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Full Length Research Paper

Isolation and identification of endophytic bacteria of bananas (*Musa* spp.) in Kenya and their potential as biofertilizers for sustainable banana production

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This study was conducted with the aim of isolating and identifying endophytic bacteria associated with bananas in Kenya and assessing their functional potentiality as biological fertilizers. Banana material was collected from two different banana cultivars in five different geographical regions and bacteria were isolated using five different isolation media. Whole-cell matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS) analysis was used for microorganism profiling. Protein from the living cells were extracted using the ethanol/formic acid extraction procedure and intact molecular weights of the ionized proteins directly measured and the pattern of the protein molecular weights used as fingerprints. Forty three isolates were selected for partial 16S rRNA gene sequencing. Isolates were characterized on the basis of their *in-vitro* plant growth-promoting activities that included abilities to fix free nitrogen, solubilize phosphates and produce siderophores. The isolates were identified as *Serratia*, *Pseudomonas*, *Rahnella*, *Enterobacter*, *Raoultella*, *Yokenella*, *Bacillus*, *Klebsiella*, *Yersinia* and *Ewingella* species. Siderophore production activity was detected with all the *Pseudomonas* isolates as determined on blue Chrome Azurol S (CAS) agar plates. Twenty seven isolates were observed to solubilize phosphates, with *Rahnella* isolates showing the highest potential as determined on NBRIP growth medium. All the isolates grew on solid nitrogen-source free medium, suggesting their ability to fix nitrogen. In conclusion, endophytic bacteria of bananas in Kenya were isolated and identified, and *Rahnella* and *Pseudomonas* isolates proposed as potential microbial biofertilizers for sustainable banana production in Kenya.

Key words: *Musa* spp., endophytic bacteria, diazotrophes, phosphate-solubilizing microorganisms, siderophores, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS).

INTRODUCTION

In Kenya, banana production is constrained by among others, declining soil fertility (Vanlauwe and Giller, 2006; Okumu, 2008). This is brought about by insufficient application of manure due to cost implications especially for the farmers without livestock, and limited use of inorganic fertilizers, which are expensive and therefore unaffordable for most banana farmers in Kenya. A sustainable complementary approach would be to increase the biological inputs of nutrients by exploitation of microorganisms, which are largely untapped natural resources for plant growth promotion (Thomas and Soly, 2009; Uribe et al., 2010).

Plants are naturally associated with mutualistic microbes that include endophytes. Endophytes are diverse microbes, most commonly fungi and bacteria (Strobel and Daisy, 2003), which spend the entire or part of their life cycle living in internal plant tissues causing no apparent or immediate disease symptoms (Hallmann et al., 1997; Bacon and White, 2000; Long et al., 2008).

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Table 1. Agro-ecological zones and agro-climatic classification of study sites.

Region	Zone belt	Agro-climatic classification	Elevation (m asl)	Soil type	Soil pH	Annual mean rainfall (mm)	Annual mean temperature (°C)
Kisii	UM1	I - Humid	1780-1880	ando-humic NITISOLS	5.1 – 6.9	1605	19.4
Meru	UM2	II - Sub-humid	1370-1750	humic NITISOLS	5.9 - 7.37	1083	20.7
Embu	UM1	I - Humid	1430-1750	ando-humic NITISOLS	4.46 - 6.6	1091	21.4
Maragua	UM3	III - Semi-humid	1300-1380	eutric NITISOLS	4.79 - 7.5	1074	20.5
Juja	UM4	IV - Semi-humid to semi-arid	1530-1542	rhodic FERRALSOLS	6.6 - 7.65	1074	20.5

Adopted and modified from World Bank Climate Change Knowledge Portal 2.0 and Niels (2006). Key: UM – Upper midland. Numbers 1 to 4 in the second column correspond to the agro-climatic classification I to IV in the third column.

Endophytes are increasingly gaining scientific and commercial interest because of their potential to improve plant quality and growth and their close association with internal tissues of host plant (Carroll, 1992; Schulz et al., 1999). Endophytes are of agronomic interest in that they can enhance plant growth in non-leguminous crops and improve their nutrition through nitrogen fixation, phosphate solubilization or iron chelation (siderophores production) (Dobereiner and Baldani, 1998; Sturz et al., 2000; Boddey et al., 2003; Iniguez et al., 2004; Ryan et al., 2008; Uribe et al., 2010).

According to Senthilkumar et al. (2011), of the roughly 300,000 plant species found on earth, each plant is a host to one or more endophytes, however only few plants have been completely studied in regard to their endophytic biology. As a result, the opportunity to find novel and unique endophytic microbes among numerous plants in different ecosystems is considerable. In bananas, genera and species identified have included: *Azospirillum brasilense* and *Azospirillum amazonense* (Weber et al., 1999), *Bacillus* spp. (Harish et al., 2008), *Burkholderia* spp. (Weber et al., 1999; Ting et al., 2008), *Citrobacter* spp. (Martínez et al., 2003), *Enterobacter* spp. (Martínez et al., 2003), *Herbaspirillum* spp. (Weber et al., 1999, 2001), *Klebsiella* spp. (Martínez et al., 2003; Rosenblueth et al., 2004), *Pseudomonas* spp. (Harish et al., 2008; Ting et al., 2008), *Rhizobium* spp. (Martínez et al., 2003) and *Serratia* spp. (Ting et al., 2008). However, to the authors' knowledge, endophytic bacteria of bananas in Kenya have not been isolated or identified. Thus, there is no information on their diversity and their functional potentiality in regard to banana growth and nutrition.

Imported microbial inoculants could be used for banana production in Kenya but because of the fitness challenge, there is need to isolate domestic bacteria from bananas in our practical farm fields and assess their functional potentiality as biological fertilizers. It is in view of this that this study was conducted with the aim of isolating and identifying endophytic bacteria associated with bananas in Kenya and evaluating their functional potentiality as biological fertilizers.

MATERIALS AND METHODS

Isolation and profiling

Sample collection

Roots(r), corms (c) and stems (s) of bananas belonging to two different banana cultivars (*Musa* AAA – Cavendish (V1) and *Musa* AAB – plantain (V2)) were collected from five different banana growing regions in Kenya; Juja (J) and Maragua (M) in February 2009, Embu (E) and Meru (ME) in March 2009 and Kisii (K) in January 2010. Study material was sampled from five sites per region except in Juja where the sites were three. Agro-ecological zones and agro-climatic classification of the five study regions are described in Table 1. Collected samples were transported in cool boxes and stored in refrigerator for further processing.

Surface sterilization

The banana samples were thoroughly washed in running tap water. They were then surface-sterilized using 70% ethanol for 2 min and immersed in 150 ml of 1.5% sodium hypochlorite plus a few drops of Tween 20 for 5 min with shaking. The samples were then rinsed thoroughly in five changes of sterile distilled water and dried in sterile paper towels.

Isolation and selection

Surface sterilized samples were macerated with a sterile mortar and pestle and then serially diluted in 12.5 mM potassium phosphate buffer at pH 7. To target a wide range of endophytes, five different isolation media were used, that is, LGI solid media (Cavalcante and Dobereiner, 1988), nitrogen-free media - NFb (Dobereiner et al., 1976), MacConkey, Congo Red (Rodriguez, 1982), YEM agar

(Vincent, 1970) and nutrient agar (HIMEDIA, HiMedia Laboratories Pvt. Ltd.). Extensive colony purification was done to attain single strain cultures. Morphological characterization was done on the basis of colony color, appearance, motility and gram staining and based on the colony morphotypes selection of representative isolates was done.

Microorganism profiling

Profiling of isolated bacteria was done using matrix-assisted laser desorption / ionization time of flight mass spectrometry (MALDI-TOF/MS, Bruker Daltonics). The bacterial cells (five colonies) grown on nutrient agar medium were placed into an eppendorf tube and 100 µl water added and mixed carefully. Three hundred microliter ethanol was added and mixed carefully. The mixture was then centrifuged at 15,000 rpm for 2 min at 4°C and supernatant was removed. The pellet was vacuum dried for 10 min to completely remove ethanol. Five microliter 70% formic acid was added to the pellet and mixed well using a vortex. Five microliter acetonitrile was then added and the mixture centrifuged at 15,000 rpm for 2 min at 4°C. The supernatant of 1 µl was transferred to MALDI-plate, allowed to dry and then overlaid with 2 µl matrix solution (saturated solution of α-cyano-4-hydroxycin-namic acid (α-HCCA) in 50% acetonitrile and 2.5% tri-fluoroacetic acid). The samples were analyzed with MALDI-TOF/MS equipped with a 50 Hz nitrogen laser (Ultraflex, Bruker Daltonics). Mass spectra were obtained using a positive linear mode in the range of mass to charge ratio (m/z) 2,000 to 20,000 (Tani et al., 2012).

Bacterial isolates identification

Approximately 1.5 kb 16S rRNA genes of the bacterial isolates were amplified by polymerase chain reaction using Eu8f (5'-AGAGTTTGATCCTGGCTCAG-3') and Eu1492r (5'-GGCTACCTTGTTACGACTT-3') primers (Weisburg et al., 1991). The PCR mixture composed of 0.1 µl Blend Taq Plus DNA polymerase (Toyobo, Japan), 2 µl dNTP (2 mM), 2.5 µl Blend Taq plus DNA polymerase buffer, 1 µl of each primer (12.5 µM), 17.4 µl sterile MilliQ water and 1 µl sample DNA. The thermal program was 2 min at 96°C and then 30 s at 95°C, 30 s at 52°C, 1.5 min at 72°C for 25 cycles, followed by final polymerization for 5 min at 72°C. The amplicons were purified with the DNA Fragment Purification kit (MagExtractor – Toyobo, Japan) and subjected to DNA sequencing according to the manufacture's protocol (BigDye Ready Reaction Mix, Applied Biosystems) using EU8f and EU518r (5'-GTATTACCGCGGCTGCTGG-3') primers. Some selected isolates were subjected to full-length sequencing using EU8f, Eu1492r, Eu518r, Eu1093r (5'-TTGCGCTCGTTGCGGGACT-3'), Eu803r (5'-CATCGTTTACGGCGTGGAC-3'), Eu1389r (5'-ACGGGCGGTGTGTACAAG-3') and Eu1092f (5'-AAGTCCCGCAACGAGCGCA-3') primers. Obtained sequences were processed using the Staden Package software (Bonfield et al., 1995) and BIOEDIT sequence alignment editor. Assembled sequences were then analyzed at EzTaxon-e database (Kim et al., 2012).

Screening for nitrogen fixation ability

To determine the isolates' ability to fix atmospheric nitrogen, qualitative screening of growth was done on solid N-free medium (1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 1 g $CaCO_3$, 0.2 g NaCl, 5 mg $FeSO_4.7H_2O$, 10 g glucose, 5 mg $NaMoO_4$ per litre, and 1.5% agar at pH 7.0). Growth on the N-free medium was used as the growth parameter and data were taken four and ten days post inoculation.

Screening for phosphate solubilization

Qualitative screening for phosphate solubilizing isolates was done using the National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal, 1999). The medium composed of 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2$, 0.25 g $MgSO_4$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ per litre and 1.5% agar at pH 7. Screening of phosphate solubilizers was based on formation of visible halo zones on agar plates, which is as a result of organism's production of organic acids into the surrounding medium. The halo size was used as a measure of relative efficiency of the isolates. The halo and colony diameters were measured at 13 and 21 days post inoculation. Halo size was calculated by subtracting colony diameter from the total diameter.

Screening for siderophore production

Siderophore production was detected using the Chrome Azurol S (CAS) agar plates as described by Schwyn and Neilands (1987). Orange halos around colonies on blue agar indicated siderophore excretion. Data were taken four and seven days post inoculation.

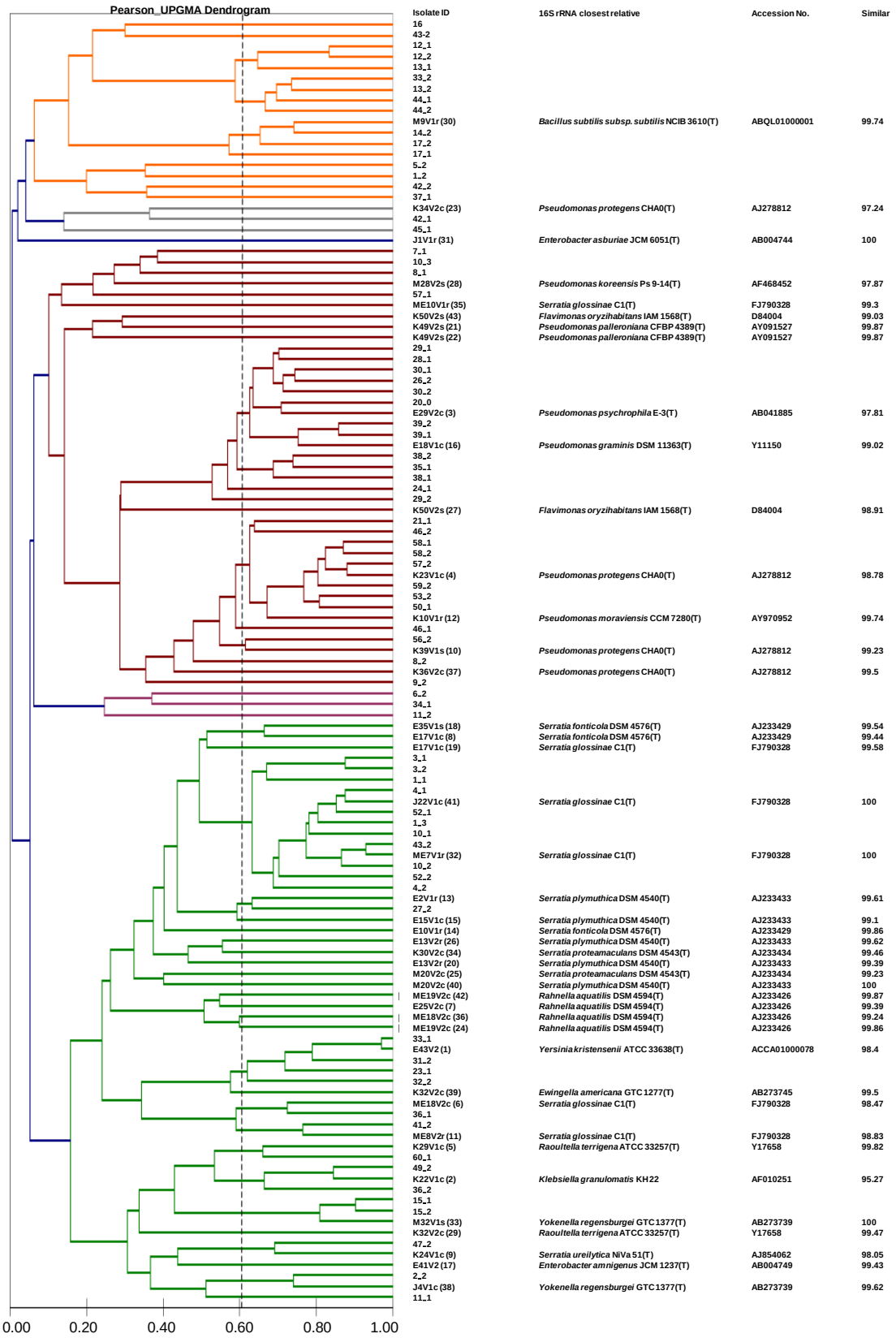
RESULTS

Isolation and profiling of bacterial isolates

Isolation of bacteria from surface sterilized plant material usually allows recovery of putative endophytic bacteria. With five different isolation media, a total of 2,717 isolates were initially obtained and 214 representative isolates selected on the basis of their colony morphotypes. The selected isolates were then profiled using MALDI-TOF/MS. Protein from the living cells were extracted, and intact molecular weights of the ionized proteins directly measured. The pattern of the protein molecular weights were used as fingerprints. Isolates that showed similarity higher than 60% were regarded as same species due to high mass spectrometric pattern similarity, and those showing less than that were regarded as different species. The profiling resulted into 53 clusters, which based on partial 16S rRNA gene sequencing could be grouped into three families *Bacillaceae*, *Pseudomonadaceae* and *Enterobacteriaceae* (Figure 1).

Identification of bacterial isolates

Partial sequencing of the 16S rRNA gene grouped 43 representative bacterial isolates into three families that is, *Enterobacteriaceae, Pseudomonadaceae* and *Bacillaceae* (Table 2). The family *Enterobacteriaceae* was the most diverse with 8 genera namely *Serratia* (17 isolates), *Rahnella* (4 isolates), *Enterobacter* (2 isolates), *Yokenella* (2 isolates), *Raoultella* (2 isolates), *Klebsiella* (1 isolate), *Yersinia* (1 isolate) and *Ewingella* (1 isolate). Both the *Pseudomonadaceae* and *Bacillaceae* families were represented by only one genus, that is, *Pseudomonas* (12 isolates) and *Bacillus* (1 isolate), respectively. *Serratia* and *Pseudomonas* species were



Isolate ID	16S rRNA closest relative	Accession No.	Similar
16			
43-2			
12_1			
12_2			
13_1			
33_2			
13_2			
44_1			
44_2			
M9V1r (30)	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)	ABQL01000001	99.74
14_2			
17_2			
17_1			
5_2			
1_2			
42_2			
37_1			
K34V2c (23)	*Pseudomonas protegens* CHA0(T)	AJ278812	97.24
42_1			
45_1			
J1V1r (31)	*Enterobacter asburiae* JCM 6051(T)	AB004744	100
7_1			
10_3			
8_1			
M28V2s (28)	*Pseudomonas koreensis* Ps 9-14(T)	AF468452	97.87
57_1			
ME10V1r (35)	*Serratia glossinae* C1(T)	FJ790328	99.3
K50V2s (43)	*Flavimonas oryzihabitans* IAM 1568(T)	D84004	99.03
K49V2s (21)	*Pseudomonas palleroniana* CFBP 4389(T)	AY091527	99.87
K49V2s (22)	*Pseudomonas palleroniana* CFBP 4389(T)	AY091527	99.87
29_1			
28_1			
30_1			
26_2			
30_2			
20_0			
E29V2c (3)	*Pseudomonas psychrophila* E-3(T)	AB041885	97.81
39_2			
39_1			
E18V1c (16)	*Pseudomonas graminis* DSM 11363(T)	Y11150	99.02
38_2			
35_1			
38_1			
24_1			
29_2			
K50V2s (27)	*Flavimonas oryzihabitans* IAM 1568(T)	D84004	98.91
21_1			
46_2			
58_1			
58_2			
57_2			
K23V1c (4)	*Pseudomonas protegens* CHA0(T)	AJ278812	98.78
59_2			
53_2			
50_1			
K10V1r (12)	*Pseudomonas moraviensis* CCM 7280(T)	AY970952	99.74
46_1			
56_2			
K39V1s (10)	*Pseudomonas protegens* CHA0(T)	AJ278812	99.23
8_2			
K36V2c (37)	*Pseudomonas protegens* CHA0(T)	AJ278812	99.5
9_2			
6_2			
34_1			
11_2			
E35V1s (18)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.54
E17V1c (8)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.44
E17V1c (19)	*Serratia glossinae* C1(T)	FJ790328	99.58
3_1			
3_2			
1_1			
4_1			
J22V1c (41)	*Serratia glossinae* C1(T)	FJ790328	100
52_1			
1_3			
10_1			
43_2			
ME7V1r (32)	*Serratia glossinae* C1(T)	FJ790328	100
10_2			
52_2			
4_2			
E2V1r (13)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.61
27_2			
E15V1c (15)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.1
E10V1r (14)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.86
E13V2r (26)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.62
K30V2c (34)	*Serratia proteamaculans* DSM 4543(T)	AJ233434	99.46
E13V2r (20)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.39
M20V2c (25)	*Serratia proteamaculans* DSM 4543(T)	AJ233434	99.23
M20V2c (40)	*Serratia plymuthica* DSM 4540(T)	AJ233433	100
ME19V2c (42)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.87
E25V2c (7)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.39
ME18V2c (36)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.24
ME19V2c (24)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.86
33_1			
E43V2 (1)	*Yersinia kristensenii* ATCC 33638(T)	ACCA01000078	98.4
31_2			
23_1			
32_2			
K32V2c (39)	*Ewingella americana* GTC 1277(T)	AB273745	99.5
ME18V2c (6)	*Serratia glossinae* C1(T)	FJ790328	98.47
36_1			
41_2			
ME8V2r (11)	*Serratia glossinae* C1(T)	FJ790328	98.83
K29V1c (5)	*Raoultella terrigena* ATCC 33257(T)	Y17658	99.82
60_1			
49_2			
K22V1c (2)	*Klebsiella granulomatis* KH22	AF010251	95.27
36_2			
15_1			
15_2			
M32V1s (33)	*Yokenella regensburgei* GTC 1377(T)	AB273739	100
K32V2c (29)	*Raoultella terrigena* ATCC 33257(T)	Y17658	99.47
47_2			
K24V1c (9)	*Serratia ureilytica* NiVa 51(T)	AJ854062	98.05
E41V2 (17)	*Enterobacter amnigenus* JCM 1237(T)	AB004749	99.43
2_2			
J4V1c (38)	*Yokenella regensburgei* GTC 1377(T)	AB273739	99.62
11_1			

Figure 1. Microorganism profiling using MALDI-TOF/MS. The profiling resulted into 53 clusters, which based on partial 16S rRNA gene sequencing, could be grouped into three families namely Bacillaceae (orange colour), Pseudomonadaceae (brown colour) and Enterobacteriaceae (green colour).

Table 2. Probable identification of 43 endophytic bacteria isolated from banana plants in Kenya in 2009/2010 based on partial sequencing of 16S rRNA gene.

Isolate ID	16S rRNA closest relative	Accession no.	Similarity
M9V1r (30)	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)	ABQL01000001	99.74
K34V2c (23)	*Pseudomonas protegens* CHA0(T)	AJ278812	97.24
J1V1r (31)	*Enterobacter asburiae* JCM 6051(T)	AB004744	100.00
M28V2s (28)	*Pseudomonas koreensis* Ps 9-14(T)	AF468452	97.87
ME10V1r (35)	*Serratia glossinae* C1(T)	FJ790328	99.30
K50V2s (43)	*Flavimonas oryzihabitans* IAM 1568(T)	D84004	99.03
K49V2s (21)	*Pseudomonas palleroniana* CFBP 4389(T)	AY091527	99.87
K49V2s (22)	*Pseudomonas palleroniana* CFBP 4389(T)	AY091527	99.87
E29V2c (3)	*Pseudomonas psychrophila* E-3(T)	AB041885	97.81
E18V1c (16)	*Pseudomonas graminis* DSM 11363(T)	Y11150	99.02
K50V2s (27)	*Flavimonas oryzihabitans* IAM 1568(T)	D84004	98.91
K23V1c (4)	*Pseudomonas protegens* CHA0(T)	AJ278812	98.78
K10V1r (12)	*Pseudomonas moraviensis* CCM 7280(T)	AY970952	99.74
K39V1s (10)	*Pseudomonas protegens* CHA0(T)	AJ278812	99.23
K36V2c (37)	*Pseudomonas protegens* CHA0(T)	AJ278812	99.50
E35V1s (18)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.54
E17V1c (8)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.44
E17V1c (19)	*Serratia glossinae* C1(T)	FJ790328	99.58
J22V1c (41)	*Serratia glossinae* C1(T)	FJ790328	100.00
ME7V1r (32)	*Serratia glossinae* C1(T)	FJ790328	100.00
E2V1r (13)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.61
E15V1c (15)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.10
E10V1r (14)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.86
E13V2r (26)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.62
K30V2c (34)	*Serratia proteamaculans* DSM 4543(T)	AJ233434	99.46
E13V2r (20)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.39
M20V2c (25)	*Serratia proteamaculans* DSM 4543(T)	AJ233434	99.23
M20V2c (40)	*Serratia plymuthica* DSM 4540(T)	AJ233433	100.00
ME19V2c (42)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.87
E25V2c (7)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.39
ME18V2c (36)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.24
ME19V2c (24)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.86
E43V2 (1)	*Yersinia kristensenii* ATCC 33638(T)	ACCA01000078	98.40
K32V2c (39)	*Ewingella americana* GTC 1277(T)	AB273745	99.50
ME18V2c (6)	*Serratia glossinae* C1(T)	FJ790328	98.47
ME8V2r (11)	*Serratia glossinae* C1(T)	FJ790328	98.83
K29V1c (5)	*Raoultella terrigena* ATCC 33257(T)	Y17658	99.82
K22V1c (2)	*Klebsiella granulomatis* KH 22	AF010251	95.27
M32V1s (33)	*Yokenella regensburgei* GTC 1377(T)	AB273739	100.00
K32V2c (29)	*Raoultella terrigena* ATCC 33257(T)	Y17658	99.47
K24V1c (9)	*Serratia ureilytica* NiVa 51(T)	AJ854062	98.05
E41V2 (17)	*Enterobacter amnigenus* JCM 1237(T)	AB004749	99.43
J4V1c (38)	*Yokenella regensburgei* GTC 1377(T)	AB273739	99.62

the most abundant with 17 isolates and 12 isolates, correspondingly. Full-length sequencing allowed identification of isolates M9V1r (30), J1V1r (31), K32V2c (39), ME19V2c (42) and K50V2s (43) as *Bacillus subtilis subsp. inaquosorum*, *Enterobacter ludwigii*, *Ewingella americana*, *Rahnella aquatilis* and *Flavimonas oryzihabitans* respectively. The sequence data generated have been deposited with the NCBI GenBank under

accession numbers AB675632 to AB675636.

Functional potentiality of bacterial isolates as biofertilizers

The bacterial isolates were qualitatively screened for abilities to fix free nitrogen, solubilize phosphates and produce siderophores *in vitro* (Table 3). All the 43 isolates showed growth on solid N-free medium, suggesting their ability to fix atmospheric nitrogen. Phosphate solubilization activity was observed with 27 isolates by the formation of visible dissolution halos (1.5 to 17 mm) on the NBRIP medium agar plates at 21 days post inoculation. These included *Pseudomonas* (11 isolates), *Serratia* (9 isolates), *Rahnella* (3 isolates), *Enterobacter* (1 isolate), *Yersinia* (1 isolate), *Yokenella* (1 isolate) and *Ewingella* (1 isolate). Isolates ME19V2c (42), ME19V2c (24), and ME18V2c (36) all *R. aquatilis* had the largest halo size of 17, 16 and 12 mm, respectively. Siderophore production activity was detected with all the 12 *Pseudomonas* isolates by the formation of distinct orange halos on the Chrome Azurol S (CAS) agar plates. Isolates K50V2s (43) and K50V2s (27) both *F. oryzihabitans* had the largest orange halos. Isolate M9V1r (30) identified as *B. subtilis subsp. subtilis* did not show growth on the CAS agar plates. *B. subtilis subsp. subtilis* is gram-positive and gram-positive bacteria are reported to be quite sensitive to HDTMA detergent used in the siderophore medium (Schwyn and Neilands, 1987). HDTMA may therefore have become toxic to *B. subtilis subsp. subtilis* causing it not to grow.

DISCUSSION

Isolation and identification of bacterial isolates

The composition of endophytic bacteria isolated from bananas in Kenya in 2009/2010 consisted of ten genera, which were identified as *Serratia*, *Pseudomonas, Rahnella*, *Enterobacter*, *Yokenella*, *Raoultella*, *Klebsiella*, *Yersinia, Ewingella* and *Bacillus*. To the authors' knowledge, *Rahnella, Yokenella*, *Raoultella, Yersinia* and *Ewingella* species have not been reported in association with bananas suggesting a larger richness of endophytic bacterial species associated with bananas than has so far been reported.

Previously reported endophytic bacteria associated with bananas include *A. brasilense* and *A. amazonense* (Weber et al., 1999), *Bacillus* spp. (Harish et al., 2008), *Burkholderia* spp. (Weber et al., 1999; Ting et al., 2008), *Citrobacter* spp. (Martínez et al., 2003), *Enterobacter* spp. (Martínez et al., 2003), *Herbaspirillum* spp. (Weber et al., 1999, 2001), *Klebsiella* spp. (Martínez et al., 2003; Rosenblueth et al., 2004), *Pseudomonas* spp. (Harish et al., 2008; Ting et al., 2008), *Rhizobium* spp. (Martínez et al., 2003) and *Serratia* spp. (Ting et al., 2008). The diverse endophytic bacteria population could be explained by the different agro ecological conditions of study sites (Table 1) and the unique East African highland banana cultivars especially the cooking banana variety (*Musa* AAB) whose endophytic biology has not been studied much. Significant variations in the populations of both indigenous and introduced endophytes have been attributed to plant source, plant age, tissue type, time of sampling, and environment (Zinniel et al., 2002).

Functional potentiality of bacterial isolates as biofertilizers

Screening for nitrogen fixation ability was qualitatively done on solid N-free medium and all the isolates tested showed growth on the medium. This may be explained by the fact that two of the isolation media used were nitrogen free (LGI and NFb). Growth of the bacterial isolates on N-free medium was suggestive of the isolates ability to fix atmospheric nitrogen. The commonly reported endophytic diazotrophic bacteria associated with bananas include *Azospirillum*, *Burkholderia, Citrobacter*, *Enterobacter, Herbaspirillum, Klebsiella* and *Rhizobium* species (Weber et al., 1999; Weber et al., 2001, 2007; Martinez et al., 2003). For a more precise determination of organisms' N fixation ability, the authors recommend a molecular approach, which is based on PCR amplification of the *nifH* gene that codes for the enzyme nitrogenase reductase (Burgmann et al., 2004).

Screening of phosphate solubilizers on NBRIP medium agar plates is based on formation of visible halo zones on the agar plates as a result of organism's production of organic acids into the surrounding medium (Nautiyal, 1999). The halo size is used as a measure of relative efficiency of the isolates. *R. aquatilis* isolates formed the largest visible dissolution halos and were therefore considered the most efficient phosphate solubilizers. These findings are consistent with those of Kim et al. (1998) who reported *R. aquatilis* having genes that are necessary for mineral phosphate solubilization. Vyas et al. (2010) also identified a phosphate-solubilizing bacterial strain from *Hippophae rhamnoides* rhizosphere as *Rhanella* spp. The use of phosphate solubilizing bacteria as inoculants increases P uptake by the plant and the crop yield as well (Rodriguez and Fraga, 1999).

A universal method to detect and determine siderophores has been developed based on their high affinity for iron (III) (Schwyn and Neilands, 1987). A highly coloured complex of chrome azurol S, iron (III), and hexadecyltrimethylammonium bromide is used as the indicator. When a strong chelator like siderophore removes the iron from the highly coloured iron dye complex, its colour turns from blue to orange. Orange halos around bacterial colonies on blue Chrome Azurol S

Table 3. Qualitative screening for bacterial isolates' ability to fix free nitrogen, solubilize phosphates and produce siderophores *in vitro*.

Isolate ID	16S rRNA closest relative	N-fixation	Phosphates solubilization	Siderophores production
M9V1r (30)	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)	+	-	-
K34V2c (23)	*Pseudomonas protegens* CHA0(T)	+	+	+
J1V1r (31)	*Enterobacter asburiae* JCM 6051(T)	+	+	+
M28V2s (28)	*Pseudomonas koreensis* Ps 9-14(T)	+	+	+
ME10V1r (35)	*Serratia glossinae* C1(T)	+	+	-
K50V2s (43)	*Flavimonas oryzihabitans* IAM 1568(T)	+	+	++
K49V2s (21)	*Pseudomonas palleroniana* CFBP 4389(T)	+	+	+
K49V2s (22)	*Pseudomonas palleroniana* CFBP 4389(T)	+	+	+
E29V2c (3)	*Pseudomonas psychrophila* E-3(T)	+	+	+
E18V1c (16)	*Pseudomonas graminis* DSM 11363(T)	+	+	+
K50V2s (27)	*Flavimonas oryzihabitans* IAM 1568(T)	+	-	++
K23V1c (4)	*Pseudomonas protegens* CHA0(T)	+	+	+
K10V1r (12)	*Pseudomonas moraviensis* CCM 7280(T)	+	+	+
K39V1s (10)	*Pseudomonas protegens* CHA0(T)	+	+	+
K36V2c (37)	*Pseudomonas protegens* CHA0(T)	+	+	+
E35V1s (18)	*Serratia fonticola* DSM 4576(T)	+	+	-
E17V1c (8)	*Serratia fonticola* DSM 4576(T)	+	+	-
E17V1c (19)	*Serratia glossinae* C1(T)	+	+	-
J22V1c (41)	*Serratia glossinae* C1(T)	+	+	-
ME7V1r (32)	*Serratia glossinae* C1(T)	+	+	-
E2V1r (13)	*Serratia plymuthica* DSM 4540(T)	+	-	-
E15V1c (15)	*Serratia plymuthica* DSM 4540(T)	+	-	-
E10V1r (14)	*Serratia fonticola* DSM 4576(T)	+	+	-
E13V2r (26)	*Serratia plymuthica* DSM 4540(T)	+	-	-
K30V2c (34)	*Serratia proteamaculans* DSM 4543(T)	+	-	+
E13V2r (20)	*Serratia plymuthica* DSM 4540(T)	+	-	-
M20V2c (25)	*Serratia proteamaculans* DSM 4543(T)	+	-	-
M20V2c (40)	*Serratia plymuthica* DSM 4540(T)	+	-	-
ME19V2c (42)	*Rahnella aquatilis* DSM 4594(T)	+	++	-
E25V2c (7)	*Rahnella aquatilis* DSM 4594(T)	+	-	-
ME18V2c (36)	*Rahnella aquatilis* DSM 4594(T)	+	++	-
ME19V2c (24)	*Rahnella aquatilis* DSM 4594(T)	+	++	-
E43V2 (1)	*Yersinia kristensenii* ATCC 33638(T)	+	+	-
K32V2c (39)	*Ewingella americana* GTC 1277(T)	+	+	-
ME18V2c (6)	*Serratia glossinae* C1(T)	+	+	-
ME8V2r (11)	*Serratia glossinae* C1(T)	+	+	-
K29V1c (5)	*Raoultella terrigena* ATCC 33257(T)	+	-	-
K22V1c (2)	*Klebsiella granulomatis* KH 22	+	-	-
M32V1s (33)	*Yokenella regensburgei* GTC 1377(T)	+	-	-
K32V2c (29)	*Raoultella terrigena* ATCC 33257(T)	+	-	-
K24V1c (9)	*Serratia ureilytica* NiVa 51(T)	+	-	-
E41V2 (17)	*Enterobacter amnigenus* JCM 1237(T)	+	-	-
J4V1c (38)	*Yokenella regensburgei* GTC 1377(T)	+	+	-

Key. Positive (+); strongly positive (++); negative (-)

(CAS) agar are indicative of siderophore excretion. Distinct orange halos were observed with all the 12 *Pseudomonas* isolates with *Flavimonas oryzihabitans* isolates having the largest orange halos. The *Pseudomonas* isolates could therefore be considered high siderophore producers. These findings are similar to

those of Gangwar and Kaur (2009) who reported *Pseudomonas* spp. isolated from ryegrass as high siderophore producer. Siderophores are responsible for the dissolution, chelation and transport of iron (III) into microbial cells (Sharma and Johri, 2003). It has also been shown that Fe chelated by microbial siderophores can also be utilized by plants (Chen et al., 1998). Siderophore-producing bacteria would therefore improve the iron nutrition of plants.

Conclusions

Endophytic bacteria of bananas in Kenya were isolated and identified as *Serratia*, *Pseudomonas, Rahnella*, *Enterobacter*, *Yokenella*, *Raoultella*, *Klebsiella*, *Yersinia, Ewingella* and *Bacillus* species. *R. aquatilis* and *F. oryzihabitans* were the most efficient in phosphate solubilization and siderophore production, respectively and since they also showed ability to fix free nitrogen, they could be proposed as potential biofertilizers in banana production. Greenhouse and field investigations are however recommended for confirmation of this potentiality.

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