PLANT GROWTH PROMOTING POTENTIAL OF BANANA (MUSA SPP.) ENDOPHYTIC BACTERIAL IN KENYA

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Abstract
In Kenya, banana production is constrained by among others, declining soil fertility. 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. Endophytes are diverse microbes, most commonly fungi and bacteria, which spend the entire or part of their life cycle living in internal plant tissues causing no apparent or immediate disease symptoms. 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). This study was conducted with the aim of assessing functional potentialities of previously isolated and identified endophytic bacteria of bananas in Kenya in regard to their plant growth promoting potential that included abilities to fix free nitrogen, solubilize phosphates and produce siderophores. The 43 bacterial isolates used in this study belonged to the genera *Serratia* (17 isolates), *Pseudomonas* (12 isolates), *Rahnella* (4 isolates), *Enterobacter* (2 isolates), *Raoultella* (2 isolates), *Yokenella* (2 isolates), *Bacillus* (1 isolate), *Klebsiella*(1 isolate), *Yersinia* (1 isolate) and *Ewingella* (1 isolate). 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 showed plant growth promoting potential, and in particular *Rahnella*and *Pseudomonas* isolates.

Key words: *Musa* spp., endophytic bacteria, diazotrophes, phosphate-solubilizing microorganisms, siderophores

1.0 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. This means that the nutrients are not adequately replenished. The most obvious solution to nutrient replenishment is increased use of 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. Additionally, use of chemical fertilizers is not environment friendly.

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). Microbial inoculants based on Arbuscularmycorrhizal fungi have successfully been used by small scale farmers on bananas in some tropical countries like Colombia, Malaysia and Cuba (Uribe *et al*., 2010). Endophytes are also 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 plants (Carroll, 1992; Schulz *et al.*, 1998; 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 (Dobereiner and Baldani, 1998; Sturz*et al*., 2000; Boddey*et al*., 2003; Iniguez*et al*., 2004; Ryan *et al*., 2008; Uribe *et al*., 2010). Imported microbial inoculants could be used for banana production in Kenya but because of the fitness challenge, there is need to isolate locally occurring bacteria from bananas in our practical farm fields and assess their functional potentiality as biological fertilizers. The objective of this study was to determine the isolates' capacity to fix free nitrogen, solubilize phosphates and produce siderophores*in vitro*.

2.0 Materials and Methods

2.1 Bacterial Isolates Tested

The 43 bacterial isolates used in this study belonged to the genera *Serratia* (17 isolates), *Pseudomonas* (12 isolates), *Rahnella* (4 isolates), *Enterobacter* (2 isolates), *Raoultella* (2 isolates), *Yokenella* (2 isolates), *Bacillus* (1 isolate), *Klebsiella* (1 isolate), *Yersinia* (1 isolate) and *Ewingella* (1 isolate).

2.2 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. Bacterial isolates were cultured 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) and their growth observed at four and ten days post inoculation. Growth on the N-free medium was used as an indication of isolates' ability to fix free nitrogen.

2.3 Screening for Phosphate Solubilization Ability

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 that dissolves inorganic phosphate resulting to a clear zone around them. 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 (mm) was calculated by subtracting colony diameter from the total diameter.

2.4 Screening for Siderophore Production Ability

Siderophore production was detected using the Chrome Azurol S (CAS) agar plates as described by Schwyn and Neiland (1987).Siderophore medium used composed of three solutions. Solution A composed of 750 ml distilled water, 100 ml MM9 (x10) salts, 15 g agar, 30.24 g PIPES, 10 g glucose and 50% NaOH solution to raise pH to the pKa of PIPES (6.8). 100 ml MM9 (x10) was made up of 0.3 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 0.2 mM (0.05 g) $MgSO_4$ and 0.1 mM (0.015 g) $CaCl_2$. Solution B was made up of 60.5 mg CAS in 50 ml water, 10 ml 1 mM $FeCl_3.6H_2O$ in 10 mMHCl and 72.9 mg hexadecyl trimethylammonium bromide (HDTMA) in 40 ml water while solution C was 30 ml 10% Casamino acid. The three solutions were autoclaved separately and mixed at the clean bench. Solution A was allowed to cool to 50°C, after which solution C was added. Solution B was then added to solutions A and C along the glass wall, with enough agitation to achieve mixing without generation of foam. Each plate received about 30 ml of the blue agar. Orange halos around colonies on blue agar indicated siderophore excretion. Data were taken four and seven days post inoculation.

3.0 Results

3.1 Nitrogen Fixation Ability of the Isolates

All the 43 isolates showed growth on solid N-free medium (Table 1); colony sizes ranged between 1 and 13 mm (Figure 1). However, the colony size may have also been a factor of the isolates ability to utilize glucose as a carbon source.

Table1: 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	Siderophore production
M9V1r (30)	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)	+	-	-
K34V2c (23)	*Pseudomonas protegens* CHA0(T)	+	+	+
J1V1r (31)	*Enterobacterasburiae* JCM 6051(T)	+	+	+
M28V2s (28)	*Pseudomonas koreensis* Ps 9-14(T)	+	+	+
ME10V1r (35)	*Serratiaglossinae* C1(T)	+	+	-
K50V2s (43)	*Flavimonasoryzihabitans* 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)	*Flavimonasoryzihabitans* IAM 1568(T)	+	-	++
K23V1c (4)	*Pseudomonas protegens* CHA0(T)	+	+	+
K10V1r (12)	*Pseudomonas moraviensis* CCM 7280(T)	+	+	+
Isolate ID	**16S rRNA closest relative**	**N-fixation**	**Phosphates solubilization**	**Siderophore production**
K39V1s (10)	*Pseudomonas protegens* CHA0(T)	+	+	+
K36V2c (37)	*Pseudomonas protegens* CHA0(T)	+	+	+
E35V1s (18)	*Serratiafonticola* DSM 4576(T)	+	+	-

E17V1c (8)	*Serratiafonticola* DSM 4576(T)	+	+	-
E17V1c (19)	*Serratiaglossinae* C1(T)	+	+	-
J22V1c (41)	*Serratiaglossinae* C1(T)	+	+	-
ME7V1r (32)	*Serratiaglossinae* C1(T)	+	+	-
E2V1r (13)	*Serratiaplymuthica* DSM 4540(T)	+	-	-
E15V1c (15)	*Serratiaplymuthica* DSM 4540(T)	+	-	-
E10V1r (14)	*Serratiafonticola* DSM 4576(T)	+	+	-
E13V2r (26)	*Serratiaplymuthica* DSM 4540(T)	+	-	-
K30V2c (34)	*Serratiaproteamaculans* DSM 4543(T)	+	-	+
E13V2r (20)	*Serratiaplymuthica* DSM 4540(T)	+	-	-
M20V2c (25)	*Serratiaproteamaculans* DSM 4543(T)	+	-	-

Isolate ID	**16S rRNA closest relative**	**N-fixation**	**Phosphates solubilization**	**Siderophore production**
M20V2c (40)	*Serratiaplymuthica* DSM 4540(T)	+	-	-
ME19V2c (42)	*Rahnellaaquatilis* DSM 4594(T)	+	++	-
E25V2c (7)	*Rahnellaaquatilis* DSM 4594(T)	+	-	-
ME18V2c (36)	*Rahnellaaquatilis* DSM 4594(T)	+	++	-
ME19V2c (24)	*Rahnellaaquatilis* DSM 4594(T)	+	++	-
E43V2 (1)	*Yersinia kristensenii* ATCC 33638(T)	+	+	-
K32V2c (39)	*Ewingellaamericana* GTC 1277(T)	+	+	-
ME18V2c (6)	*Serratiaglossinae* C1(T)	**+**	**+**	**-**

ME8V2r (11)	*Serratiaglossinae* C1(T)	+	+	-
K29V1c (5)	*Raoultellaterrigena* ATCC 33257(T)	+	-	-
K22V1c (2)	*Klebsiellagranulomatis* KH 22	+	-	-
M32V1s (33)	*Yokenellaregensburgei* GTC 1377(T)	+	-	-
K32V2c (29)	*Raoultellaterrigena* ATCC 33257(T)	+	-	-
K24V1c (9)	*Serratiaureilytica*NiVa 51(T)	+	-	-
Isolate ID	**16S rRNA closest relative**	**N-fixation**	**Phosphates solubilization**	**Siderophore production**
E41V2 (17)	*Enterobacteramnigenus* JCM 1237(T)	+	-	-
J4V1c (38)	*Yokenellaregensburgei* GTC 1377(T)	+	+	-

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

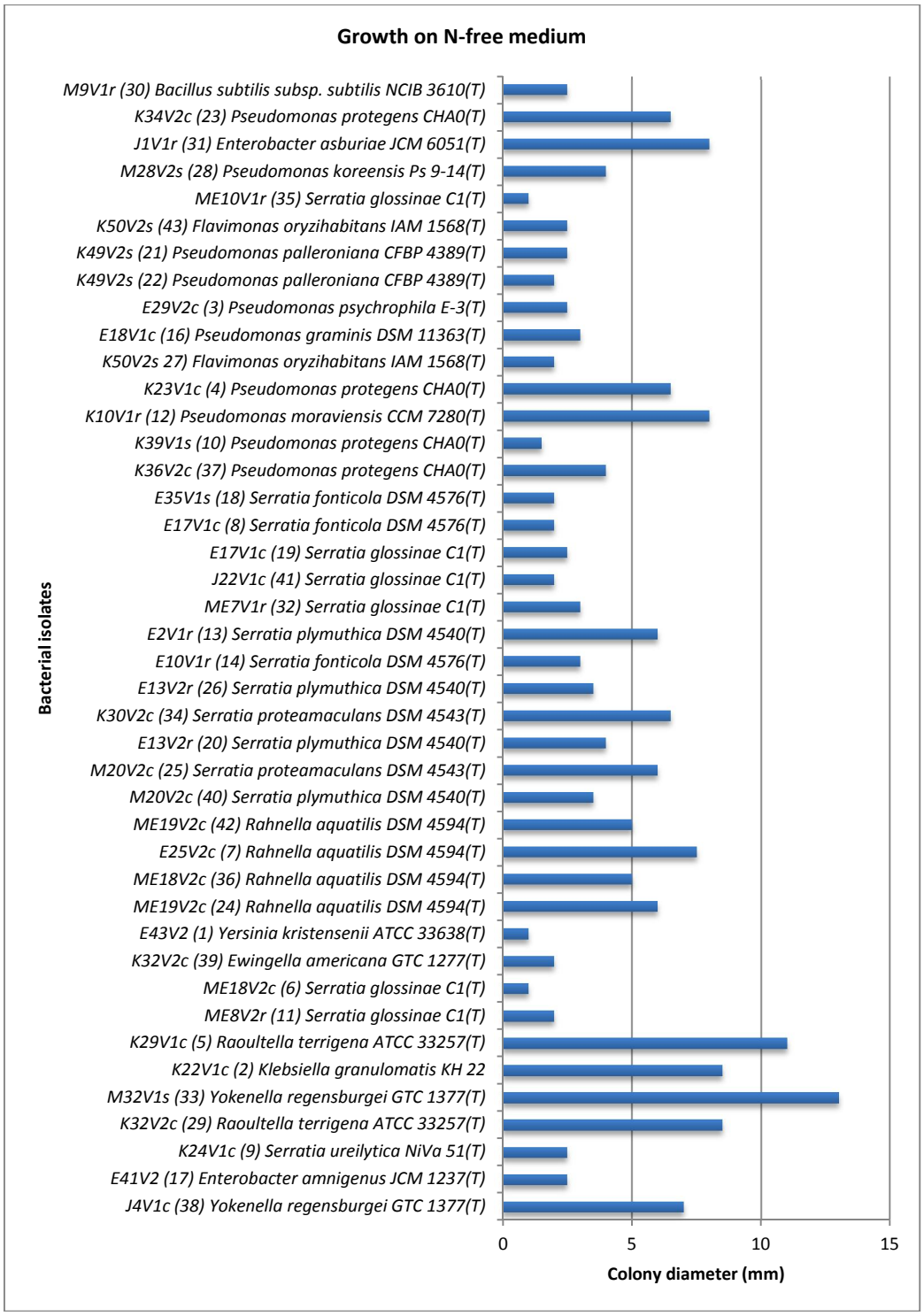

Figure1: Growth of banana endophytic bacterial isolates on solid N-free medium 10 days after inoculation. All the 43 strains grew on N-free medium and colony sizes ranged between 1 and 13 mm

3.2 Phosphate Solubilization Ability of the Isolates

All the 43 isolates grew on NBRIP medium albeit some colonies being very small (1.5 mm diameter). However, not all the isolates were observed to form visible dissolution halos on the NBRIP medium agar plates. Screening of isolates' phosphate solubilizing ability is based on formation of visible halo zones on agar plates. At 13 days post inoculation (DPI) some strains had started forming visible dissolution halos but most (63%) started forming visible dissolution halos at 21 DPI (**Figure 2**). The halo sizes ranged between 1.5 and 17 mm. The strains that showed positive activity 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 *Rahnellaaquatilis*had the largest halo size of 17 mm, 16 mm and 12 mm, respectively (Plate 1). A qualitative summary of the isolates' phosphate solubilization ability is shown on Table 1.

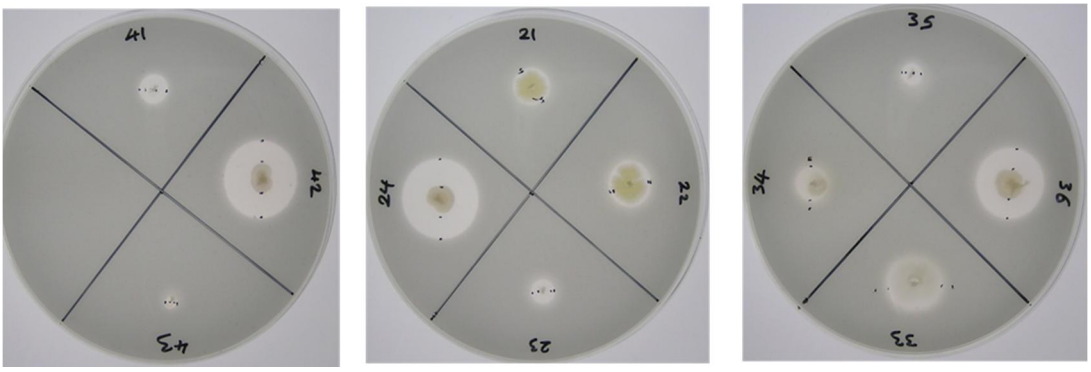

Plate 1: Qualitative screening for phosphate solubilizing isolates on National Botanical Research Institute's phosphate (NBRIP) growth medium agar plates (Nautiyal, 1999), 21 days post inoculation

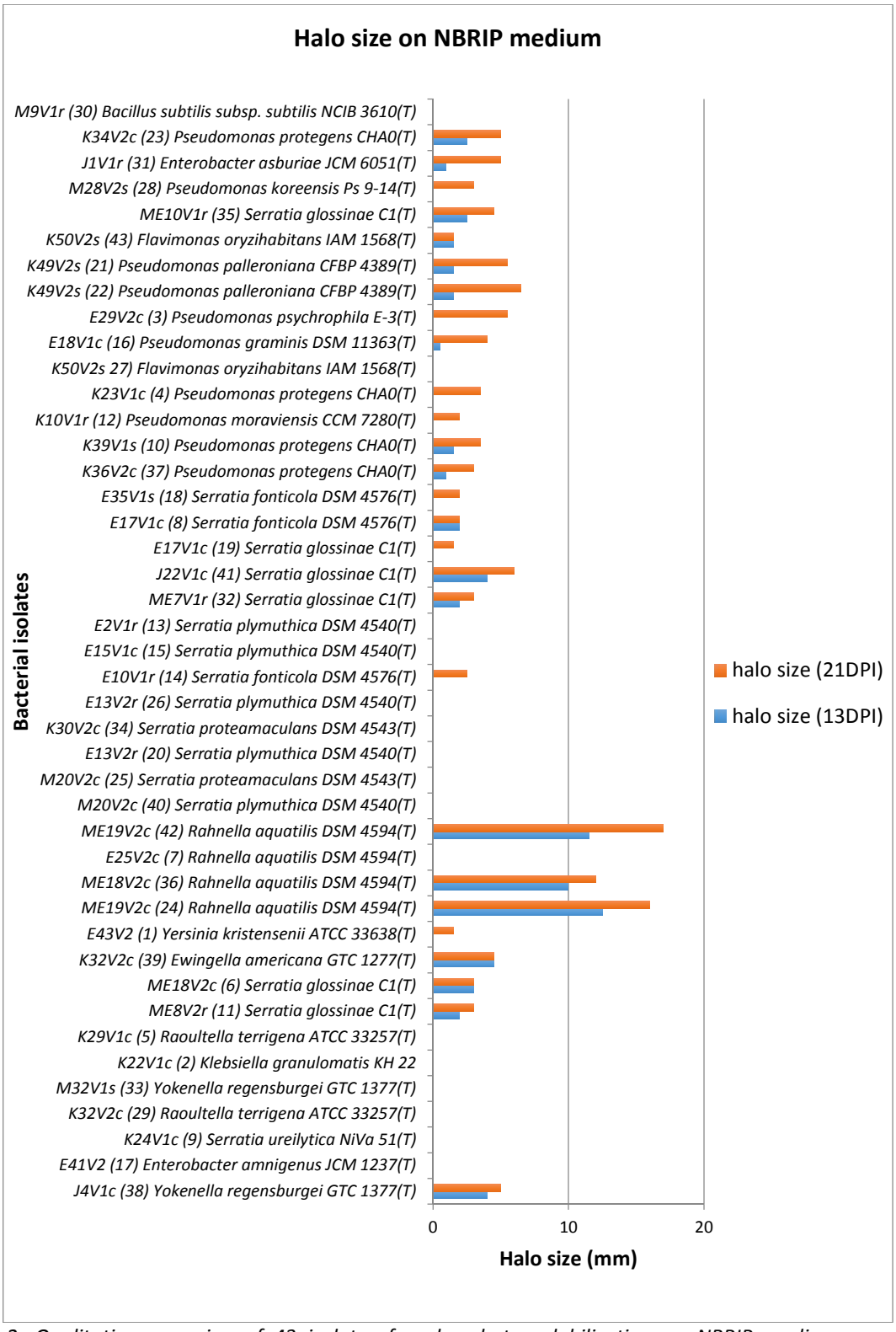

Figure 2: Qualitative screening of 43 isolates for phosphate solubilization on NBRIP medium agar plates (Nautiyal, 1999). Halo and colony diameters were measured at 13 &21 days post inoculation (DPI)

3.3 SiderophoreProduction Ability of the Isolates

All the 43 isolates except M9V1r (30) - *Bacillus subtilis subsp. subtilis*grew on CAS agar plates, however not all had orange halos around their colonies (**Plate 2**). Distinct orange halos were observed with all the 12 *Pseudomonas* isolates with isolates K50V2s (43) and K50V2s (27) both identified as *Flavimonasoryzihabitans* having the largest orange halos, that is strongly positive for siderophore production (Plate 2&Table 1). In

addition *Enterobacterasburiae* (J1V1r) and *Serratiaproteamaculans* (K30V2c) showed positive siderophore production activity (Table 1).

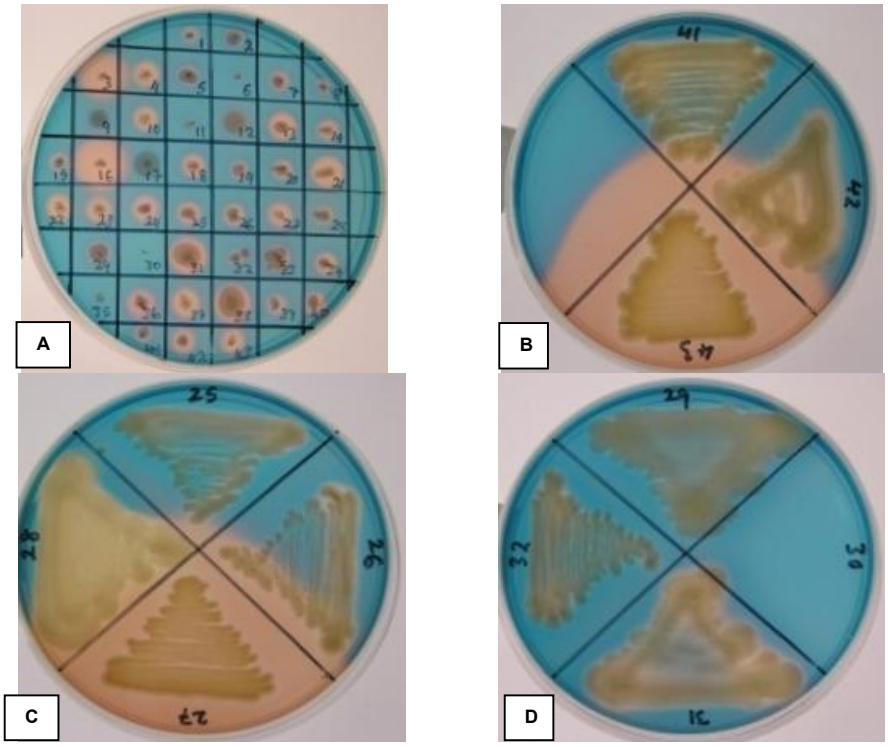

Plate 2: Qualitative screening for siderophore production on Chrome Azurol S (CAS) agar plates. A: All the 43 strains on one plate at one DPI. B and C: Isolates K50V2s (43) and K50V2s (27) with the largest orange halos at 7 DPI, respectively. D: Isolate M9V1r (30) - Bacillus spp., which did not grow on CAS agar plate

4.0 Discussion

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.Nitrogen-fixing endophytic bacteria have been isolated from several groups of plants (Ladha and Reddy, 2000) since the isolation of the endophyticdiazotrophic bacterium *Gluconacetobacterdiazotrophicus* from a Brazilian variety of sugarcane (James and Olivares, 1997). The commonly reported endophyticdiazotrophic bacteria associated with bananas include *Azospirillum*, *Burkholderia, Citrobacter*, *Enterobacter, Herbaspirillum, Klebsiella*and *Rhizobium* species (Weber *et al*., 1999; Weber *et al*., 2001; Martinez *et al*., 2003; Weber *et al*., 2007). Biological nitrogen fixation (BNF) accounts for 65% of the nitrogen utilized in agriculture (Dakora and Keya, 1997). Since BNF is not limited to legumes only, increasing the amount of biologically fixed N in non-legume crops like bananas is of paramount importance for sustainable production.

Rahnellaaquatilis(ME19V2c and ME18V2c)formed the largest visible dissolution halos and were therefore considered the most efficient phosphate solubilizers. 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 organic acids dissolve inorganic phosphate resulting to clear zones around them. The halo size is used as a measure of relative efficiency of the isolates. The findings of this study are consistent with those of Kim *et al.* (1998) who reported *Rahnellaaquatilis*having genes (pyrroloquinolinequinone) that are necessary for mineral phosphate solubilization. Vyas *et al.* (2010) also identified a phosphate-solubilizing bacterial strain from *Hippophaerhamnoides*rhizosphere as *Rahnella* 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). Utilization of identified *Rahnella* species as microbial inoculants in banana production in Kenya would therefore enhance P uptake and hence banana productivity.

Distinct orange halos were observed with all the 12 *Pseudomonas* isolates with *Flavimonasoryzihabitans* (K50V2s) having the largest orange halos. Orange halos around bacterial colonies on blue Chrome Azurol S (CAS) agar are indicative of siderophore excretion (Schwyn and Neiland, 1987).When a strong chelator like siderophore removes iron from the highly coloured iron dye complex of chrome azurol S, iron (III), its colour turns from blue to orange. The *Pseudomonas* isolates and especially *Flavimonasoryzihabitans* (K50V2s) 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. Siderophores can also promote plant growth indirectly by reducing or preventing harm caused by plant-pathogenic microorganisms (Leong, 1986).

Isolate M9V1r (30), identified as *Bacillus subtilis subsp. subtilis*did not show growth on CAS agar plates. This could be explained by the fact that *Bacillus subtilis subsp. subtilis*is Gram-positive and Gram-positive bacteria are reported to be sensitive to HDTMA detergent used in the siderophore medium (Schwyn and Neilands, 1987). HDTMA may therefore have become toxic to *Bacillus subtilis subsp. subtilis* (M9V1r) causing it not to grow.

5.0 Conclusions

From this study,it is apparent that many diazotrophic microbes inhabit the tissues of banana plants and there is the potential of exploiting them once conditions for their use is optimized. *Rahnellaaquatilis* (ME19V2c and ME18V2c) and*Flavimonasoryzihabitans* (K50V2s) having showed ability to solubilize phosphate and produce siderophore, respectively and also ability to fix free nitrogen could be proposed as potential biofertilizers for sustainable banana production in Kenya.

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