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Potentially beneficial rhizobacteria associated with banana plants in Juja, Kenya

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Abstract

Climate and its changes have a direct impact on the development of the different hydrobiont species. These effects on aquatic organisms could be either positive or negative. Every species adapts specifically to natural periodic and seasonal changes, however, the response to unexpected climate changes is inconsistent and not always adequate. Climate-related factors could influence food safety via numerous pathways, namely changes in temperature and rainfall, increased frequency and intensity of extreme meteorological phenomena, ocean warming and increased acidity of aquatic habitats, higher pollution level. Climate change could also have a socioeconomical impact on population feeding i.e. agriculture, animal production (aquaculture), global trade, demographic factors and human behaviour. The paper is aimed at describing some of current and future climatic changes and their possible impact on aquatic organisms in general. Global climate influences the ocean, but the ocean also plays an essential role in global climate patterns. Aquatic organisms are actively involved in the turnover of carbon dioxide and other compounds, hence hydrobionts should not be ignored.

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Introduction

Rhizosphere is the thin layer of soil that immediately surrounds the plant roots (Saharan and Nehra, 2011) and has an abundant nutritious elements because of the continuous rot activities of the plants. This zone is mixed with solid particles and an active community of free living beneficial bacteria (Haghighi *et al*., 2011), usually known as plant-growth-promoting rhizobacteria (PGPR). The banana plant (*Musa* sp.) has adventitious and horizontal roots proliferating the topsoil and cannot get water and nutrients from the deeper soil profile unlike other fruit crops. The undeveloped root system inhibits the utilization of essential mineral nutrients thus limiting the large scale production of bananas under adverse tropical soil conditions. In the recent past there has been an increasing interest in soil microorganisms due to their importance in maintaining soil fertility since it has been shown that plant growth may be stimulated by vitamin-related and pathogen suppressing phytohormones produced by rhizospheric bacteria (Mia *et al*., 2010).

The mutual association where the bacteria obtain the nutrients released from the roots of the plants for growth as they in turn secrete metabolites to the rhizosphere (Van Loon, 2007), is perceived to augment plant growth by the rhizobacteria directly acting as biofertilizers to provide nutrients to the host plant or indirectly by their positive influence on the growth and morphology of roots (Haghighi *et al*., 2011). Previously, diverse banana endophytic bacteria with potential of utilizing the limiting nitrogen and phosphorous nutrients in banana plant growth have been reported (Ngamau *et al*., 2012; Reena *et al*., 2013). The banana rhizosphere may harbor a wide diversity of PGPR that may not only aid in beneficial symbiotic relationships but may stimulate the plant growth by suppressing pathogenic organisms. Biofertilizers are widely accepted as a source of fertilizers with significant increase in crop yields (Vessey, 2003). This study sought to characterize the PGPR associated with banana roots in Juja, Kenya and determine their potential use as a biofertilizer in banana growth.

Materials and methods

Sample collection, isolation and profiling

Soil samples from the rhizosphere region were collected during the month of June 2012 from the cooking and ripening varieties of *Musa* sp. from seven farms in Juja, Kenya by digging at 20-40 cm surrounding the banana plant roots and scrubbing the soil attached to the roots. The rhizosphere soil was scrubbed off the roots, serial diluted fivefold and inoculated on nitrogen-free media, yeast extract mannitol agar and nutrient agar (HIMEDIA) for 24 hours at 30°C. Individual colonies were aseptically picked and streaked on sterile fresh media to purify them and generate pure cultures for morphological, biochemical and molecular characterization as previously described (Cappuccino and Sherman, 2002).

Characterization of rhizobacteria for plant growth promoting traits

The isolates were assessed for nitrogenase activity based on their ability to reduce acetylene (C_2H_2) to ethylene (C_2H_4) by acetylene reduction assay by growing an isolate for 72 hours at 30°C in 5ml nitrogen free semisolid agar in 10ml vials. Acetylene was added at 12% v/v concentration and the ethylene produced was determined on a Shimadzu Gas Chromatograph (GC-9A, Japan) after 12 hours (Eckert *et al*., 2001). Using a syringe, 1ml of ethylene produced was injected into the GC machine and the retention time for each isolate measured. The isolates were screened for phosphate solubilisation using the National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal, 1999). Formation of visible halo zones on agar plates, indicating the organism's production of organic acids into the surrounding medium, 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 described by using solubilization index [ratio of total diameter (colony +

halo zone) to the colony diameter]. Indole acetic acid production was tested using the calorimetric Salkowski reagent (Glickmann and Dessaux, 1995).

Genomic DNA extraction and amplification of the 16S rRNA

The 16S rRNA gene of the bacterial isolates was amplified by polymerase chain reaction using universal primers 27F 5'-GAGTTTGMTCCTGGCTCA-3' and 1492R, 5'-TACGGYTACCTTACGACT-3' (Bioneer, USA) as described (Embley and Stackebrandt, 1994) using an Eppendorf AG, model 22331 thermal cycler (Hamburg, Germany). The PCR mixture comprised of 0.2 Units of Taq polymerase, 20pmol of 27F forward primer, 20pmol of 1492R reverse primer, 1.25mm dNTPs mix (QIAGEN), 10x PCR buffer (QIAGEN), 1 µl of template DNA and 29.8 µl of PCR water. The thermal program was initial denaturation at 94˚C for 5 min, denaturation at 94˚C for 30 s, primer annealing at 43˚C for 2 min, chain extension at 72˚C for 1.5 min for 35 cycles, followed by final extension at 72˚C for 5 min. The amplicons were purified with the Gene JET Purification kit (Thermo Fisher Scientific Inc.) and subjected to DNA sequencing according to the manufacture's protocol (Macrogen, South Korea) using 27F (5'-GAGTTTGMTCCTGGCTCA-3') and 1492R (5'-TACGGYTACCTTACGACT-3') primers.

The sequences were processed using the Chromas Pro software (www.technelysium.com.au) and aligned using CLUSTAL Omega program (http://www.clustal.orgused). BLAST searched against sequences hosted at the National Center for biotechnology Information (NCBI) database to determine their closest neighbours using (BLASTn algorithm, http://www.ncbi.nih.gov as described (Altschul *et al.*, 1990). A phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei, 1987) by using MEGA V5.10 package (www.megasoftware.net) while evolutionary distances were computated using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). To obtain statistical support values for the branches, bootstrapping was conducted with 1000 replicates and all sites, including gaps in the sequence alignment, were excluded pairwise in the phylogenetic analysis (Felsenstein, 1985). The partial sequences for the 16S rRNA genes were deposited in the GenBank sequence database and assigned accession numbers KP797914 to KP797931. The taxonomic assignment was confirmed using the naïve Bayesian rRNA classifier on the RDP website at a 95% confidence level (Cole *et al.*, 2005).

Assessment of growth enhancement effect on tissue culture banana plantlets

Based on plant growth promoting traits screened previously, five strains namely *Chryseobacterium* sp., *Bacillus megaterium*, *Bacillus flexus*, *Pseudomonas monteilii* and *Streptomyces* sp. were assessed for plant growth enhancement under controlled greenhouse conditions. Bacterial cultures prepared as previously described by Mia *et al.* (2009) were infected on tissue culture banana plantlets by dipping the 3 month old plantlets into the specific bacterial isolate treatment for 48 hours at room temperature while distilled water was used for the control experiment. The plantlets were grown in a sterile nutrient inert Cocopeat media (Amiran, Kenya) supplemented with nitrogen, phosphorus and potassium [NPK] slow release fertilizer at 5 levels [0gms, 2.5gms, 5gms, 7.5gms and 10gms] in a random block design in three replicates. Agronomical features of the plantlets: height, diameter and number of green and dry leaves were assessed at 14 days intervals for 3 months (Mia *et al.*, 2009) after which the plants were destructively sampled by gently uprooting, washing off the media under running tap water and measuring the fresh weight. Dry weight was taken after oven drying the plantlets at 80°C for 14 days then essential nutrient levels of nitrogen, phosphorus, potassium, calcium, iron and magnesium was assessed (Plank,1992).

Statistical analyses

The agronomical data, mean±SD from three experimental replicates were collected for analysis. Differences in agronomical parameters among the bacterial treatments were tested by analysis of

variance procedure (ANOVA) using SPSS software. The student t-test was used to assess the variation between each of the treatments taken against the control experiment (Mia *et al.*, 2009). A probability of 5% was considered significant ($P \leq 0.05$).

Results and discussion

Isolation and profiling of banana rhizosphere isolates

After isolation and culturing, 20 pure bacterial isolates were obtained from 67 soil samples based on colony morphological and biochemical characterization revealing relative bacterial diversity. Nitrogenase activity was recorded in 19 isolates, 18 solubilized phosphates while none of the bacterial strains produced the auxin indole acetic acid hormone. Catalase activity was observed in all the isolates (Table 1). The *Bacillus* and *Pseudomonas* sp. isolates showed stronger phosphorus solubilization activity than the rest of the isolates. *Pseudomonas* sp. had phosphate solubilization activity at 1.6 being the highest, followed by *Bacillus* sp. at 1.5 and 1.45 on the phosphate solubilization index on agar assay (Table 2). Phosphate solubilization ability of the plant growth promoting rhizobacteria is considered to be one of the most important traits associated with plant phosphorous nutrition. Soil or seed inoculations with phosphate solubilizing microbes (PSM) have been used for the improvement of crop growth and production by the solubilization of fixed and applied phosphates (Banerjee *et al.*, 2010).

Table 1. Morphology, physiological and biochemical characterization of rhizobacteria isolated from banana farms in Juja, Kenya.

Serial no	Isolate code	Gram test	Morphology	Catalase test	Urease test	Nitrate reduction	Citrate utilization	Hydrogen sulfide production	Phosphate solubilisation	Methyl Red	Voges Proausker	Acetylene Reduction Assay	Indole Acetic Acid	Gelatin hydrolysis	Casein hydrolysis	Starch hydrolysis	Probable identity
1	F1RWL	+	Cocci	+	+	-	-	-	+	+	+	+	-	+	+	+	*Staphylococcus* sp.
2	F1RWL	-	Rods	+	-	-	-	-	-	-	-	+	-	+	+	+	*Chryseobacterium* sp.
3	F2RNY	+	Cocci	+	+	-	-	-	-	+	-	+	-	-	+	-	*Staphylococcus* sp.
4	F1RWL	+	Cocci	+	+	-	-	-	+	+	+	+	-	+	+	+	*Staphylococcus* sp.
5	F5RNY	+	Rods	+	-	-	-	-	+	+	-	+	-	+	+	+	*Bacillus* sp.
6	F6RIS	+	Rods	+	-	+	-	-	+	+	-	+	-	+	+	+	*Bacillus* sp.
7	F5RMS	+	Rods	+	-	-	+	-	+	+	-	+	-	+	+	-	*Bacillus* sp.
8	F4RKB	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
9	F5RMS	-	Rods	+	+	-	-	-	+	-	-	-	-	+	+	+	*Chryseobacterium* sp.
10	F1RGC	+	Rods	+	-	-	+	-	+	+	+	+	-	+	+	-	*Bacillus* sp.
11	F1RWL	+	Rods	+	+	-	+	+	+	-	-	+	-	+	+	+	*Streptomyces* sp.
12	F1RGN	+	Rods	+	-	+	+	-	+	+	+	+	-	+	-	-	*Paenibacillus* sp.
13	F1RGC	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
14	F4RNY	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
15	F6DGG	+	Rods	+	-	-	+	+	+	+	+	+	-	+	+	+	*Bacillus* sp.
16	F6RKB	+	Rods	+	-	-	+	-	+	+	+	+	-	+	+	-	*Bacillus* sp.
17	F1RVL	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
18	F1RWL	+	Rods	+	-	+	+	-	+	+	+	+	-	+	+	+	*Bacillus* sp.
19	F6RMB	-	Rods	+	-	-	+	-	+	-	-	+	-	-	+	-	*Pseudomonas* sp.
20	F5RNY	+	Rods	+	+	-	+	+	+	-	-	+	-	+	+	+	*Streptomyces* sp.

Key: F1-Farm 1; F2-Farm 2; F3-Farm 3; F4-Farm 4; F5-Farm 5; F6-Farm 6; F7-Farm 7; R-Ripening; D-Dessert; WL- Williams; NY-Nyoro; IS-Israel; MS-Mshirain; GC- Giant Cavendish; GN- Grand naine; KB- Kibuu; GG-Gichagara ; VL- Vallary ; MB- Mboo ; + (positive); - (negative).

All *Bacillus* isolates were found to have nitrogenase activity with one of the *Bacillus* sp. showing the highest concentration of 0.7547 µl/ml followed by another with 0.1515 µl/ml (Table 2). *Chryseobacterium* sp. showed nitrogenase activity of 0.069 µl/ml and 0.0145 µl/ml. This could be a potential nitrogen fixer since members of these genus exhibit plant-growth promoting activities as they are universal symbionts of some higher plants (Montero-Calasanz *et al.*, 2013, Weller and Thomashow, 1994).

Two *Staphylococcus* sp. isolates with nitrogenase and phosphate solubilisation abilities were isolated which to our knowledge has not been isolated from banana rhizosphere.

Nitrogen is a crucial element for bananas as it may be a limiting factor in the production and growth of bananas. Nitrogen is among the nutrients commonly required by the banana plant for their optimal growth and productivity (Ahemad and Khan, 2011).

The spore forming *Paenibacillus xylanilyticus* showed promising plant growth promoting nitrogenase and phosphate solubilisation activities. Some species of *Paenibacillus* have consistently shown ability to fix atmospheric nitrogen *in vitro* (Jin *et al.*, 2011).

Though reported to be one of the dominant species in banana rhizosphere, the present study isolated only one *Pseudomonas monteilii* with nitrogenase and phosphate solubilisation ability.

This bacterium has been suggested to belong to the sub group of *P. putida* which are ecologically important in colonizing the banana plant roots rhizosphere (Naik *et al.*, 2008). Only two *Streptomyces* sp. isolates were isolated despite being shown as important microorganisms present in the banana root rhizosphere that have antagonism to phytopathogenic fungi (Cao *et al.*, 2005). *Streptomyces* strains promote banana plantlets' growth by supplying iron to them.

Table 2. Phosphate solubilisation on agar and ethylene production by bacterial isolates from Juja, Kenya.

Serial No.	Isolate code	Phosphate solubilization index on agar assay	Concentration of Ethylene (µl/ml)
1	F1RWL	1.33	0.0309
2	F1RWL	-	0.0145
3	F2RNY	-	0.0113
4	F1RWL	1.25	0.0474
5	F5RNY	1.33	0.0252
6	F6RIS	1.2	0.0635
7	F5RMS	1.25	0.0706
8	F4RKB	1.25	0.0510
9	F5RMS	1.33	-
10	F1RGC	1.33	0.0067
11	F1RWL	1.11	0.1459
12	F1RGN	1.2	0.0611
13	F1RGC	1.33	0.0339
14	F4RNY	1.2	0.0669
15	F6DGG	1.25	0.1515
16	F6RKB	1.5	0.7547
17	F1RVL	1.11	0.0466
18	F1RWL	1.42	0.0631
19	F6RMB	1.6	0.0508
20	F5RNY	1.2	0.0508

Key: F1-Farm 1; F2-Farm 2; F3-Farm 3; F4-Farm 4; F5-Farm 5; F6-Farm 6; F7-Farm 7; R-Ripening; D-Dessert; WL- Williams; NY-Nyoro; IS-Israel; MS-Mshirain; GC- Giant Cavendish; GN- Grand naine; KB- Kibuu; GG- Gichagara ; VL- Vallary ; MB- Mboo.

Bacterial identification using the amplification of the 16S rRNA

Phylogenetic relationship of the bacterial isolates sequenced was inferred using the Neighbour Joining method. Eighteen (18) sequences of the 20 isolates in this study were included while two (2) were excluded in subsequent analysis since they had chimeras. The bacterial isolates clustered with sequences from different genera. Three isolates were categorized as *Staphylococcus* sp., two as *Streptomyces* sp., another two as *Chryseobacterium jejuense* strain NW50 while 11 belonged to the genus *Bacillus*. Two isolates showed 100% sequence similarity to *Paenibacillus* sp. and *Pseudomonas monteilii* strain IHB B 2329 (Fig. 1).

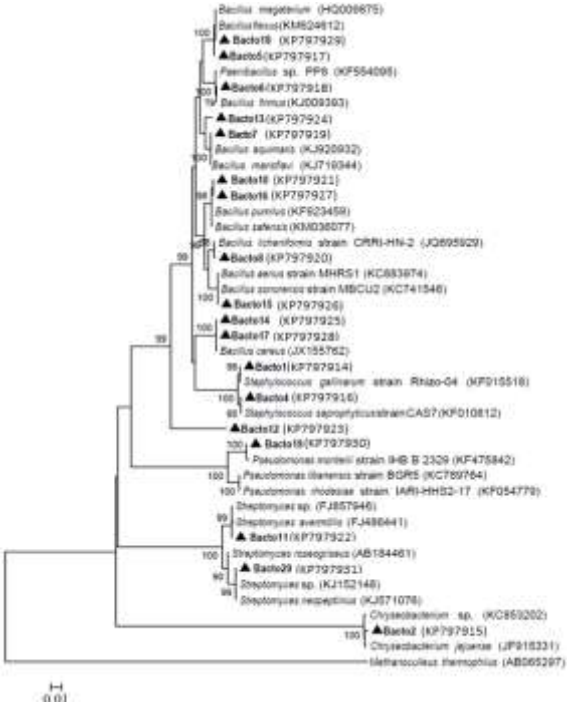

Fig. 1. Neighbour joining phylogenetic tree showing the position of the banana rhizospheric isolates from Juja, Kenya. Eighteen strains from seven different farms in Juja that were isolated in this study are indicated in bold font. The accession numbers of the sequences are indicated in parantheses. The bootstrap values are the numbers seen at the nodes based on 1000 replications. The 16S rRNA gene sequence of *Methanoculleus thermophiles* was an outgroup used.

Assessment of growth enhancement effect on tissue culture banana plantlets

Inoculation of the plants with five selected isolates had a positive growth influence on tissue cultured banana plantlets treated with 5gms, 7.5gms and 10gms levels of NPK fertilizer levels in height, diameter, number of green leaves and mass in dry weight compared with their respective controls. The direct effect of the inoculants on the plantlets could not be established because of the confounding fertilizer level effect. The mean plant diameter (cm) and number of green leaves in isolates plantlets treated with *Pseudomonas monteilii* and *Streptomyces* sp. were higher than their controls, as similarly observed in the mean diameter for plantlets treated with *Chryseobacterium* sp., *Bacillus megaterium* and *Bacillus flexus* strains at 10 gms fertilizer level. Plantlets treated with *Streptomyces* sp. isolate registered a significantly higher growth in all the parameters ($p<0.05$). Height and dry weight were significantly higher in all the five strains tested ($p=0.000$) except *Pseudomonas monteilii* in height ($p=0.007$). Diameter was significantly higher for plantlets treated with *Streptomyces* sp. ($p=0.000$) at 10gms fertilizer level (*Fig*. 2). The total accumulation of nitrogen, phosphorus, magnesium and calcium was significantly higher ($p=$ 0.00000684, 0.000163, 4.46E-09 and 3.77E-05 respectively) in all the treatments following inoculation with the isolates. This could be attributed to the effect of the inoculation on the uptake of these nutrients. However, the intake of potassium, zinc and iron was not influenced by inoculants since there were no differences observed between the treatments and the controls. Plant growth promoting rhizobacteria inoculation on bananas substantially increases the development and growth of tissue-cultured banana plantlets (Mia *et al.*, 2009).

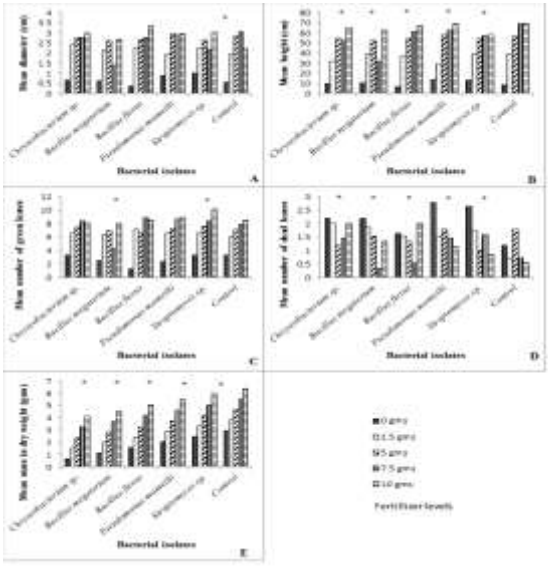

Fig. 2. Effect of selected bacteria isolates from Juja, Kenya on banana plant growth (A: Mean diameter, B: Mean height, C: Mean number of green leaves, D: Mean number of dead leaves, E: Mean mass in dry weight). An asterisk (*) means significantly different to the control ($p<0.05$).

Conclusion

The present study showed that banana rhizospheric bacterial isolates from *Pseudomonas*, *Bacillus, Staphylobacterium, Chryseobacterium, Streptomyces and Paenibacillus genera* in Juja, Kenya harbor plant growth promoting traits and therefore these isolates could be used as biofertilizers in agriculture to promote the growth and production of bananas.

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