Abundance and Symbiotic Potential of Common Bean (*Phaseolus vulgaris*) Nodule Associated Bacteria in Western Kenya Soil

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Abstract

Plant growth-promoting Rhizobacteria are beneficial native soil bacteria that colonize plant roots and result in increased plant growth. Those that colonise the nodules of legumes are known as nodule associated bacteria. The aim of this study was to determine the effect of chemical soil factors on the abundance of nodule associated bacteria and the symbiotic efficiency of these bacteria when coinoculated with Phaseolus vulgaris in Western Kenya soils. The soil samples were collected from cultivated lands in Kisumu near Lake Victoria, slopes of Mt. Elgon and Kakamega. In each of these regions, the soil samples were collected from four regions. 1ml of soil solution at 10 fold dilution for seven dilution steps (10-1to 10-7) and three replications for each dilution was used to inoculate common bean seedling in the Leonard jars. They were harvested after four weeks to determine abundance of nodule associated bacteria using most probable number plant infection method. Mt. Elgon region had the highest population of nodule associated bacteria (120000 cells per gram of the soil), followed by Kisumu (1290 cells per gram of the soil) and Kakamega region had the lowest (17 cells per gram of the soil). The effect of plant growth-promoting Rhizobacteria on the yield of common beans was significantly higher ($p < o.oo1$) when co-inoculated with Rhizobia compared to the yield of Rhizobia inoculated alone or negative control (not inoculated) ($p < 0.05$). This study therefore provides knowledge on the factors that favour the survival of common bean symbiotic bacteria and their symbiotic capability which is necessary for production of plant growth-promoting Rhizobacteria inoculants suitable to the soils of Western Kenya.

I. INTRODUCTION

Recent census findings on the population of Western region shows that more than 80 % of the population is involved in agricultural activity [1]. These farmers repeatedly cultivate their land causing a reduction in soil fertility and hence crop productivity. Chemical fertilizers currently being used in this region are very expensive and also cause soil and water pollution. The economic implications on the use of the inorganic fertilizers together with

their negative impacts to the environment have become a global concern, therefore there is need to shift to sustainable farming practices [2]. Some of these practices include utilization of natural microorganisms in enhancing soil fertility and plant protection from pathogenic attack. This paper therefore determines factors that favour the survival and distribution of this common bean nodulating symbiotic microorganisms in the soils of western Kenya.

The use of plant growth promoting Rhizobacteria (PGPR) in agricultural practices has increased tremendously in many parts of the world. Significant increases in growth and yield of agricultural crops due to inoculation with PGPR have been reported severally [3, 4, 5]. PGPR affect plant growth by various direct and indirect mechanisms [6]. Some of these mechanisms, are enhanced phosphate solubilization and nitrogen fixation, making nutrients available for the plant; inhibition of soil borne phytopathogens (by the production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients); enhancing plant tolerance to stress, drought, salinity, and metal toxicity; and production of phytohormones such as indole-3-acetic acid (IAA) [7]. In addition, some PGPR have 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme, which usually hydrolyse ACC, immediate precursor of plants ethylene [8]. By reducing the concentration of ethylene in seedlings and therefore its inhibition ability, these PGPR enhance the seedlings' root length [9]. Biological control of phytopathogens and deleterious microorganisms, through the production of lytic enzymes, antibiotics, siderophores, hydrogen cyanide, and through competing for nutrients and space can enhance significantly the plant health and promote its growth.

Improvement of nitrogen fixation in legumes by coinoculation of *Rhizobia* with plant growth promoting Rhizobacteria (PGPR) is a way of enhancing nitrogen availability in affordable agricultural systems. Various studies show that simultaneous inoculation with *Rhizobia* and rhizosphere bacteria enhances nodulating capacity and growth in a wide range of leguminous plants [9, 10]. These nodule promoting bacteria are likely to be free-living bacteria at the rhizosphere or endophytic. Endophytic bacteria are intercellular or intracellular within the host tissues and are therefore at advantage when compared to the free-living counterparts as they are cushioned from stresses of the environment and competition from other microorganisms. Depending on how they relate to the host plant, these endophytes can be put in three groups: plant growth inhibiting, plant growth promoting, and plant growth neutral [11]. It is however known that a larger proportion of endophytic bacteria have plant growth promoting ability [12].

Contrary to the believe that only *Rhizobia* reside in the nodules, presence of other bacteria other than *Rhizobium* in the root nodules has been reported [13, 14] in the Leguminosae. This study described the isolation of bacteria of different genera from legume tissues, such as *Aerobacter, Agrobacterium, Bacillus, Chryseomonas, Pseudomonas, Curtobacterium, Erwinia, Enterobacter, Flavimonas and Sphingomonas*.

Available reports have shown improved plant health and plant yield under greenhouse conditions in terms of increase in root weight and nodulating ability when coinoculated with nodule associated bacteria, compared to inoculating with *Rhizobia* alone [6]. PGPR that have been coinoculated with *Rhizobia* include the strains of the following well-known Rhizobacteria: *Azospirillum* [15], *Azotobacter* [16], *Bacillus* [17] and *Pseudomonas* [16]. Coinoculation of specific strains *of Pseudomonas* and *Bacillus* together with highly effective *Rhizobium sp*. is shown to stimulate chickpea growth, nodulation, and nitrogen fixation [11]. Some *Serratia* strains, for example, *S.*

proteamaculans and *S. Liquefaciens*, have desired effects on leguminous plant growth [9]. *Bacillus sp.* is a spore-forming, gram-positive rod-shaped bacterium which comprises one of the highly distributed soil bacteria and they are commonly isolated from rhizospheric region of plants. *Bacillus* species, common endophytes are readily adaptable to field applications because of their spore-forming capability. *Exiguobacterium sp*. fall in the class *Bacilli*, order *Bacillales* and family *Bacillaceae*. These bacteria are non-spore-forming gram-positive. The objectives of this study are to determine: abundance and symbiotic efficiency of common bean nodule associated bacteria.

II. MATERIALS AND METHODS

A. Site of study and soil sample collection

The soils was collected from farmers' fields in which beans had been grown frequently but for which there is no history of inoculation with PGPR . The soils were collected from four sites of Lake Victoria shore, Kakamega and Mt. Elgon slopes.

After clearing the surface debris, soils were sampled with a spade that is sterilized with alcohol to a depth of 30 cm following a W pattern across the selected fields. About 10g of the soils were taken at intervals of 6 metres along the W pattern running across the whole plot. Homogenous soil particles for analysis were obtained by passing the soil through 2mm sieve. The soil samples from each farm were thoroughly mixed, bulked and sub-sampled. One sub-sample was used to determine the population of soil *Rhizobacteria*, the other for symbiotic efficiency of Rhizobacteria while the other sub-sample was used for the chemical analyses of the soil.

Soil sample for *Rhizobacteria* analysis was loosely closed immediately in sterile khaki bags and stored at 4^0C while soil samples for chemical analysis was air-dried and stored at room temperature until it was used. The bulked samples was analysed for soil pH, percent carbon, nitrogen, phosphorus, potassium and organic carbon using established procedures at the College of agriculture & veterinary sciences, Department of land resource management & agricultural technology, University of Nairobi.

B. Soil chemical analysis

The bulked soil samples was analysed for soil pH, percent carbon, nitrogen, aluminium, potassium and Phosphorus at the University of Nairobi, College of agriculture & veterinary sciences using established protocols. Available nitrogen was determined by Cadmium reduction method [18], soil pH by glass electrode method [19], organic carbon by Walkley black method [20], Phosphorus by Mehlich method [21], aluminium was extracted with KCl [22] and read with AAS and potassium was extracted with NH_4OAc [23] and read with flame photometer.

C. NAB count to determine the abundance

The numbers of NAB present in Western Kenya soils which nodulates with *P. vulgaris* was estimated by the use of most probable-number, plant infection technique, modified by Ref. [24]. Seeds of *P. vulgaris* cv. Roseccoco were surface sterilized by 95% ethanol and then submerged in 0.2% acidified mercuric chloride for 3 min. They were then germinated on sterile water-agar plates for 2 days. Single seedlings were transplanted into Leonard jars [25]

containing vermiculite and the jars were placed in a growth room at 24^0C for a few days. The seedlings were inoculated with 1 ml of 10-fold soil dilutions. In these tests, seven soil dilution steps (from $10^{-1}$to 10^{-7}) and three jars (seedlings) for each dilution, and the plants were watered as required with sterile N-free nutrient solution [26, 27]. Nitrogen free nutrient solution contained in g/L: $CaCl_2$ 0.1, $MgSO_4.7H_2O$ 0.12, KH_2PO_4 0.1, $Na_2HPO_4.2H_2O$ 0.15, Ferric citrate 0.005, and 1.0 ml of trace elements stock solution. The trace elements stock solution contained: H_3BO_3 2.86, $MnSO_4.7H_2O$ 2.03, $ZnSO_4.7H_2O$ 0.22, $CuSO_4.5H_2O$ 0.08, and $NaMoO_2.2H_2O$ 0.14 in g/L. The pH of the nutrient solution was adjusted to 6.8 with 1M NaOH and 1M HCl. After 4 weeks of growth, the roots of the seedlings were gently washed in tap water and inspected for the presence of nodules in each dilution, and the total number of positive cases counted. Based on these scores, the most probable numbers (MPN) of NAB in the test soils and 95% confidence limits was calculated using MPNES program. Log-transformed numbers of NAB in the test soils was expressed per 1g of soil dry matter.

D. Determining symbiotic efficiency of the NAB isolates

The plant inoculation tests with Common beans were carried out as described in Ref. [28]. Seedlings were planted in the autoclaved vermiculite systems comprising of the following: autoclaved vermiculite planted with seedlings with no bacterization as the negative control (NC); seedlings with *Rhizobium sp.* strain NUM466 (from the Nairobi Microbiological Resources Center collection) as positive control (PC) and the other seedlings were co-inoculated with *Rhizobia* and NAB isolates as the experimental systems. All the experiments were performed in triplicates and plants were harvested after 56 days of inoculation and various parameters such as number of pods per plant, number of seeds per pod, weight of pods per plant, and total dry matter of shoot per plant were recorded.

E. *Data analysis*

Data collected was reported as Mean ± SD. The association between the size of the population of NAB and concentrations of chemicals in the soils was analysed using Pearson's correlation analysis implemented in Graphpad Prism (Version 5). Comparisons of various parameters with plants inoculated with *Rhizobia* and other NAB, *Rhizobia* alone and negative control was done by one way ANOVA followed by Turkey's post hoc test implemented in Graphpad Prism (Version 5). Findings of the study were reported as significant at $p< 0.05$).

III. RESULTS

A. *Chemical characteristics of the soil*

The sites from which the soils were collected varied in pH, Nitrogen, Phosphorus, Aluminium and potassium concentrations (Table 1). Kakamega site had lowest concentration of pH, followed by Kisumu and Mt. Elgon region had the highest. Mt. Elgon region had the lowest levels of aluminium followed by Kisumu and eventually Kakamega had the highest. Kakamega had the highest amount of nitrogen and inorganic carbon while Kisumu had the highest amount of phosphorus and potassium.

B. *NAB population characteristics in the soils*

All the soil samples collected from the three field sites contained common bean nodule associated bacteria (NAB). The population size of indigenous NAB in the field sites varied from 17 to 120000 bacterial cells per gram of dry

soil. Mt. Elgon soils produced higher amount of bacteria cells per gram, followed by Kisumu and eventually Kakamega produced the least number. The population of NAB from Mt. Elgon soils was significantly higher than those of Kisumu and Kakamega ($p< 0.05$) but there was no significant difference between NAB population for Kakamega and Kisumu ($p> 0.05$).

C. Association between soil characteristics and NAB population

Aluminium, nitrogen and phosphorus was negatively correlated to the population size of NAB while pH, carbon and phosphorus was positively correlated to the soil NAB population but only aluminium and pH had a statistically significant correlation ($p< 0.05$) while the rest were statistically insignificantly ($p> 0.05$) correlated with NAB population (Table 1).

	ELGON	KISUMU	KAKAMEGA	
NAB Population	120000±86409	12920±10893	17±12.83	
pH	6.09±0.44	5.48±0.22	4.61±0.46	
% Nitrogen	0.2±0.03	0.15±0.03	0.27±0.07	
% Carbon	1.89±0.22	1.085±0.07	2.27±0.3	
Potassium (Cmol/Kg)	0.89±0.1	1.54±0.05	0.31±0.13	
Phosphorus (PPM)	18.53±7.75*	118.71±94.17	19.75±7.91	
Aluminium (Cmol/Kg)	3.7±0.78*	5.78±0.88	9.43±1.86*	

Table 1: Chemical and population characteristics of the selected study sites

D. Determination of symbiotic efficiency

The means of number of pods per plant for the plants inoculated with *Rhizobia* and other NAB were significantly higher ($p<0.05$) than those inoculated with *Rhizobia* alone and the uninoculated plants while there was no significant difference between the means for the plants inoculated with *Rhizobia* alone and the uninoculated ones ($p>0.05$) (Table 2).

The means of number of seeds per pod for the *Rhizobia* and other NAB were significantly higher than the means of both from plants inoculated with *Rhizobia* alone and uninoculated ones ($p<0.05$) while the mean of number of seeds per pod from plants inoculated by *Rhizobia* alone was not significantly higher than the uninoculated ones ($p>0.05$) (Table 2).

The means of weight of pods per plant for the plants inoculated with *Rhizobia* and other NAB was significantly higher than those of plants inoculated with *Rhizobia* alone and the uninoculated ones ($p<0.05$) and the mean of the weight of pods per plant for the plants inoculated with *Rhizobia* alone and the mean for the plants that were not inoculated were not significantly different ($p<0.05$) (Table 2).

The means of total shoot dry matter of plants inoculated with *Rhizobia* and other NAB was significantly higher than the uninoculated and those inoculated with *Rhizobia* alone ($p<0.05$) while the means of total shoot dry matter

for plants inoculated with *Rhizobia* alone and the uninoculated ones were not significantly different (p>0.05) (Table 2).

	Inoculated with NAB	Inoculated with Rhizobia	Uninoculated
Number of pods per plant	13.4±1.17*	10.3±1.00	10.1±3.07
Number of seeds per pod	4.8±1.32*	3.4±0.8	3.3±1.34
Weight of pods per plant	25.01±1.68*	23.32±0.85	22.53±1.94
Total dry matter per plant	26.19±0.62*	23.6±1.99	23.13±3.09

Table 2: Effect of NAB and Rhizobia on number of pods, weight of pods and total dry matter per plant and number of seeds per pod

IV. DISCUSSION

A. Abundance of nodule associated bacteria

The indigenous populations of NAB associated with common bean ranged from 17 bacteria cells per gram of dry soil to 120000 bacteria cells per gram of dry soil. These observations provide evidence that indigenous common bean associated bacteria are widespread in Western Kenya. The high population levels of common bean associated indigenous bacteria in the field sites could be attributed to the legumes' widespread integration in the cropping system in Kenya [29]. Variation among the field sites in population sizes of root nodule bacteria (i.e. 17 to >120000 cells per gram of dry soil) observed in this study is a common phenomenon. This can be attributed to differences in levels of soil pH, plant nutrients, soil type, soil moisture, temperature and crop/soil management, among other factors [30]. The results are in agreement with the findings of several researchers, Ref. [31] reported inhibition of NAB by high aluminium concentration and low pH. The low population of NAB and legume nodulation noted with the Kakamega soil could be attributed to its low pH (4.56), high aluminium concentration (9.425 Cmol/kg), and low organic carbon content (0.3125 Cmol/kg) that have been shown to adversely affect both survival of NAB and nodulation process in legumes [32].

In this study, aluminium and low pH significantly reduced the population of soil *Rhizobacteria* similar to other reported findings [33, 34]. Kakamega soils with the highest concentration of aluminium and lowest pH had the lowest population of NAB (17cells/g) as compared to other regions. The similarity in the NAB population in the soils from Kisumu and Kakamega can be attributed to the small difference between soil aluminium concentration and pH between these two regions. It is therefore advisable that farming practices that reduces the concentration of soil aluminium and raises its pH should be encouraged to improve the survival of soil microflora including liming which is known to increase the pH. Studies have demonstrated that aluminium solubilization is higher when soil acidity increases [34] showing that increasing the soil pH by liming is an important practice in lowering soil aluminium concentration. Intensive use of inorganic fertilizers in farming inhibits the growth of soil *Rhizobacteria* [36, 29]. Probably, they have a role to play in soil acidification. The application of the inorganic fertilizers in agriculture therefore should be very minimal because apart from being a potential soil pollutant [37], it also inhibits the soil microbial growth by interfering with the solubilization of phosphorus [38] and production of antibiotics

necessary in reducing the growth of phytopathogens [39]. Plant growth promoting Rhizobacteria inoculants that are adapted to Western Kenya region are therefore recommended.

B. Symbiotic efficiency of the root nodule associated bacteria

Rhizobia was able to increase the number of seeds per pod, the number of pods per plant, weight of seeds per plant, and total dry matter of plants in seed filling stage when compared to uninoculated plants, however, these increases were not significant. Co-inoculation of the common bean with *Rhizobium* and PGPR resulted in significantly higher yield when compared to yield resulting from plants inoculated with *Rhizobia* alone or the negative control. This is in agreement with previous reports demonstrating the enhanced symbiotic efficiency of *Rhizobia* when coinoculated with PGPR belonging to *Pseudomonas spp*. and *Azotobacter spp*. nodulating different legume crops [37, 40]. The results indicated that application of PGPR together with *Rhizobium* improved the growth and seed production by inoculated beans. Beneficial effects of PGPR on common bean have been described in several other studies with different climatic and soil conditions. In Ref. [25], inoculation of chickpeas with *Azospirillum brasilense* and native *Rhizobia* resulted in a significant increase in nodulation, root and shoot growth, and crop yield as compared with non-inoculated controls. Ref. [41] also investigated the synergy in the interaction between *Rhizobium* and arbuscular mycorrhizal fungi for improving the growth of faba bean that was grown in the alkaline soil. Findings demonstrated that there was a significant increase in the number and nodule mass, leghaemoglobin content of nodule, nitrogenase activity, mycorrhizal colonization, shoot and dry mass of root in dual inoculated crops than plants with individual inoculated with *Rhizobia* alone when compared to the control.

The mechanisms of growth and nitrogen fixation promotion by PGPR are not well demonstrated and findings are still inconclusive [42]; however, it has been suggested that there could be both direct and indirect mechanisms of action of growth and nitrogen fixation promotion [7, 43, 8, 44, 45]. Root system development in plants is regulated by auxins activity and in legume root nodules, indole acetic acid produced by most of PGPR, activates the enzyme H^+-ATPase, which is important for energy production in the nodules [20]. It is also understood that plant root flavonoids are the inducers of nodulation gene (nod genes) expression in *Rhizobium* [41]. *Rhizobacteria* have also been found to produce phytoalexins, a class of fluorescent compounds, closely related to flavonoids and isoflavonoids in roots of several crop plants. These phytoalexins have a direct effect on plant protection mechanisms against phytopathogens, which are necessary in root development [46]. Mobilization of insoluble nutrients such as phosphorus followed by enhancement uptake by plants, and production of phytopathogens inhibiting substances [47] can also positively affects the *Rhizobium*-legume symbiosis.

V. CONCLUSION

This study found that Mt. Elgon soils had the highest population of nodule associated bacteria. This might have been due to relatively low concentration of aluminium. Now that low pH contributes to aluminium solubilization, practices that reduce soil acidity such as liming are recommended to reduce soil aluminium in order to increase colonization of the soil by PGPR.

Inoculation of common beans with PGPR significantly increased the yield when compared to plants inoculated by *Rhizobia* alone and when plants were not inoculated by any bacteria. However, there was no significant difference in the yield between plants inoculated by *Rhizobia* alone and those that were not inoculated. This therefore shows that that inoculants need to include both *Rhizobia* and other nodule associated bacteria rather than *Rhizobia* alone.

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