of the photosynthetic active radiation (PAR).

5.1 Conclusion

The results show the drought tolerant properties of tepary bean. This plant therefore provides a viable alternative to the crops commonly grown in Makueni District. That this crop can form symbiotic relationships with rhizobia for nitrogen fixation is an important attribute that can improve the soils in this region. On the strength of its superior biomass production within a short period in the semi arid Makueni District, the symbiotic

relationship between treatment 3254 and tepary bean should be exploited further in order to boost food productivity in this region that is often beset by drought and the attendant food shortage. This symbiotic pairing also resulted in superior nitrogen fixation as indicated by the % N and total N of beans inoculated with strain 3254. To this end a programme could be set up to mass produce inoculum from the 3254 rhizobium strain for use by farmers. Though on the whole the common bean produces more grain than the tepary bean, the latter would provide a viable option for farmers in the semi – arid seasons when the the common bean would not survive.

5.2 Recommendations for further research

There is need to assess the productivity of tepary bean under other equally arid regions considering that a high proportion of the country is classified under ASALs. This is with a view of extending the growth of the crop to other marginal areas to supplement land use patterns and alleviate the chronic starvation prevalent in these lands. The possibility of utilizing TB leaves as vegetable supplements as with other beans could also be explored. There is also need to further differenciate the rhizobia used in this study on the basis of their genetic constitution. This would enable us to know for certain whether we are actually dealing with different rhizobial strains. A further search could be mounted for more indigenous rhizobial strains considering that the local isolate KTB1 performed better than all the other commercial strains in terms of the mean %N fixed (1.92) except for treatment 3254 which it matched (Table 4.18). Research should also be carried out in order to establish the persistence of introduced rhizobia in ASALs. If it is established that there is need for frequent re-introduction of rhizobia, it would render the exercise beyond the financial need for most farmers and an alternative would then be sought.

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APPENDICES

Appendix 1. Meteorological data for the KARI field station, Kiboko, during the growth season November 1999 – December 1999.

September 1999

Date	Max. Temp. (^{0}C)	Min. Temp. (^{0}C)	Relative humidity (%)	Rainfall (mm)
1	27.5	17.0	78	0.0
2	28.5	16.0	77	0.0
3	28.5	16.0	59	0.0
4	30.0	14.0	18	0.0
5	30.5	14.0	64	0.0
6	30.5	15.0	63	0.0
7	28.0	16.5	70	0.0
8	27.7	12.5	58	0.0
9	27.0	17.0	64	0.0
10	27.0	12.0	73	0.0
11	32.3	17.0	64	0.0
12	31.0	18.5	65	0.0
13	30.0	10.0	68	0.0
14	31.0	13.0	52	0.0
15	30.0	12.5	60	0.0
16	31.0	17.0	59	0.0
17	30.5	16.0	63	0.0
18	30.5	13.0	61	0.0
19	32.0	11.0	64	0.0
20	31.5	12.0	59	0.0
21	33.0	19.5	63	0.0
22	32.2	13.0	60	0.0
23	33.0	14.5	61	0.0
24	31.5	16.0	66	0.0
25	33.0	11.5	59	0.0
26	32.0	13.0	59	0.0
27	33.5	16.5	62	0.0
28	33.0	20.0	59	0.0
29	32.5	15.5	79	0.0
30	31.0	12.5	68	0.0
Mean	30.66	14.73	62.5	0.0
Total				0.0

October 1999

Date	Max. Temp. (0C)	Min. Temp. (^{0}C)	Relative humidity	Rainfall (mm)
1	31.0	19.0	73	0.0
2	31.0	12.0	69	0.0
3	32.5	13.5	56	0.0
4	33.5	19.0	55	0.0
5	30.0	15.5	79	0.0
6	31.0	15.0	59	0.0
7	30.0	14.0	62	0.0
8	31.0	18.5	75	0.0
9	30.5	14.0	62	0.0
10	28.0	18.5	68	0.0
11	30.0	17.0	70	0.0
12	32.0	18.5	82	0.0
13	30.5	16.0	81	0.0
14	31.5	11.0	79	0.0
15	31.5	18.0	59	0.0
16	31.0	18.0	65	0.0
17	31.0	12.0	82	0.0
18	33.0	16.5	62	0.0
19	30.5	18.5	60	0.0
20	32.5	18.0	72	0.0
21	34.0	16.0	62	0.0
22	35.0	17.5	62	0.0
23	36.5	14.0	59	0.0
24	37.0	16.5	57	0.0
25	37.0	17.0	59	0.0
26	38.5	20.0	63	0.0
27	34.0	20.5	61	0.0
28	34.5	20.0	64	0.0
29	34.7	20.5	66	0.0
30	35.5	18.5	70	0.0
31	33.5	12.5	63	0.0
Mean	32.65	16.63	66.3	0.0
Total				0.0

November 1999

DATE	MAX TEMP.(^{0}C)	MIN.TEMP (^{0}C)	RELATIVE HUMIDITY (%)	RAIN (mm)
1	34.0	16.5	61	0.0
2	34.0	18.0	56	0.0
3	33.5	20.0	63	0.0
4	33.0	20.0	70	0.0
5	33.0	20.0	76	0.0
6	34.0	13.5	70	66.0
7	33.0	18.0	91	4.0
8	33.0	19.0	80	0.0
9	33.0	17.0	76	0.0
10	30.0	20.0	70	0.0
11	30.5	17.5	73	18.5
12	30.5	17.5	91	5.0
13	30.0	18.5	78	0.0
14	30.0	19.0	76	0.2
15	28.5	18.5	84	54
16	27.5	18.0	84	0.0
17	28	19.0	80	56.5
18	25.9	19.0	91	2.0
19	31.0	18.0	84	41.5
20	28.5	18.0	87	4.0
21	29.0	18.0	80	3.0
22	28.5	18.0	84	6.0
23	27.0	17.0	84	13.5
24	28.0	18.0	93	16.5
25	28.0	18.5	87	32.5
26	27.5	18.0	96	18.5
27	30.0	17.5	93	13.0
28	29.0	18.0	95	49.0
29	27.5	18.5	91	2.0
30	28.0	18.0	80	0.0
MEAN	30.23	18.15	80.8	13.51
TOTAL				405.2

December 1999

Date	Max. Temp. (^{0}C)	Min. Temp. (^{0}C)	R.H (%)	Rainfall (mm)
1	31.0	18.0	73	0.0
2	28.5	18.5	76	3.0
3	28.5	19.0	87	0.0
4	30.0	16.5	83	0.0
5	31.0	18.5	83	20.5
6	29.0	18.2	87	0.0
7	30.0	18.5	77	0.0
8	28.5	18.5	78	0.5
9	30.0	18.0	70	0.0
10	31.0	19.0	75	0.0
11	29.0	18.0	87	5.0
12	29.5	16.5	96	1.4
13	31.0	15.2	77	0.0
14	30.5	17.0	88	0.0
15	32.0	18.5	85	0.0
16	29.5	15.5	76	0.0
17	30.0	18.0	91	1.8
18	28.0	19.0	84	3.5
19	29.0	18.0	90	10.0
20	26.5	18.5	93	11.5
21	27.0	18.0	84	0.7
22	27.0	18.0	87	0.0
23	27.5	18.0	80	0.0
24	27.5	18.0	91	18.0
25	27.5	18.5	96	1.0
26	29.0	18.5	89	16.8
27	28.5	18.5	80	4.0
28	28.0	18.0	84	0.7
29	27.0	18.0	83	0.5
30	28.6	19.0	96	0.0
31	30.5	17.5	86	0.6
Mean	29.05	17.97	84.3	3.19
Total				1.98

Appendix 2. Results of N analysis

C + N analysis

		Date	**01-Jan**	**Total No. of samples**		**28**			
	GNM	**29/01/01**							
No.	**Sample**	**Weight**	**%N**	**Area N**		**%C**	**Area C**		
	bb N	1281	**KN**	**7.77 * 10-5**		**KC**	**2.78 * 10-5**		
	bb C	1408							
1	ATR		**7.7**			**2.79**			
2	ATR		**7.72**			**2.79**			
3	G-NF1 a	0.523	**1.43**	10877	9596	**40.15**	756729	755321	
4	G-NF1 a	0.598	**1.42**	12188	10907	**40.17**	865461	864053	
5	G-NF1 b	0.893	**1.50**	18476	17195	**39.86**	1281805	1280397	
6	G-NF1 c	0.801	**1.55**	17256	15975	**40.05**	1155407	1153999	
7	F-UNC 2 a	0.7	**1.53**	15020	13739	**39.52**	996557	995149	
8	F-UNC 2 b	0.611	**2.20**	18615	17334	**40.17**	884259	882851	
9	F-UNC 2 c	0.622	**2.03**	17547	16266	**40.50**	907473	906065	
10	579 3 a	0.541	**2.32**	17468	16187	**41.37**	806492	805084	
11	579 3 b	0.748	**1.97**	20240	18959	**40.96**	1103600	1102192	
12	579 3 c	0.562	**1.45**	11779	10498	**40.99**	830081	828673	
13	ATR	7.67				**2.74**			
14	ATR	7.7				**2.75**			
15	E-RLS 4 a	0.821	**2.51**	27841	26560	**41.40**	1224191	1222783	
16	E-RLS 4 b	0.701	**1.81**	17586	16305	**41.51**	1048051	1046643	
17	E-RLS 4 c	0.63	**1.64**	14538	13257	**41.17**	934483	933075	
18	578 a	1.059	**1.81**	25903	24622	**40.84**	1557025	1555617	
19	578 b	0.654	**1.95**	17729	16448	**39.90**	939991	938583	
20	578 c	0.771	**1.73**	18439	17158	**40.84**	1134072	1132664	
21	446 a	0.442	**1.52**	9929	8648	**41.04**	653877	652469	

22 446 b	0.692	**1.68**	16240	14959	**41.62**	1037362	1035954
23 446 c	0.787	**1.59**	17392	16111	**41.62**	1179769	1178361
24 3254 7 a	0.689	**2.61**	24446	23165	**40.63**	1008427	1007019
25 3254 7 b	0.616	**2.27**	19266	17985	**41.98**	931515	930107
26 3254 7C	0.809	**2.76**	29976	28695	**41.37**	1205448	1204040
27 ATR		**7.94**			**2.78**		
28 ATR		**7.83**			**2.75**		