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Biological Nitrogen Fixation in Selected Legumes of the Semi-Arid Makueni District of Southeast Kenya

Maingi, J.M.*, Shisanya, C.A.**, Gitonga, N.M.* and Hornetz, B.***

1 Introduction

Approximately 506,000 km^2 or 88 % of Kenya's total area is comprised of both arid and semi-arid rangelands (Darkoh, 1990). The people living in these areas are faced with a myriad of problems, for example shortage of fuelwood, inadequate fodder for animals, and decreased soil fertility. Currently, food production in the arid and semi-arid lands (ASALs) has lagged behind population growth and therefore there is an urgent need to increase food production for the ever increasing population (Bohlool et al., 1992). Recently, an influx of settlers (agropastoralists) from the overpopulated more humid highlands of central and western Kenya into the semi-arid fringe of the ASALs has been observed (Hornetz, 1997).

One of the major steps towards increasing food production is by use of modern agricultural technologies. Their use in the ASALs has been restricted because of socio-economic constraints (Shisanya, 1996; Graham, 1981). Most farmers in the ASALs are resource poor and can hardly afford the required inputs mainly in form of chemical nitrogen (N) fertilizers. Legume-Rhizobium technology which involves Biological Nitrogen Fixation (BNF) has been exploited elsewhere as a substitute for N fertilizers. It should be pointed out that besides the work of Pilbeam et al. (1995), no systematic research work on BNF in the ASALs of Kenya, particularly SE-Kenya has been undertaken. It has been estimated that the annual contribution of dinitrogen fixation to agricultural production worldwide exceeds four to five fold all the N fertilizers produced by fertilizer industries in a given year (Brill, 1977).

Given the aforementioned problems facing the resource poor farmers of the ASALs and the gap in research, the primary objective of this study was to investigate BNF in two major legumes, i.e. green gram (*Vigna radiata*) and common bean (*Phaseolus vulgaris*/ kathika variety) grown in semi-arid Makueni District. Secondary ojectives included assessment of nodulation of green gram and common bean with resident rhizobia in the soils of the study area; estimate the population of rhizobia specific to both legumes and finally, isolate, authenticate and assess the effectiveness of the indigenous rhizobia in nitrogen fixation compared with commercial strains using dry weight.

* Department of Botany, Kenyatta University, P. O. Box 43844, Nairobi, Kenya

** Department of Geography, Kenyatta University, P. O. Box 43844, Nairobi, Kenya

*** Department of Geosciences, University of Trier, D-54286, Trier, Germany (Corresponding author)

2 Materials and methods

2.1 Materials

Seeds of common bean were obtained from local farmers and those of green gram from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) at Kiboko, SE-Kenya. Uniformity in seed size and colour were the criteria used in seed selection. Rhizobia material (*Rhizobium leguminosarum* bv. *phaseoli* strain 446 and *Bradyrhizobium sp.* strain CB-1015) were obtained from the Microbiological Resource Centre (MIRCEN), University of Nairobi. Isolates C_1S and C_2L were isolated from common bean and GG-T from green gram grown in soils from the study area. Modified Leonard jar assemblies described by Vincent (1970) were used as the growth containers while growth media was vermiculite.

2.2 Methods

Nodule assessment was carried out in the greenhouse using the procedure described by Vincent (1970). A soil sample from Kiboko study site (10 samples, 0-15 cm, bulked and thoroughly mixed and subsampled) was used in estimating the nodulating population of rhizobia. Estimation of rhizobia population was done using the Most Probable Number (MPN) plant infection technique (Beck et al., 1993). The rhizobia were routinely grown in either yeast extract manitol agrar (YEMA) or yeast extract manitol broth (YEMB). Isolation, presumptive and authentication tests were conducted according to the methods described by Somasegaran et al. (1985). The tests carried out were Gram staining, growth of isolates on YEMA, growth on YEMA plus Bromothymol blue (BTB) and growth of isolates YEMA plus Congo red media. The plates were incubated in darkness at 28 °C for 3-5 days.

A total of five treatments were used in common bean and four in green gram experiments. The treatments in common bean were: Isolates C_1S, C_2L, commercial B-446, material control B-MC and nitrogen control B-NC. For the green gram, the treatments were: Isolate GG-T, commercial CB-1056, material control GG-MC and nitrogen control GG-NC. The experimental design was complete randomized with four replicates per treatment. All data were subjected to analysis of variance and the means separated by Duncan's multiple range tests (Steel and Torrie, 1960).

3 Results

3.1 Nodule assessment

All green gram and common bean plants nodulated (Table 1). Nodulation was poor in green gram (18 nodules/plant) compared to common bean (80 nodules/plant). In common bean, nodules occured mainly on the lateral and finer roots and very few were

located on the tap root. 68 % of the nodules in common bean were white in colour compared to 23 % in green gram. The proportion of nodules which had an inner pink colouration was very low in common bean but high in green gram.

Table 1: Nodulation status of green gram and common bean grown in the greenhouse in soils obtained from Kiboko

Legume	Nodulation	% number of plants nodulated
Green gram	+	100
Common bean	++	100

++ indicates good nodulation + indicates poor nodulation

3.2 Estimation of the number of rhizobia in the soil

The number of rhizobia resident in Kiboko soils and specific to green gram and common bean was determined using the MPN plant infection technique. Table 2 and 3 show the nodulation status at various soil dilutions for the common bean and green gram, respectively. From the MPN tables, it was calculated that the number of rhizobia specific to common bean and green gram ranged from 2,037 to 14,850 and 519 to 3,780 rhizobia cells per gram of soil, respectively.

Table 2: Nodulated units in common bean MPN experiment

Dilution level	Nodulation (+) or (-) and replications	Total number of nodulated units
10^{-1}	+ + + +	4
10^{-2}	+ + + +	4
10^{-3}	+ + + +	4
10^{-4}	+ + + +	4
10^{-5}	+ + + +	4
10^{-6}	+ + - -	2
10^{-7}	+ - - -	1
10^{-8}	+ - - -	1
10^{-9}	- - - -	0
10^{-10}	- - - -	0
Total		24

Number of replications (n) = 4 Lowest dilution = 10^{-1} Dilution steps (s) = 10 + units = 24

3.3 Presumptive tests

Presumptive tests were carried out to establish the cultural characteristics of the rhizobia isolated from green gram and common bean grown in soils obtained from Kiboko. All the isolates were Gram negative rods (Table 4). On Congo red medium, the colonies were milky to translucent hence showing very little or no absorption of the dye. On BTB, growth of colonies of the isolates from common bean was accompanied by a colour change of the medium from deep green to yellow indicating the production of

acidic substances which diffused into the medium. Colonies of isolates from green gram were accompanied by colour change of the medium from deep green to blue indicating the production of alkaline substances which diffused into the medium.

Table 3: Nodulated units in green gram MPN experiment

Dilution level	Nodulation (+) or (-) and replications	Total number of nodulated units
10^{-1}	+ + + +	4
10^{-2}	+ + + +	4
10^{-3}	- + + +	3
10^{-4}	+ - + +	3
10^{-5}	- + + -	2
10^{-6}	+ - - -	2
10^{-7}	+ + - -	2
10^{-8}	- - - -	0
10^{-9}	- - - -	0
10^{-10}	- - - -	0
Total		20

Number of replications (n) = 4 Lowest dilution = 10^{-1} Dilution steps (s) = 10 + units = 20

Two types of isolates of rhizobia were observed in common bean. One type had small dry colonies (ca. 2 mm in diameter). For the purpose of this study, this type was given the reference number C_1S. The other type had mucoid large colonies (ca. 3-5 mm in diameter). This type was referenced as C_2L. Only one type of *Rhizobium* was isolated in green gram and was given the reference number GG-T.

Table 4: Gram stain reaction and performance of *Rhizobium leguminosarum* bv. *phaseoli* and *Bradyrhizobium* sp. on various media

Isolate reference number	Bromothymol Blue	Congo red	Gram stain
C_1S	Y	X	-
C_2L	Y	X	-
GG-T	B	X	-

Y = Yellow colour B= Blue colour X = Poor absorption of Congo red

- = Gram negative

3.4 Authentication of isolates as rhizobia

The isolates from common bean and green gram conformed to the cultural characteristics of rhizobia (VINCENT, 1970) and were further confirmed using plant nodulation tests. There was very poor nodulation observed in common bean plants inoculated with isolate C_1S. Growth of the plants treated with this *Rhizobium* isolate was poor and leaves were yellow in colour. There was poor nodulation of common bean plants inoculated with isolate C_2L (13 nodules/plant) on average. The nodules were very small with diam-

eter ranging from 1 mm to 2 mm and were located on the lateral roots.

Nodulation was good in plants inoculated with *Rhizobium leguminosarum* bv. *phaseoli* strain 446 (52 nodules/plant) on average. These plants were deep green in colour and bigger compared to the yellow stunted non-nodulated uninoculated controls and the poorly nodulated plants. Nodulation was crown, i.e. nodules formed near the stem in all the bean plants in association with strain 446. Results from cross-sectioning of the nodules revealed a dark red to light red colouration of the interior. Plant shoot dry weight was higher in plants inoculated with *Rhizobium* strain 446 as compared with isolate C_1S and C_2L (Table 5a). However, there was no significat difference in shoot dry weight of plants inoculated with 446 and C_2L.

For the green gram, nodulation was very poor in plants inoculated with isolate GG-T. The plants had stunted growth and chlorosis was exhibited in the leaves of the plants. There was good nodulation in the plants inoculated with *Bradyrhizobium sp.* strain CB-1015. The plants inoculated with this strain had deep green leaves. On average, the number of nodules per plant was 79 and nodulation was of the crown type. The interior of the nodules had dark red to light red colouration. The trend in nodule number was also observed in nodule dry weight. Plant shoot dry weight was highest in plants inoculated with *Bradyrhizobium sp.* strain CB-1015 (Table 5b).

Table 5a: Effectiveness of rhizobia in N fixation in common bean

Treatment	Shoot dry weight (g)
C_1S	1.36 a
C_2L	1.61 ab
B-446	1.74 ab
B-MC	1.58 ab
B-NC	1.93 b

Means (n = 4) followed by the same letter are not significantly different by Duncan's multiple range test at P = 0.05 significance level. Treatments: C_1S - common bean inoculated with isolate C_1S; C_2L - common bean inoculated with isolate C_2L; B-446 - common bean inoculated with *Rhizobium* strain 446; B-MC - material control; B-NC - nitrogen control

Table 5b: Effectiveness of rhizobia in N fixation in green gram

Treatment	Shoot dry weight (g)
GG-T	0.15 a
CB-1015	0.40 b
GG-MC	0.19 a
GG-NC	0.52 c

Means (n = 4) followed by the same letter are not significantly different by Duncan's multiple range test at P = 0.05 significance level. Treatments: GG-T - green gram inoculated with isolate GG-T; CB-1015 - green gram inoculated with *Bradyrhizobium sp.* strain CB-1015; GG-MC - material control; GG-NC - nitrogen control

4 Discussion and conclusion

Results obtained on nodulation indicated that green gram had very few nodules per plant. However, these nodules were effective in N fixation as evidenced by the pink colouration of the nodules which is indicative of the presence of leghaemoglobin (AMARA et al., 1995; SPRENT and SPRENT, 1990). Unlike green gram, nodulation was good in common bean in terms of nodule abundance and distribution. However, a high proportion of the nodules lacked leghaemoglobin, indicating that they were not effective in N fixation. This lack of leghaemoglobin in the nodules of common bean points to the need for seed or soil inoculation since indigenous rhizobia are ineffective in N fixation (AMARA et al., 1995; VINCENT, 1970). Nodule number is frequently used as a measure of infectiveness (BECK et al., 1993). The high number of nodules per plant in common bean plants was an indication of high infectiveness of *Rhizobium* in the soil. Although adequate nodulation was observed in common bean plants, ineffective nodules exceeded the number of effective nodules hence little nitrogen fixation took place (WANI et al., 1995).

The enumeration of specific *Rhizobium* in the soils is required to predict the need for inoculation, rate of inoculation and to study the fate of the inoculum in the soil (BECK et al., 1993). The MPN plant infection technique is a reliable method known to microbiologists since it avoids the antagonistic effects of other microorganisms that hamper the counting of rhizobia in the soils. The rhizobia populations specific to green gram and common bean were adequate for satisfactory nodulation results. These results were in agreement with the findings of Nambiar et al. (1988) that most tropical soils have a rhizobial population of more than 100 rhizobia cells per gram of soil capable of nodulating the legumes grown in such soils. Thies et al. (1991) identified the critical rhizobial population sizes in soils which preclude observed responses to inoculation as >50 cells per g of soil for an individual host.

The results obtained from Gram staining and growth of isolates in YEMA conformed with the standard cultural and morphological characteristics of *Rhizobium sp.* described by Vincent (1970) and Somasegaran et al. (1985). Isolates from green gram and common bean nodules did not absorb Congo red at all. On BTB medium, a colour change to yellow indicated the production of acidic substances which diffused into the alkaline medium. Change to blue colour indicated production of alkaline substances which diffused into the medium. Production of acid or alkaline is common with fast growing *Rhizobium sp.* and slow growing *Bradyrhizobium sp.* (SOMASEGARAN et al., 1985). These tests helped in the screening of rhizobial material for contamination (VINCENT, 1970) and enabled the rejection of contaminated cultures.

The plant test is the only confirmatory test for rhizobia studies (VINCENT, 1970). Modified Leonard jar assemblies were used for the plant tests. This method has become a standard for testing nodulation and nitrogen fixation under greenhouse conditions (BECK et al., 1993). In green gram, poor nodulation was observed in plants inoculated with

isolate GG-T. This poor nodulation could be attributed to the loss of viability of some of the *Bradyrhizobium* cells during culturing and sub-culturing processes in the laboratory. Plants inoculated with strain CB-1015 had good nodulation. Nodules were large in size and active in N fixation. This justifies the use of strain CB-1015 for inoculation of green gram in the study area. In common bean, plants which were inoculated with isolate C_1S and C_2L did not nodulate well compared to the plants inoculated with strain 446. Like the case of green gram, this could again be attributed to loss of viability of the indigenous rhizobia isolates during the culturing and sub-culturing process. Besides, erratic symbiotic performance in common bean has been reported before (GRAHAM, 1981).

Plant dry weight was used to estimate nitrogen fixation. This method is accurate for screening large numbers of plants for nitrogen fixation in nitrogen free media (BROCKWELL et al., 1995; HALLIDAY, 1984). The method is inexpensive and easy to use. However, this method is not sensitive enough to be used in soils with a high nitrogen content (DANSO, 1985). Sometimes, other factors besides nitrogen do not permit the nitrogen fixed to be translated into increased dry matter yield (DANSO, 1985). In laboratory tests, strain 446 for common bean significantly contributed to a higher increase in plant dry matter yield compared with isolates C_1S and C_2L and the uninoculated control (Table 5a). In green gram, dry weight of plants inoculated with strain CB-1015 was significantly higher than that of all the other plants. Variation in nodule number and the total plant dry weight of plants inoculated with different strains were the factors that were considered in selecting the *Rhizobium/Bradyrhizobium* to be used in the field. These two factors are among the essential characteristics recommended for strain selection to ensure that a legume seed inoculant contains strain(s) of *Rhizobium/Bradyrhizobium* capable of forming fully effective nitrogen fixing nodules on the legume species for which it is recommended (HALLIDAY, 1984). Strains 446 for common beans and CB-1015 for green gram proved to be superior to the isolates obtained from the legumes and are therefore strongly recommended for inoculation of these legumes in the study area.

5 Summary

The major objective of this study was to investigate biological nitrogen fixation in two main legumes (green gram and common bean) grown in semi-arid southeast Kenya. Nodulation experiments on the two legumes were carried out in the greenhouse of the Botany Department, Kenyatta University with soil samples that had been obtained from Kiboko. Indigenous rhizobia were isolated from the two legumes and screened for the ability to fix nitrogen in comparison with commercially available strains from MIRCEN, University of Nairobi. The population of indigenous rhizobia specific to the two legumes was determined using the Most Probable Number (MPN) plant infection technique.

Results showed that infectivity in common bean was better (80 nodules/plant) than in green gram (18 nodules/plant). Bisection of the nodules showed that 32 % and 77 % of them had an inner pink colour in common bean and green gram, respectively. This is an indication that a majority of the nodules in green gram were effective nitrogen fixers compared to those in common bean. The results of the MPN counts indicated that the number of indigenous rhizobia resident in Kiboko soils, and specific to green gram and common bean were 519-3,780 and 2,037-14,850 rhizobia cells per gram of soil, respectively. Two different isolates of rhizobia for common bean (C_1S and C_2L) and one for green gram (GG-T), were isolated. Presumptive and authentication tests confirmed these isolates as rhizobia. Greenhouse trials showed that isolate C_1S and C_2L was not as effective in nitrogen fixation, as C_2L, and *Rhizobium leguminosarum* bv. *phaseoli* strain 446 from MIRCEN although no significant difference in Shoot dry weight was recorded for C_2L and 446. Further, isolate GG-T from green gram was not as effective in nitrogen fixation as the commercial strain *Bradyrhizobium* sp. strain CB-1015.

Biologische Stickstoffixierung ausgewählter leguminosen in den semiariden Gebieten des Makueni District, SE-Kenya

Zusammenfassung

Die vorliegende Studie beschäftigt sich mit der N_2-Fixierung von zwei angepaßten Leguminosen im Trockengrenzbereich des Anbaues in SE-Kenya (Green grams, *Vigna radiata; Phaseolus vulgaris*, lokale Varietät Kathika). Dazu wurden verschiedene Gewächshausexperimente im Botany Department der Kenyatta University Nairobi mit Böden aus dem Projektgebiet Kiboko durchgeführt. Endemische Rhizobienpopulationen wurden aus den Pflanzsubstraten (und Pflanzen) isoliert und ihre Effektivität in der N_2-Fixierung im Vergleich zu kommerziell verfügbaren Rhizobienstämmen des MIRCEN (University of Nairobi) getestet. Die Populationsdichte der isolierten pflanzenspezifischen Rhizobien wurde mit Hilfe der MPN-Methode untersucht.

Die Ergebnisse zeigen, daß *P.vulgaris* stärker infiziert wurde als *V.radiata* (80 Noduli/Pflanze gegenüber 18). Gewebeschnitte der Noduli ergaben in 32 (*P.vulgaris*) bzw. 77% (*V.radiata*) der Proben eine Pinkfärbung, was auf eine effektive N_2-Fixierung besonders an *V.radiata* hinweist. Die MPN-Tests erbrachten eine Anzahl von 519-3780 (*V.radiata*) bzw. 2037-14850 (*P. vulgaris*) endemischer Bakterien pro g Boden; zwei unterschiedliche Linien konnten für *P.vulgaris* (C_1S, C_2L) und eine für *V.radiata* (GG-T) isoliert werden, die eindeutig als Rhizobien identifiziert wurden. In Gewächshausexperimenten konnte nachgewiesen werden, daß C_1S weniger effektiv N_2 fixieren konnte als C_2L und der kommerzielle Stamm 446 des MIRCEN (*Rhizobium leguminosarum* bv. *phaseoli*), obwohl kein signifikanter Unterschied im Trocken gewicht zwischen C_2L und 446 festgestellt werden konnte. Ebenso konnte herausgefunden werden, daß GG-T an *V.radiata* nicht so effektiv war wie der kommerzielle Stamm CB-1015 (*Bradyrhizobium sp.*).

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