ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA OF BANANAS IN KENYA

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Abstract
This study was conducted with the aim of isolating and identifying banana endophytic bacteria on the basis of their potential as biological fertilizer. Banana materials were collected from five different geographical regions to enhance diversity. Isolation of bacteria was done using five (5) different isolation media and the isolates were characterized on the basis of their morphology, biochemical and molecular characteristics. A total of 214 bacterial isolates were obtained and characterized. Microorganism profiling was done using MALDI-TOF/MS and the isolates were clustered into 53 genotypes. Based on their functional characteristics, 43 isolates were selected for 16S rRNA gene sequencing. The 43 strains showed varied levels of positive nitrogenase activity as measured by the acetylene reduction assay and 37 strains were observed to solubilize phosphates by the formation of visible dissolution halos on agar plates (NBRIP medium). Siderophore production of the isolates was determined using Chrome Azurol S (CAS) agar plates and all the isolates were observed to be positive for siderophore production with 3 strains showing distinctively high level of production. Using the 16S rRNA gene sequencing, the 43 strains were identified as *Serratia spp*. (17 strains), *Pseudomonas spp*. (12 strains), *Enterobacter spp*. (4 strains), *Rahnella spp*. (4 strains), *Raoultella spp*. (2 strains), *Bacillus spp*. (1 strain), *Klebsiella spp*. (1 strain), *Yersinia spp*. (1 strain) and *Ewingella spp*. (1 strain). In conclusion, banana endophytic bacteria were successfully isolated and identified, and some of the isolates showed potential of being biological fertilizers. However, greenhouse and field investigations are necessary to confirm this potentiality.

Key words: Endophytic bacteria, diazotrophic endophytes, phosphate-solubilizing microorganisms (PSM), banana

1.0 Introduction

Plants are naturally associated with mutualistic microorganisms that include endophytes. These are diverse microbes, including fungi and bacteria. They spend the entire or part of their life cycle living inside the plant causing no apparent symptoms of disease (Hallmann *et al*., 1997 and Long *et al.*, 2008). Although the interaction between endophytic bacteria and host plants has not been fully understood, it is well established that some of these interactions are beneficial to the plant (Long *et al.*, 2008; Rosenblueth and Martinez-Romero, 2006). The endophytes' close association with internal tissues of host plant has increasingly gained them scientific and commercial interest due to their potential to improve plant quality and growth (Carroll, 1992; Schulz *et al.*, 1998; Schulz *et al.*, 1999). Endophytic bacteria 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 (Long *et al*., 2008).

Banana belongs to the family *Musaceae* in genus *musa* (Rossel, 2001). It is an excellent source of nutrients with a high consumer demand worldwide. It is the third world's most important starchy staple after cassava and sweet potato, and increased trade in local, regional and international markets has made it an important cash crop, and in some cases the only source of income for rural populations (Frison and Sharrock, 2001). In Kenya, area under banana production is about 84,000ha (MoA, 2006) with an estimated yield of 10 tonnes per hectare as opposed to a potential yield of over 40 tonnes per hectare (Karamura *et al*., 1998). Banana production in Kenya is constrained by, among others, declining soil fertility (Vanlauwe *et al*., 2005). 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. Soil erosion and export of nutrients in harvested banana parts e.g. bunches and pseudostems also contribute to the declining soil fertility. This problem can be addressed using chemical fertilizers. However, these are expensive and out of reach for most resource-poor farmers, who constitute the vast majority of banana farmers in Kenya.

Sustainable production of bananas in Kenya will therefore mean increasing their yield without the mass use of chemical fertilizers. It thus becomes of paramount importance to consider biological alternatives, which are cost effective and environment friendly. Some of these biological alternatives are the endophytic bacteria, which have beneficial characteristics to the cultivation of plants (Long *et al*., 2008, Ting *et al*., 2008). In Kenya, to the authors' knowledge, endophytic bacteria of banana have not been isolated or identified. Thus, there is no information on how they contribute, either individually or in mixtures, to banana growth and nutrition. A positive endophyte-banana association would reduce the use of agricultural inputs, such as fertilizer and pesticides, consequently saving on costs and reducing environment pollution.

The aim of this study was to isolate and identify endophytic bacteria from banana plants growing in Kenya and to determine their capacity to fix nitrogen, solubilize phosphates and produce siderophore *in-vitro*.

2.0 Materials and Methods

2.1 Sample Collection

Banana samples (roots, corm and stem) of two different banana cultivar groups (AAA - Cavendish & AAB - plantain) were collected from five different banana growing regions in Kenya namely Embu, Juja, Kisii, Maragua and Meru.

2.2 Surface sterilization

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

2.3 Isolation and Characterisation

Surface sterilised samples were macerated with a sterile mortar and pestle and then serially diluted in 12.5 mm potassium phosphate buffer at pH 7.1 (Zinniel *et al*., 2002). To target a wide range of endophytes, five different isolation media were used i.e. LGI solid media (Cavalcante and Dobereiner, 1988), nitrogen-free media (Dobereiner *et al.*, 1976), MacConkey (Rodriguez Caceres, 1982), YEM agar (Vincent, 1982) and nutrient agar. Morphological characterization was done on the basis of colony color, appearance, motility and gram staining. Biochemical characterization included Potassium hydroxide (KOH), catalase production, starch hydrolysis, gelatin hydrolysis, growth in 10% and 7% NaCl, citrate utilization, methyl red, urease and TSI agar tests.

2.4 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 ethanol/formic acid extraction procedure was used. Cluster dendrogram analysis was done and a MALDI tree constructed.

2.5 Screening for Nitrogenase Activity

The nitrogenase activity of the isolates was measured using the acetylene reduction assay (ARA). Ethylene production was determined on a Shimadzu Gas Chromatograph as described by Rogel *et al*., (2001).

Screening for phosphate solubilization: Qualitative screening of phosphate solubiliSation was done using the NBRIP growth medium (Nautiyal, 1999).

2.6 Detection of Siderophores

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.

2.7 16S rRNA Gene Sequencing

Partial 16S rRNA gene amplification was done on the isolates that showed some functional potentiality using Eu8f AGAGTTTGATCCTGGCTCAG & Eu1492r GGCTACCTTGTTACGACTT primers. The PCR mixture composed of 0.1 µl Blend Taq Plus DNA polymerase, 2 µl dNTP, 2.5 µl Blend Taq Plus DNA polymerase buffer, 1 µl of each primer (12.5 µmol/ µl), 17.4 µl sterile MilliQ water and 1 µl sample DNA. The amplified 1.5 kb-product was purified using the MagExtractor and sequenced using EU8f AGAGTTTGATCCTGGCTCAG and EU518r GTATTACCGCGGCTGCTGG primers. The reaction mixture composed of 1.5 µl BigDye Ready Reaction Mix, 2.5 µl sequencing buffer (5x), 2 µl of 0.9 pmol/µl of each primer (EU8f and EU 518r [separately]), 3.5 µl sterile MilliQ water and 0.5 µl template DNA (Mag Extractor - purified PCR product).

2.8 Processing and Analysing of DNA Sequences

Obtained sequences were processed using the Staden Package software and BIOEDIT sequence alignment editor. Assembled sequences were analyzed at the Ribosomal Database Project site (http://rdp.cme.msu.edu/). Phylogenetic analysis was done using the Molecular Evolutionary Genetics Analysis (MEGA) software (http://www.megasoftware.net/).

3.0 Results

3.1 Isolation and Selection

To target a wide range of endophytes, five (5) different isolating media were used. With this range of isolating media, a total of 2,717 isolates were initially obtained. On the basis of colony morphotypes, 214 representative isolates were selected. The selected isolates were further profiled using MALDI-TOR/MS (Figure 1). From the clusters obtained and on the basis of their functional characteristics, 43 isolates were selected for further characterization and identification.

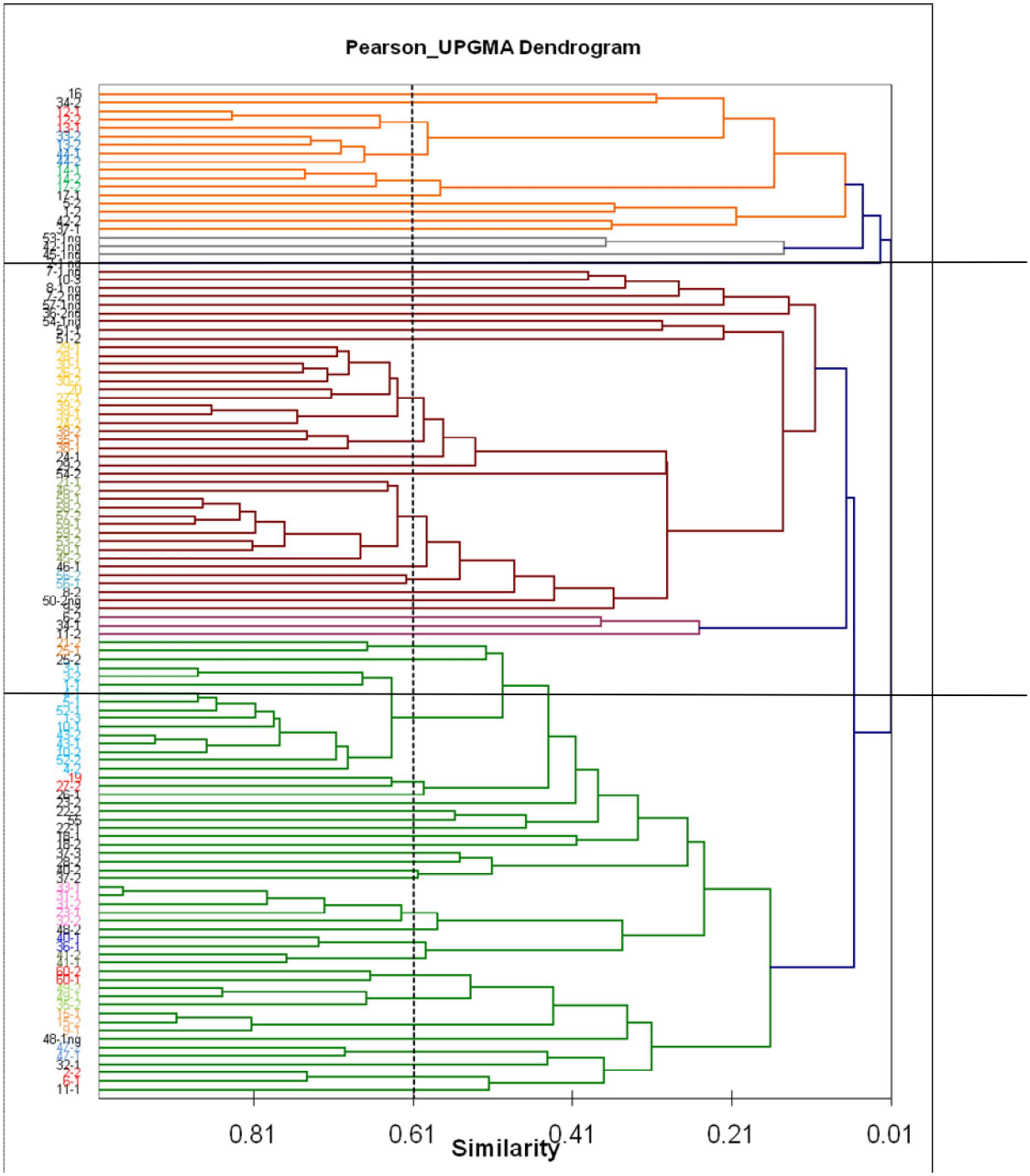


Figure 1: MALDI tree - microorganism profiling was done using MALDI-TOF/MS and isolates that showed similarity of up to 61% were clustered together. The isolates were clustered into 53 genotypes, which based on partial 16S rRNA gene sequencing could be grouped into three (3) families (Bacillaceae at the top, Pseudomonadaceae at the middle and Enterobacteriaceae at the bottom)

3.2 16S rRNA Gene Sequencing

Phylogenetic analysis based on partial 16S rRNA gene sequencing allowed identification of the 43 selected isolates as *Serratia* spp. (17 strains), *Pseudomonas* spp. (12 strains), *Enterobacter* spp. (4 strains), *Rahnella* spp. (4 strains), *Raoultella* spp. (2 strains), *Bacillus* spp. (1 strain), *Klebsiella* spp. (1 strain), *Yersinia* spp. (1 strain) and *Ewingella* spp. (1 strain) (Table 1). The selected endophytic bacteria isolated from banana plants grown in Kenya in 2008/2009 can therefore be grouped into three (3) families namely *Bacillaceae, Enterobacteriaceae* and *Pseudomonadaceae* (Figure 1 and Table 1).

Table 1:Probable identification of 43 endophytic bacteria strains isolated from banana plants grown in Kenya in 2008/2009 based on partial sequencing of 16S rRNA gene. Similarity values with their 16S rDNA closest relatives ranged between 96% and 100%

Strain ID	*16S rDNA closest relative*	*Similarity*	*Strain ID*	*16S rDNA closest relative*	*Similarity*
14-1 (M9)	*Bacillaceae - Bacillus spp. "subtilis" subsp. "subtilis"*	99.7	26-1 (E15)	*Enterobacteriaceae - Serratia spp. "plymuthica"*	99.6
32-1 (E41)	*Enterobacteriaceae - Enterobacter spp. "amnigenus"*	99.4	22-1 (E13)	*Enterobacteriaceae - Serratia spp. "plymuthica"*	99.3
2-1 (J1)	*Enterobacteriaceae - Enterobacter spp. "hormaechei"*	99.2	22-2 (E13)	*Enterobacteriaceae - Serratia spp. "plymuthica"*	99.6
6-1 (J4)	*Enterobacteriaceae - Enterobacter spp. "ludwigii"*	100	18-2 (M20)	*Enterobacteriaceae - Serratia spp. "plymuthica"*	100
48-2 (K32)	*Enterobacteriaceae - Ewingella spp. "americana"*	99.5	18-1 (M20)	*Enterobacteriaceae - Serratia spp. "proteamaculans"*	98.6
49-1 (K22)	*Enterobacteriaceae - Klebsiella spp. "granulomatis"*	95.3	55 (K30)	*Enterobacteriaceae - Serratia spp. "proteamaculans"*	99.6
28-2 (E25)	*Enterobacteriaceae - Rahnella spp. "aquatilis"*	99.4	47-1 (K24)	*Enterobacteriaceae - Serratia spp. "ureilytica"*	98.1
37-2 (ME19)	*Enterobacteriaceae - Rahnella spp. "aquatilis"*	99.9	31-1 (E43)	*Enterobacteriaceae - Yersinia spp. "kristensenii"*	98.4
37-3 (ME19)	*Enterobacteriaceae - Rahnella spp. "aquatilis"*	99.9	9'-1 (M32)	*Enterobacteriaceae - Yokenella "regensburgei"*	99.5
40-2 (ME18)	*Enterobacteriaceae - Rahnella spp. "aquatilis"*	99.6	54-1 (K50)	*Pseudomonadaceae - Pseudomonas (Flavimonas) spp."oryzihabitans"*	99
60-2 (K29)	*Enterobacteriaceae - Raoultella spp. "terrigena"*	99.5	54-2 (K50)	*Pseudomonadaceae - Pseudomonas (Flavimonas) spp."oryzihabitans"*	98.9
48-1 (K32)	*Enterobacteriaceae - Raoultella spp. "terrigena"*	99.5	24-2 (E18)	*Pseudomonadaceae - Pseudomonas spp. "japonica"*	98.8
23-2 (E10)	*Enterobacteriaceae - Serratia spp. "fonticola"*	99.9	7'-2 (M28)	*Pseudomonadaceae - Pseudomonas spp. "koreensis"*	97.9
21-2 (E35)	*Enterobacteriaceae - Serratia spp. "fonticola"*	99.5	45-2 (K10)	*Pseudomonadaceae - Pseudomonas spp. "moraviensis"*	99.7
5-1 (J22)	*Enterobacteriaceae - Serratia spp. "fonticola"*		51-1 (K49)	*Pseudomonadaceae - Pseudomonas spp. "palleroniana"*	99.8
36-2 (ME10)	*Enterobacteriaceae - Serratia spp. "glossinae"*	99.3	51-2 (K49)	*Pseudomonadaceae - Pseudomonas spp. "palleroniana"*	99.8
40-1 (ME18)	*Enterobacteriaceae - Serratia spp. "glossinae"*	99	27-1 (E29)	*Pseudomonadaceae - Pseudomonas spp. "psychrophila"*	97.8
25-1	*Enterobacteriaceae - Serratia*	99.6	50-2	*Pseudomonadaceae - Pseudomonas*	98.5

(E17)	*spp. "glossinae"*		(K36)	*spp. "saponiphila"*	
25-2	*Enterobacteriaceae - Serratia*	99.6	56-1	*Pseudomonadaceae - Pseudomonas*	98.5
(E17)	*spp. "glossinae"*		(K39)	*spp. "saponiphila"*	
41-1	*Enterobacteriaceae - Serratia*	99.2	59-1	*Pseudomonadaceae - Pseudomonas*	97.3
(ME8)	*spp. "glossinae"*		(K23)	*spp. "savastanoi"*	
43-1	*Enterobacteriaceae - Serratia*	100	53-1	*Pseudomonadaceae - Pseudomonas*	96.4
(ME7)	*spp. "glossinae"*		(K34)	*spp. "segetis"*	
19 (E2)	*Enterobacteriaceae - Serratia spp. "plymuthica"*	99.6			

3.3 Functional Potentiality

The 43 isolates were selected on the basis of their potential ability to fix free nitrogen, solubilize phosphates and produce siderophore (Table 2). All selected isolates showed ability to fix free nitrogen, having grown in nitrogen-free media, but at varied degree as indicated by different ethylene peak areas in the Acetylene Reduction Assay (data not shown). 37 isolates were found to be positive for P solubilization with isolates 48-2 (K32) (*Ewingella* spp.), 37-2 (ME19) (*Rahnella* spp.), 40-2 (ME18) (*Rahnella* spp.) and 37-3 (ME19) (*Rahnella* spp.) showing the highest ability to solubilize P (Figure 2). All the isolates produced siderophore with highest production being observed with isolates 2-1 (J1) (*Enterobacter* spp.), 54-2 (K50) (*Pseudomonas* spp.) and 54-1 (K50) (*Pseudomonas* spp.) (Figure 3).

Table 2: Summary of the isolates' functional potentiality based on qualitative screening

Strain	*N-fixation ability*	*P-solubilization ability*	*Siderophore Production*
Bacillus spp. (1)	+	+	+
Enterobacter spp. (4)	+	+	+++
Ewingella spp. (1)	++	+++	+
Klebsiella spp. (1)	++	+	+
Rahnella spp. (4)	+	+++	+
Raoultella spp. (2)	++	+	+
Serratia spp. (17)	+	+	++
Yersinia spp. (1)	+	+	+
Pseudomonas spp. (12)	++	+	+++

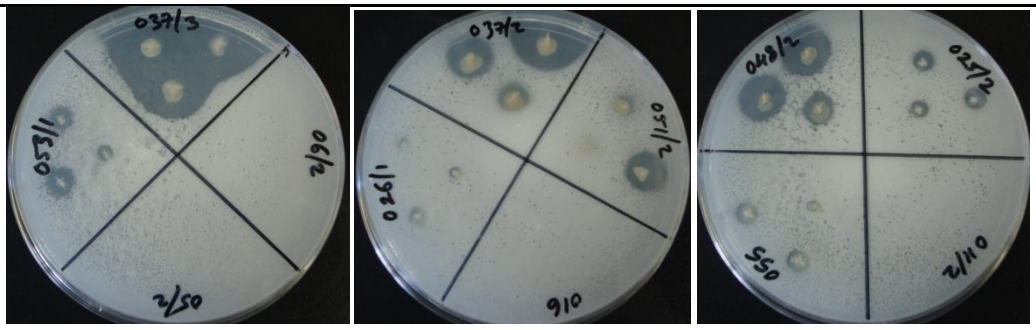


Figure 2: Qualitative screening for phosphate solubilization using the NBRIP medium. Isolates 37-3 (ME19), 37-2 (ME19), 48-2 (K32) and 40-2 (ME18) showed the highest P-solubilization ability. Isolates 6-2 (J4), 5-2 (J22), 16 (M10) and 11-2 (M6) were found to be negative for P-solubilization and hence not among the 43 selected

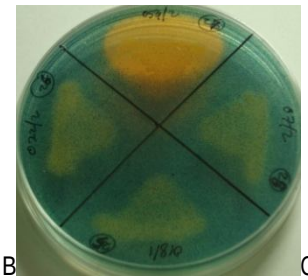
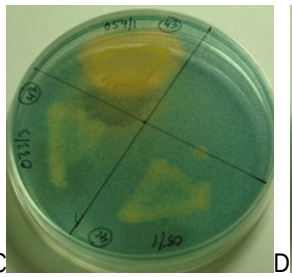
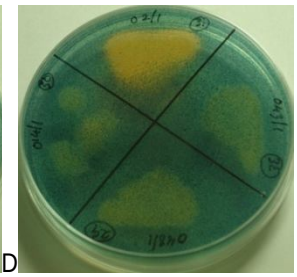
A A.B  C  D 

Figure 3: Qualitative screening for siderophore production on Chrome Azurol S agar plates. Plate A: All the 43 isolates on one agar plate for easy screening. Plates B to D: Showing the 3 isolates that showed highest siderophore production i.e. isolates 2-1 (J1), 54-2 (K50) and 54-1 (K50)

4.0 Discussion and Conclusions

Endophytic bacteria of bananas grown in Kenya were isolated and identified in respect to their potential as biofertilizers. The isolates could be grouped into three families i.e. *Enterobacteriaceae, Pseudomonadaceae* and *Bacillaceae*. The family *Enterobacteriaceae* was the most diverse with 7 genera i.e. *Serratia* (17 strains), *Enterobacter* (4 strains), *Rahnella* (4 strains), *Raoultella* (2 strains), *Klebsiella* (1 strain), *Yersinia* (1 strain) and *Ewingella* (1 strain). Both the *Pseudomonadaceae* and *Bacillaceae* family were represented by only one genus namely *Pseudomonas* (12 strains) and *Bacillus* (1 strain), respectively. *Serratia* and *Pseudomonas* species were the most abundant with 17 strains and 12 strains out of the 43 identified isolates, respectively. *Azospirillum, Burkholderia, Citrobacter, Herbaspirillum, Klebsiella, Pseudomonas* and *Serratia* species are among the bacterial endophytes that have been isolated from banana plants (Weber *et al*., 1999; Martinez *et al.*, 2003; Rosenblueth *et al.*, 2004; Ting *et. al*., 2007 and Weber *et. al*., 2007).

Isolates 48-2 (K32) (*Ewingella* spp.), 37-2 (ME19), 40-2 (ME18) (*Rahnella* spp.) and 37-3 (ME19) (*Rahnella* spp.) showed the highest potential as P solubilizers. According to Kim and others (1998b), *Rahnella aquatilis* has genes that are necessary for mineral phosphate solubilization. Isolates 2-1 (J1) (*Enterobacter* spp.), 54-2 (K50) (*Pseudomonas* spp.) and 54-1 (K50) (*Pseudomonas* spp.) showed high siderophore production. Gangwar & Kaur (2009) also reported *Pseudomonas spp*. isolated from ryegrass as high siderophore producer. None of the 43 isolates showed exceptionally high nitrogen fixing ability. The commonly reported endophytic diazotrophic bacteria of banana include; *Azospirillum*, *Burkholderia,* and *Herbaspirillum* species (Weber *et al*., 1999 and Weber *et al*., 2007), of which none was identified in the current study.

In conclusion, isolates 48-2 (K32) (*Ewingella* spp.), 37-2 (ME19) (*Rahnella* spp.), 40-2 (ME18) (*Rahnella* spp.), 37-3 (ME19) (*Rahnella* spp.), 2-1 (J1) (*Enterobacter* spp.), 54-2 (K50) (*Pseudomonas* spp.) and 54-1 (K50) (*Pseudomonas* spp.) can be qualified as potential biofertilizers and greenhouse and field investigations are recommended for confirmation of this potentiality.

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