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Distribution and phenotypic characteristics of common bean (*Phaseolus vulgaris L.*) nodulating bacteria in diverse soils

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ABSTRACT

Bacteria were isolated from the root nodules using common bean as a trap host. Growth and morphological characteristics of the bacterial isolates were described on yeast extract mannitol mineral salts agar and broth media. The results showed that over 67% of the isolates produced extracellular polysaccharide with an entire margin and convex elevation. The others differentiated into cream yellow, cream white and milky white colouration with colony area ranging from 0.8 to 26 mm^2. The population levels of bacteria nodulating common bean varied in soils and were not affected by the cropping system. However, selected soil parameters greatly influenced the occurrence and distribution of these bacteria. The isolation of indigenous bacteria in all the soils with different cropping systems is an indication that the soils are favourable for nitrogen fixation. Based on the differences in cultural characteristics of the isolates, our data demonstrate the presence of high diversity of bacteria associated with bean nodules.

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Trap host; isolates; indigenous; cropping systems; diversity

Introduction

Nitrogen fixation in leguminous plants involves a symbiotic relationship between nitrogen-fixing bacteria and legume roots and occurs within specialised root nodules. Due to their considerable agricultural and environmental significance, these legume symbionts have been extensively studied (Keet et al. 2017; Kawaka et al. 2018a). In the recent past, assessment of diversity within the natural population in various regions of the world has received increased attention (Agrawal et al. 2012; Wekesa et al. 2016). These studies confirm that biological nitrogen fixation is an important and integral component of a sustainable agricultural system. Bacteria nodulating legumes are facultative symbionts that have adapted to persist in soil for long periods in a free-living state if the suitable legume host is absent (Chhetri et al. 2019). These are widely distributed, growing from low to high altitudes and represented in nearly all the eco-climatic zones (Odee et al. 1997). Although legume nodule bacteria seem to be widely distributed in the soil, soil characteristics in different places affect their populations. Some soil may have effective strains, but their numbers may be too low or containing higher number of ineffective strain (Graham 2008).

Several factors can influence the number and diversity of indigenous legume bacteria, including nutrient availability, soil type, acidity and agricultural management regimes (Laguerre et al. 2006). Although there are numerous factors limiting their survival in the soil, these bacteria are common soil inhabitants in all climatic zones of the world (Fagerli and Svenning 2005; Wielbo et al. 2012). These factors affect the potential success of individual strains to persist in local natural populations. New introduced strains may quickly disperse in the native population in bacteria-rich soils or remain as a significant proportion of a population for years in bacteria-depleted soils (Masson-Boivin et al. 2009; Wielbo et al. 2012).

Production of common bean in Kenya is mainly in the highland and midlands with approximately 75% of the annual cultivation taking place in Rift valley, Eastern and Eastern regions (Katungi et al. 2009). In terms of output, the rift valley contributes the biggest share, accounting for 33% of the national output followed by Nyanza and Western regions accounting for 22% each (Abate et al. 2012). The output from Eastern parts of the country and the coast is constrained by adverse climatic conditions (Katungi et al. 2009). Although Kenya has two seasons for common bean, a significant number of farmers grow the crop once a year because of adverse climatic conditions (Kimani et al. 2019).

In western Kenya, studies have reported that Common bean is a promiscuous host that is nodulated

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by a wide variety of rhizobia for N_2 fixation (Kawaka 2016). The promiscuity involved in common bean symbiosis represents an interesting model for studies of rhizobial species. Cropping system and/or nodulation history can have a significant impact on rhizobial diversity in soils (Nkot et al. 2008). The use of fertilisers, pesticides and herbicides can also alter rhizobial diversity in different agroecologies with different cropping history (Farooq and Vessey 2009).

However, few studies have focused on the effect of diverse soil types on the distribution and cultural characteristics on native bacteria nodulating legumes in Kenya (Kawaka 2016). The aim of the current study was to establish the distribution and morphological characteristics of common bean nodulating bacteria in different soils of western Kenya.

Materials and methods

Study site

The current study was done in Kisumu (0° 05′35″ S, 0° 34° 41.32″E) and Kakamega (0° 17′ 25.57″ N, 34° 45′ 50.02″) counties. Kisumu and Kakamega are located at an altitude of 1300 and 1585 m above sea level with an annual rainfall of 1200–2100 mm and 1200–1300 mm, respectively. The study sites and cropping system for the current study were selected based on the agroclimatic conditions and prevalence of crops that are commonly grown in the region respectively. The farms had no previous inoculation or external input of either organic and or inorganic fertilisers.

Soil sampling and analysis:

Sampling of soil was carried out in beans, maize, maize–bean intercrop and fallow farm in Kisumu while in Kakamega, soil was taken from maize, beans, nappier and fallow farms. In every farm, 20 soil cores were randomly taken from each farm to a depth of 15 cm using 2.5 cm diameter soil probe and thoroughly mixed to form a composite sample. The samples were asceptically collected and then divided into two for determination of the population of bacteria and chemical and physical analyses using standard procedures (Okalebo et al. 2002).

Determination of population of bacteria in soil

Common bean seeds were used as the trapping host and pre-tested to determine the germination period for synchronised germination (Chemining'wa et al. 2012). Seeds were surface sterilised in 3% sodium hypochlorite solution for 3–5 min, rinsed in 95% alcohol to remove waxy material on the surface and trapped air. After that, the seeds were soaked in water in a refrigerator for 4 h to imbibe water then placed in a germination chamber for pre-germination. After germination, two to three seedlings were planted in every Leonard jar inoculated with the diluted soil samples and later thinned to one plant per jar (Chemining'wa et al. 2012). Soil inocula was prepared by suspending 10 g of soil in 90 mL of sterile water in a 160 mL dilution bottle and shaken for 20 min in a wrist-action shaker at room temperature (25°C). Then 1 mL of each suspension was aseptically pipetted into 9 mL sterile water diluent in McCartney bottle and shaken for 2 min. The resulting suspension was serially diluted 10-fold from 10^{-1} to 10^{-6} with four replications at each dilution level. An aliquot of 1 mL of diluent was used to inoculate each seedling in the Leonard jars (Broughton and Dilworth 1971). The seedlings were watered using N-free plant nutrient solution prepared using a standard method and sterilised at 121°C for 15 min (Beck et al. 1993). Regular checking of N-free nutrient solution was done to ensure the seedlings were adequately moistened and plants scored for the presence or absence of nodules 28 days. Using the presence of a single nodule as a positive score, most probable number (MPN) technique was used to determine bacterial cells in 1 g of dry soil (Somasegaran and Hoben 1994).

Isolation of the nodule bacteria and authentication

Nodules were surface sterilised in 1% NaOCl for 6 min, rinsed and crushed with a flame-sterilised blunt-tipped pair of forceps. As previously described, a loopful of the crushed nodule was streaked on Yeast Extract Mannitol mineral salts agar in Petri dish (Vincent 1985). Repeated streaking was done to check on purity of the colonies and verify their cultural characteristics and absorption of the Congo red dye (0.00125 mg kg^{-1}) containing bromothymol blue (0.00125 mg kg^{-1}). The isolates were incubated at 28°C and stored at −20°C in 25% glycerol-YEM broth. The pure isolates were authenticated as bean nodulating bacteria by re-inoculating 1 mL on the host plant grown in a controlled environment (Somasegaran and Hoben 1994). The plants were watered with N-free nutrient solution with treatments without inoculation and inorganic N-supplement serving as a negative control. Plants without inoculation but with N supplement applied at a rate of 70 µg N ml^{-1} as KNO_3 solution served as a positive control.

Data analysis

The MPN technique was used to determine the population of soil bacteria (cells per gram of soil) nodulating

bean. Pearson correlation coefficients were calculated to establish the relationship between selected soil characteristics and the population of bacteria nodulating bean (BNB).

Results

Soil characteristics

Soil pH of the farms in Kakamega was highly acidic (4.98) compared to those of Kisumu (6.10) as shown in Table 1. Levels of moisture, sand, silt and clay were high in clay soils of Kakamega than loamy soil in Kisumu. Across the two regions, high acidity in soils was characterised with elevated levels of Electrical Conductivity, Nitrogen, organic Carbon, Magnesium, Aluminium, Copper and Manganese. Sandy loam soil had high sand, bulk density, Phosphorus, Iron, Zinc and Sodium content.

Population levels of BNB

The soils across the two sites exhibited different soil characteristics even in farms growing similar crops (Table 2). For example, levels of Nitrogen (N), Organic Carbon (OC), Aluminium (Al), Copper (Cu) and Manganese (Mn) were high in soils with maize in Kakamega compared to the same farms in Kisumu. Fallow soil in Kakamega had high Aluminium (1.9 cmol kg^{-1}) and Copper (5.4 ppm) content. Soil from bean farms recorded lower EC, N, OC and Cu compared to similar farms in Kakamega. Across the two sites, soils from maize and nappier farms in Kakamega had high levels of N. Bacteria nodulating Common bean was isolated from all the soil samples collected from the farms at the two sites. The bacterial population ranged from 3.2×10^1 gm^{-1} (fallow) to 1.25×10^4 (bean and maize bean intercrop). Soil from land-use systems with crops recorded high populations of bacteria compared non-cultivated fallow plots. The results further indicated that all the crop types did not influence the occurrence of the bacteria. Soil from farms growing similar crops did not record the same abundance of bacteria.

Table 1. Surface soil (1–20 cm) in Kakamega and Kisumu.

Kakamega		Kisumu	
Soil characteristics	Value	Soil characteristics	Value
pH	4.98	pH	6.10
EC dS/m	0.7	EC dS/m	0.2
% Nitrogen (N)	0.24	% Nitrogen (N)	0.11
% Organic Carbon (OC)	2.66	% Organic Carbon (OC)	1.32
Potassium (cmol/kg)	0.93	Potassium (cmol/kg)	1.39
Sodium (cmol/kg)	0.7	Sodium (cmol/kg)	0.6
Magnesium (cmol/kg)	1.39	Magnesium (cmol/kg)	1.17
Calcium (cmol/kg)	3.08	Calcium (cmol/kg)	3.02
Aluminum (cmol/kg)	2.56	Aluminum (cmol/kg)	0.62
Zinc (ppm)	7.6	Zinc (ppm)	9.8
Copper (ppm)	5.1	Copper (ppm)	1.6
Iron (ppm)	10.1	Iron (ppm)	22
Manganese (ppm)	75.2	Manganese (ppm)	38.3
Phosphorus (ppm)	28	Phosphorus (ppm)	35
Bulk density	1.07	Bulk density	1.42
Moisture	35.31	Moisture	11.32
% Sand	15.68	% Sand	71.84
% Silt	17.44	% Silt	10.0
% Clay	66.88	% Clay	18.16
Texture	Clay	Texture	Sandy Loam

Table 2. Population of bacteria nodulating common bean in different land use systems in Kisumu.

	Land use systems			
Soil characteristics	Bean	Maize	Fallow	Maize/Bean
Bacterial population	1.25×10^4	3.5×10^4	3.2×10^1	1.25×10^4
pH	6.1	6.06	5.98	5.24
EC (dS/m)	0.2	0.2	0.4	0.4
Total N (%)	0.11	0.13	0.15	0.2
Organic carbon (%)	1.32	1.15	1.78	1.39
K (cmol/kg)	1.39	1.42	1.39	1.42
Na (cmol/kg)	0.6	0.7	0.6	0.8
Mg (cmol/kg)	1.17	1.4	1.08	1.77
Ca (cmol/kg)	3.02	2.66	2.56	5.31
Al (cmol/kg)	0.6	0.7	0.6	0.9
Zn (ppm)	9.8	6	6.2	1.7
Cu (ppm)	1.6	1.8	1.4	1.6
Fe (ppm)	22	12.5	20.4	43.2
Mn (ppm)	38.3	48.6	32.3	94.1
P (ppm)	35	62	11.8	15
Soil texture	SL	SL	SL	SL

Representative bacterial isolates from the nodules of common bean from Kakamega (M004 and M006) and Kisumu (K001 and K004) are shown in Figure 1(a–d). The shiny or dull isolates differentiated into cream yellow, cream white and milky white colours that were either transparent or opaque. The colonies had an entire margin and convex elevation with over 67% of the isolates producing extracellular polysaccharide. The circular and oval colonies had firm dry or smooth viscous texture with colony area ranging from 0.8 to 26 mm^2. On YEMA media with Congo red dye, the isolates either fully absorbed the red dye or slightly absorbed it under incubation in the dark. However, YEMA media containing bromothymol blue (BTB), turned the colonies into moderately yellow to deep yellow colour.

Simple correlations demonstrated that selected soil properties had direct effect on the abundance on the bacteria in soil (Figure 1(a–d)). The results show that bacterial population had moderate negative correlation with total N ($r = -0.54$, $p = .0004$), OC ($r = -0.65$, $p = .00009$) and Al ($r = -0.57$, $p = .002$). On the other hand, there was a strong positive relationship between bacterial population and P ($r = 0.78$, $p = .01$).

Among the 14 pure isolates evaluated during authentication experiments, 85.7% initiated nodulation in common bean and were hence confirmed as root nodule bacteria. The morphologically and culturally described isolates initiated nodulation in common bean on re-inoculation. The nodules from the inoculated

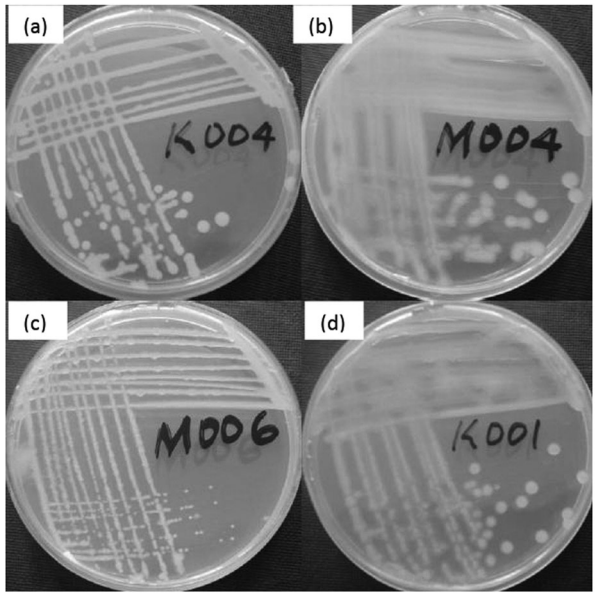


Figure 1. Representative common bean nodulating bacteria isolates.

plants were pink with dark-green leaves (Figure 3(a,d)). After 21 days, the non-inoculated control plants with and without nitrogen input did not form nodules and leaves turned yellow (Figure 3(b,c)).

Discussion

The variation in soil characteristics observed across the two sites could be attributed to differences in soil management practices on individual smallholder farms. As shown in Table 1, the low-soil pH in Kakamega farms shows that the soils are highly withered and leached. Okalebo et al. (2005) reported that acid soils are widely distributed in most smallholder farms of western Kenya. Acid soils can occur naturally or develop as the result of continuous application of acid-forming fertilisers (Okalebo et al. 2005; Goulding 2016). Farms with low-soil pH recorded high Aluminium, Copper and Manganese levels which are a characteristic of a highly withered soil. Elsewhere, analysis of highly withered soil showed high contents of Fe and Al oxides often with large amounts of soluble Mn (Rengel 2015). Lack of external nitrogen input could be responsible for the low levels of soil N recorded in Kisumu farms (Table 1).

Population of BNB ranged from $3.2 \times 10^{1-}3.5 \times 10^{4}$ cells per gram of soil in all the farms, indicating the presence of native bacteria in soils of western Kenya. Katungi et al. (2009) reported that the occurrence of BNB in different land-use systems could be attributed to widespread integration of legumes in the cropping system. As indicated in Table 3, the clay texture and acidic pH of soil in Kakamega-suppressed bean nodulation thus reducing population of BNB. Similar studies have reported that clay soil (Loureiro et al. 2007; Mothapo et al. 2013) and high hydrogen ion concentration (Ferguson et al. 2013) reduce nodule bacterial population. High hydrogen concentration increases solubility of toxic metal ions such as Al^{3+} Cu^{2+} and Mn^{2+} that cause intercellular pH instability leading to growth inhibition in



Figure 3. Leaves of common bean in a greenhouse during authentication experiment (a, b, c and d).

Table 3. Population of bacteria nodulating common bean in different land use systems in Kakamega.

	Land use systems			
Soil characteristics	Maize	Fallow	Bean	Napier
Bacterial population	7.8×10^1	1.98×10^2	4.102×10^3	3.9×10^1
pH	5.12	5.01	4.98	5.4
EC (dS/m)	0.3	0.7	0.7	0.4
Total N (%)	0.35	0.18	0.24	0.31
Organic Carbon (%)	2.6	1.58	2.66	2.8
K(cmol/kg)	1.75	0.75	0.93	1.2
Na (cmol/kg)	0.6	0.5	0.7	0.8
Mg (cmol/kg)	1.19	1.01	1.39	1.53
Ca (cmol/kg)	2.74	2.84	3.08	3.7
Al (cmol/kg)	2.8	1.9	2.6	2.5
Zn (ppm)	7.2	4.5	7.6	20
Cu (ppm)	5.6	5.4	5.1	5.6
Fe (ppm)	16.1	13.3	10.1	24.6
Mn (ppm)	72.4	43.3	75.2	91.1
P (ppm)	24	6	28	35
Soil texture	Clay	Clay	Clay	Clay

low pH soils (Lin et al. 2012). However, other authors have isolated acid-tolerant BNB species with pH tolerance facilitated by proton exclusion, increased cytoplasmic buffering and acid-shock response mechanisms (Bhat et al. 2015). The occurrence of high populations of BNB in most farms in Kisumu (Table 2) could have resulted from the light sandy soil texture. Light sandy soil with pH above 6.0 promotes the proliferation and survival of root nodule bacteria in most cropping systems (Martyniuk and Oron 2008). High nitrogen levels in soils from maize and nappier farms in Kakamega contributed to the low population of BNB. Reports have shown that N levels delay symbiosis process and multiplication of free-living BNB (Gage 2004). The isolation of BNB in soils from fallow and nappier land-use systems is an indication that these bacteria are present

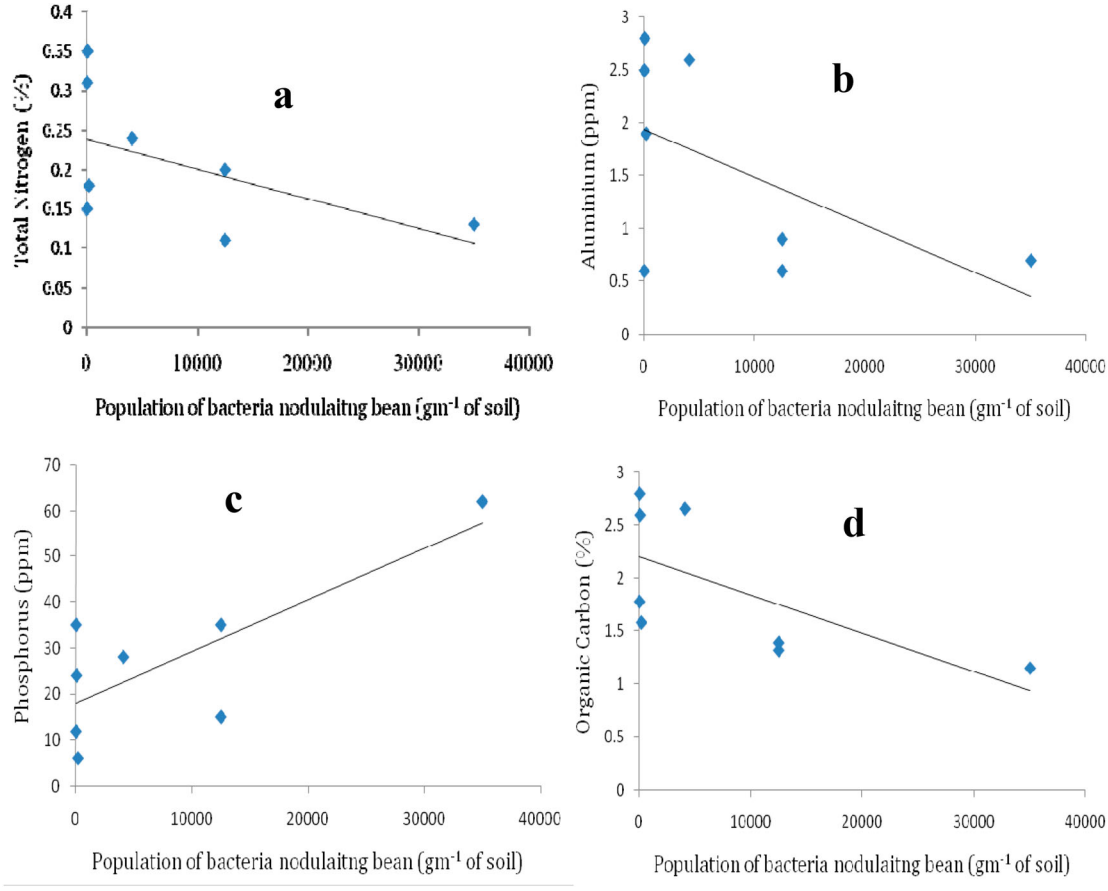


Figure 2. Effect of selected soil characteristics on the population of soil BNB.

in soils independent of the host crop or deliberate inoculation. The study further demonstrated that the type of crop grown has no influence on the population of bacteria.

Differences in the morphological and characteristics observed in isolates (Figure 1) are an indication of their diversity. Other studies have reported a high diversity of the bacterial isolates from nodules sampled from plants inoculated with soil dilution grown in a greenhouse (Alberton et al. 2006; Kawaka et al. 2014). Absorption of Congo red dye has been used by many authors to distinguish BNB from other root nodule bacteria in leguminous plants (Abere et al. 2009).

Typical growth characteristics of the isolates on yeast extract mannitol agar (YEMA) have been reported for BNB (Somasegaran and Hoben 1994). Turning of YEMA containing BTB into moderately yellow to deep yellow colour by the isolates is a typical colour change for fast-growing and acid-producing bacteria. Fast-growing bacteria have a shorter time of establishment in soil and are good candidates for the development of inocula for local environmental conditions (Werner and Newton 2006). The cream yellow, milky white and cream white colony colours that were either opaque or translucent are cultural characteristics for BNB (Somasegaran and Hoben 1994). The production of surface polysaccharides by the isolates could be an adaptive feature by the bacteria against temperature, salinity and pH fluctuations in the soil (de carvalho and Fernandes 2010; Miranda-Ríos et al. 2015).

In Figure 2(a–d), our results show correlation between selected soil characteristics and bacterial population in soil. For example, the study demonstrated a significant positive correlation between bacterial population and soil Phosphorus (P). Soil P is an important nutrient that is required for the growth and development of BNB. On the other hand, soil N, OC and Al showed a negative relationship with the occurrence of BNB in different farms.

The initiation of nodulation by the isolates on re-inoculation on a host crop grown on a sterile vermiculite is a confirmatory test for BNB. Several authors have reported that no bacterial isolate is regarded as BNB before it is confirmed through plant infection test on an appropriate host crop (Brockwell 1980; Howieson and Brockwell 2005; Weir 2006). Control bean plants grown with and without N supplementation failed to form nodules. Failure to nodulate on a host crop by the isolates confirms the absence of external contamination during the experiment. This authentication method is widely accepted as a standard for testing nodulation under greenhouse conditions. The pink nodules and had dark-green leaves observed in inoculated plants and yellow leaves of uninoculated (Figure 3) are similar to the findings reported by Chouhan et al. (2008). Typical colour change is an indication of effective symbiotic leghaemoglobin content in the nodules required for N fixation in root nodules (Chouhan et al. 2008; Kawaka et al. 2018b).

In conclusion, our study demonstrates that land-use systems had no influence on the soil characteristics and the variation observed could be due to individual farm management practices. The population levels of bacteria nodulating common bean varied in soils of western Kenya and were independent of the crops grown in the farms. However, selected soil parameters such as pH, N, Al and OC negatively affected the population of nodule bacteria in the soil, whereas P increased their numbers.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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