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Isolation and characterization of culturable bacteria from bulk soil samples and the rhizosphere of arid-adapted *Tylosema esculentum* (Burchell). A. Schreiber (Marama bean) in Namibia

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Plant growth promoting (PGP) bacteria are microorganisms living in association with plants. PGP bacteria have various physiological activities that they perform which are beneficial to plants. For example, phosphate solubilisation, nitrogen fixation, phytostimulation and formation of siderophores. The aim of this investigation was to determine the diversity of PGP bacteria and to characterise them for possible promotion of plant growth from the rhizosphere of *Tylosema esculentum*, a nutritious arid adapted legume. For that purpose, in this study, bacteria were isolated from marama bean rhizosphere and bulk soil. The bacteria were screened for their ability to solubilise phosphates, for aminocyclopropane-1-carboxylate (ACC) deaminase activity, production of catalase, hydrogen cyanide, ammonia and protease activity. Efficiency of phosphate solubilising activity by bacteria was determined by phosphate solubilisation index. DNA was extracted from bacterial cultures and used to obtain 16S rDNA amplicons for bacterial molecular identification. A total of eight bacterial strains were isolated from the rhizosphere and 19 strains from bulk soil with potential plant growth promoting traits. The 27 bacterial isolates showed phosphate solubilising activity and five of the isolates had a solubilisation index of at least 6. A total of 23 isolates showed ACC deaminase activity. Hydrogen cyanide was produced by 16 isolates; 26 isolates had catalase activity, 23 isolates showed protease activity and all the isolates produced ammonia. The identified genera include *Bacillus, Raoultella, Klebsiella, Acinetobacter, Arthrobacter, Kosakonia* and *Burkholderia.* From this study, we conclude that there is a community of bacteria living in *T. esculentum* rhizosphere and proposed that in future these native bacterial strains can be used as biofertilisers for this arid agro-ecological area after verifying their suitability for inoculum development.

Key words: Rhizobacteria, plant growth promoting bacteria, *Kosakonia, Burkholderia*.

INTRODUCTION

Tylosema esculentum (marama bean) is a long-lived perennial, non-nodulation and non-nitrogen fixing legume native to arid areas of Southern Africa in the Kalahari sandy regions. It produces a raceme up to 25 mm long, containing many yellow-orange flowers and it has circular pods with large brownish-black oil and protein-rich seeds (Holse et al., 2010). It has an underground water storage tuberous root that can grow very large to at least 20 kg.

The tuberous root enables it to thrive in environments with high temperatures (typical daily maximum of 37°C in the growing season), low rainfall (50 to 500 mm) and long periods of drought (Jackson et al., 2011). The edible and nutritious seeds from marama bean are underutilized as food, but constitute a part of the traditional diet for the San people and other indigenous groups in southern Africa (Jackson et al., 2011). The marama beans are gathered from the wild and are mostly eaten as a snack after roasting in hot sand (Jackson et al., 2011). The seeds have a high lipid and protein content (Holse et al., 2010) which gives them a socio-economic value. Besides the high nutritional value of the roasted seeds, the marama bean also has potential as a source of oil production and other healthy food products such as marama milk and defatted marama flour (Jackson et al., 2011). Hence, this neglected legume may be applied in food systems and has a potential to improve both human nutrition and increase food availability in arid ecological zones.

It is intriguing to imagine where this plant could be getting nutrients to survive and thrive in a soil environment which is nutrient deficient especially nitrogen-poor, phosphorrous-poor and also dry. For a long time, plant growth promoting (PGP) bacteria have been proposed to provide these nutrients to plants in a symbiotic relationship (Bulgarelli et al., 2013). However, such studies have been neglected for the nutritionally-rich marama bean. PGP bacteria are a group of bacteria that live in association with plants, while enhancing and stimulating plant growth and development using various mechanisms (Banik and Dey, 1983; Bulgarelli et al., 2013; Ali et al., 2014). Microorganisms can solubilise insoluble phosphates while maintaining a high quality and healthy soil (Richardson, 2001). They use several direct and indirect mechanisms of action to improve plant growth and health. These mechanisms can be active simultaneously or independently at different stages of plant growth. Direct mechanisms such as phosphate solubilisation (Kim et al., 1998) and indirect methods include hydrogen cyanide, catalase production and aminocyclopropane-1-carboxylate (ACC) deaminase activity and formation of siderophores (Penrose and Glick, 2002; Grönemeyer et al., 2012; Bulgarelli et al., 2013; Ali et al., 2014). Phosphate solubilisation provides phosphorus which is an essential nutrient to plants. It is involved in several key plant functions, including energy transfer, photosynthesis, transformation of sugars and starch, nutrient movement within the plant and transfer of genetic characteristics from one generation to the next (Kim et al, 1998). Similarly, ACC deaminase is an enzyme that enhances seed emergence, promotes root elongation, lowers ethylene levels and enhances plant growth (Leidi and Rodriguez-Navarro, 2000; Penrose and Glick, 2002; Bulgarelli et al., 2013). Phosphorous deficiency is a major limiting factor to plant growth, as well as crop production. Plants can only absorb inorganic phosphorus, provided that it is soluble. Most phosphorus added as fertiliser becomes insoluble and thus unavailable to plants. It accumulates due to excessive use of chemical fertilisers, resulting in soil contamination with lethal consequences to beneficial microbes, poor growth rate and low crop production. Large proportions of added fertilisers are converted to the insoluble form, becoming unavailable to plant uptake and accumulates in the soil (Rodriguez and Fraga, 1999; Borch et al*.,* 1999).

The phosphorus content in soil is usually much higher than plant requirements, however, bioavailability of phosphorus to plant is one of the major plant growth limiting constrains. Thus, there is direct need to mobilize this big pool of soil phosphorus to improve crop yields on a sustainable basis and one of the strategies useful for this purpose is the use of specific microorganisms applied in biofertiliser inoculants. Some PGB bacteria are known to promote growth of plants by solubilizing these unavailable/insoluble phosphates in soil while others enhance phosphorus acquisition by plants indirectly through promoting extensive root growth because of their ACC-deaminase activity (Higa and Wididana, 1991; Arshad and Frankenberger, 2002; Dudeja and Giri, 2014).

Bacterial ACC deaminase plays a significant role in the regulation of a plant hormone, ethylene, and thus, enhances the growth and development of plants. Low levels of ethylene like 10 µg l^{-1} have been found to enhance root initiation and growth while the higher levels like 25 µg l^{-1} may lead to inhibition of root growth (Mattoo and Suttle, 1991). Bacterial strains with ACC-deaminase activity can at least partially eliminate the stress-induced ethylene-mediated negative impact on plants by converting the germinating seed/roots ACC into α-ketobutyrate and ammonia (Glick et al., 1998; Dudeja and Giri, 2014). Plants grown under natural soil conditions are generally exposed to environmental stresses and more ethylene is produced by the plant in response to various kinds of stress.

Microbial inoculants as biofertilisers promote plant growth, crop production, increase the nutrient status of the plants, maintain a healthy environment and have been accepted worldwide as an alternative source for chemical fertilizers (Vessey, 2003; Dudeja and Giri, 2014). The use of microorganisms to improve plant growth is increasing every year in various parts of the

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Abbreviation: PGP, Plant growth promoting.

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world. Plant growth promoting bacteria affect both growth and development of plants by direct and indirect mechanisms. Indirectly, the bacteria may exert positive influence on plant growth by lessening certain deleterious effects of pathogenic organisms by inducing host resistance to the pathogen or by knocking out the pathogen from root surfaces or producing chitinases or other pathogen suppressing substances. Although, scientists have reported both direct and indirect ways of growth stimulation by plant growth promoting bacteria, there is no clear distinction. A bacterium influencing plant growth by regulating synthesis of plant hormones can also play a role in controlling plant pathogens and diseases and vice versa (Gray and Smith, 2005). The aim of this study was to examine the diversity of rhizospheric bacteria in the arid-adapted and non-nitrogen fixing legume, *T. esculentum* and to screen for potential phosphate solubilizing bacteria and/or ACC deaminase activity associated with marama bean.

MATERIALS AND METHODS

Sample collection

The rhizospheric soils from five uprooted 2-year old plants and five bulk soil samples randomly from marama growing field were collected from the Omaheke and Otjozondjupa regions which are the native lands for *T. esculentum* in Namibia. Samples were randomly collected and carefully labelled from sites located at Okatumba, Ehungiro, African Wild Dog Conservancy and Okahamupurunga. For rhizospheric soils, the roots were carefully washed in distilled water to collect all the soil tightly attached to the roots, and for the bulk soil samples, a 500 g sample was carefully dug out from top soil of random locations of marama growing fields. The samples were put in zip lock bags, wrapped in foil paper and transported to the laboratory in liquid nitrogen. In the laboratory, samples were immediately processed for culturing bacteria.

Isolation of bacteria

Each bulk soil sample, 1 g was suspended in 9 ml of double distilled water containing 0.1 M phosphate buffered saline (PBS) buffer and vortexed for 2 min, and for rhizospheric soil samples a similar treatment was done but to 1 ml of soil solution. The resulting suspensions were serially diluted to 10^{-10}. Each dilution (0.1 ml) was spread on pre-solidified tryptone soy agar (TSA) plates. Growth was monitored on a daily basis and a single colony was sub-cultured onto TSA plates. Sub-culturing was done until pure colonies were obtained. All the subsequent *in vitro* plate assay analyses were done in triplicate with positive and negative controls.

Screening of phosphate solubilising bacteria

All the isolated pure culture strains were screened for their ability to solubilise insoluble phosphates by an agar assay. The National Botanical Research Institute's phosphate (NBRIP) medium, supplemented with 15 g of bacteriological agar was used (Nautiyal, 1999). The NBRIP growth medium contained in grams per litre: glucose (10 g), $Ca_3(PO_4)_2$ (5 g), $MgCl_2.6H_2O$ (5 g), $MgSO_4.7H_2O$ (0.25 g), KCl (0.2 g) and $(NH_4)_2SO_4$ (0.1 g) (Nautiyal, 1999). One strain was stabbed four times on a plate using sterile loops. The plates were incubated at 29°C for 14 days. The colony and halo diameters were measured. Solubilisation index was used to determine the strains abilities to solubilise insoluble phosphates. Solubilisation index is the ratio of the total diameter (colony + halo zone) to the colony diameter (Edi Premono et al., 1996).

Production of plant growth promoting enzymes and hormones

Aminocyclopropane-1-carboxylate (ACC) deaminase

The ACC deaminase activity was determined using Dworkin and Foster (DF) minimal salts medium containing ACC as sole nitrogen source (Dworkin and Foster, 1958). The composition of the solidified salt minimal media containing ACC as sole nitrogen source in grams per litre (sterile distilled water) was as follows: agar (10 g), KH_2PO_2 (1.36 g), Na_2HPO_4 (2.13 g), $MgSO_4.7H_2O$ (0.2 g), $CaCl_2.2H_2O$ (0.7 g), $FeSO_4.7H_2O$ (0.2 g), $CuSO_4.5H_2O$ (0.04 g), $MnSO_2.H_2O$ (0.02 g), $ZnSO_4.7H_2O$ (0.02 g), H_3BO_3 (0.003 g), $CoCl_2.6H_2O$ (0.007 g), $Na_2MoO_4.2H_2O$ (0.004 g), substrate ACC (5 mM), and glucose (10 g). Strains were inoculated using the streaking method. The plates were incubated at 29°C and growth was monitored daily for three days.

Catalase and protease activity

The catalase test was performed to study the presence of the catalase enzyme in the phosphate solubilising bacteria. A drop of 3% hydrogen peroxide was added to a colony on a sterile glass slide and mixed well using a sterile loop. The effervescence indicated catalase activity. The protease activity was determined using skim milk agar medium, which contained per litre: pancreatic digest of casein (5 g), yeast extract (2.5 g), glucose (1 g), 7% skim milk solution and bacteriological agar (15 g). Bacterial cultures were spot inoculated and incubated for 48 h at 29°C. Clear zones around the cultures indicated protease activity (Smibert and Krieg, 1994).

HCN and ammonia production

The bacterial isolates were screened for hydrogen cyanide (HCN) production (Castric, 1975). The bacterial cell cultures were streaked on nutrient agar medium, which contained 4.4 g per litre of glycine. A Whatman filter paper was soaked in 0.5% picric acid solution and it was placed inside the lid of the plate. The plates were sealed with parafilm and incubated at 29°C for four days. The appearance of a light brown to dark brown colour of the filter paper indicated HCN production. The bacterial isolates were tested for the production of ammonia (Cappuccino and Sherman, 1992). Bacterial cultures were inoculated in 10 ml peptone broth and incubated at 29°C for 48 h. Shaking using a shaker at 1000 rpm for 1 h was done daily. After incubation, 0.5 ml of Nessler's reagent (0.09 $moll^{-1}$ solution of potassium tetraiodomercurate (II) ($K_2[HgI_4]$) in 2.5 $moll^{-1}$ potassium hydroxide) was added. The development of faint yellow to dark brown colour indicated the production of ammonia.

Molecular identification of bacterial strains

DNA extraction 16S rRNA gene amplification and sequencing analysis

The DNA of each isolate was extracted from fresh broth cultures grown for 24 h using the Zymo Research ZR Soil Microbe DNA MiniPrep™ Catalog No. D6001 extraction kit. The protocol followed was enclosed in the instruction manual of the manufacturer (Zymo Research, California, USA) http://www.zymoresearch.com. Purified

Table 1. Description of the bacterial isolates.

Sample	Type of soil	No. of isolates	Isolate designation code
1	Bulk*	Three	BP1, BP2, BP3
2	Bulk	Two	BP4, BP5
3	Bulk	Eight	BP6, BP7, BP8, BP9, BP10, BP11, BP12, BP13
4	Bulk	Three	BP14, BP15, BP16
5	Bulk	Three	BP17, BP18, BP19
6	Rhizosphere	Two	RP1, RP2
7	Rhizosphere	Two	RP3, RP4
8	Rhizosphere	Two	RP5, RP6
9	Rhizosphere	One	RP7
10	Rhizosphere	One	RP8

*soil obtained in the marama bean growing fields. BP means bulk soil portion, RP means rhizosphere soil portion.

DNA quality was confirmed by electrophoresis on a 1% agarose gel stained with 5 µl of 10 mg/ml ethidium bromide and visualised under ultraviolet light. Amplification of the 16S rDNA was carried out using the universal primers Bac8uf (5′-AGAGTTTGATNHTGGYTCAG-3′) and Univ1492r (5′-GGNTCCTTGTTACGACTT-3′) as reported in Grönemeyer et al. (2012). The 50 µl amplification mixture consisted of: water (33.5 µl), 10xDream *Taq* buffer (5 µl), 500 mM dNTPs (10 µl), 500 nM of each primer, DNA template (1 µl) and 1 unit of Mol*Taq* polymerase (Molzym, Germany). The amplification profile was carried out with an initial denaturation at 95°C (4 min) followed by 35 cycles of denaturation at 95°C (1 min), annealing at 50°C (30 s), extension at 72°C (1 min) and a final extension at 72°C (10 min) in a thermocycler (BioRad, England). Sequencing of amplicons was done at Inqaba Biotech (Pretoria, South Africa) with the primers Bac8uf and Univ1492r. The quality of the sequences obtained was assessed by eye using BioEdit. The sequences were used to do similarity searches with BLAST in the National Centre of Biotechnology Information (NCBI) genbank (http://blast.ncbi.nlm.nih.gov).

RESULTS

A total of 27 bacterial strains were isolated from a total of five rhizospheric soil samples associated with the marama bean plants and five bulk soil samples (Table 1). All the isolates were given designation codes as shown in Table 1. The characterisation assays revealed that these strains had various plant growth promoting activities including, phosphate solubilisation, production of ACC deaminase and protease activity. The partial 16S rDNA sequencing managed to determine the identity of the isolated bacteria. The isolates were identified as belonging to seven genera namely *Bacillus, Raoultella, Klebsiella, Acinetobacter, Arthrobacter, Kosakonia* and *Burkholderia.* Some of the well-known PGP bacterial genera like *Rhizobium*, *Azospirillum* and *Herbaspirillum* could not be isolated, suggesting the possible need to use alternative media to isolate them.

Isolation and screening of phosphate solubilizing bacteria on agar assay

All the isolates formed halozones (data not shown) around the colonies which was a characteristic signature of phosphate solubilising activity. The halos indicated the solubilisation of the phosphate source used in the media, which was tri calcium phosphate (Gaur, 1990). Isolates BP18 (*A. calcoaceticus*), BP6 (*Acinetobacter calcoaceticus*) and BP7 (*Klebsiella oxytoca*) showed the highest phosphate solubilising activity with solubilisation indices of 10, 11 and 12, respectively (Table 2). Isolates BP16 and BP17 (both *A. calcoaceticus*) showed good phosphate solubilising activity with a solubilisation index of 6 each. Isolate BP13 (*Kosakonia ludwigii*) had the lowest solubilisation activity with a solubilisation index of 2.07. The solubilisation index is directly proportional to the solubilisation activity. Isolates with high solubilisation indices are good candidates to be included in the design of biofertilizer inoculants.

Production of plant growth promoting enzymes

ACC deaminase activity

The ACC deaminase activity of the isolates was observed in 23 isolates (85% of the total isolates) (Table 3). Only four isolates were negative for the production of ACC deaminase. These were identified to be closest to *Arthrobacter mysorens,* 2 *K. oxytoca* isolates and *Burkholderia ferrariae*. It was, however, observed that some isolates of *K. oxytoca* were ACC deaminase positive. It was observed that most of the isolates of *Klebsiella* were positive for protease activity. The PGP characteristics displayed by isolates closest to *K. oxytoca* are clearly indicative for the need to use another gene region to decipher conclusively the species identity of the 10 isolates since 16S rDNA may not have conclusively resolved their identity. The possibility of new species cannot be ruled out.

Catalase and protease activity

It was observed that 96% of the isolates were catalase

Table 2. Phosphate solubilisation activity of the bacterial isolates.

Isolate	*Name of bacterial species closest to the isolate	Colony diameter (cm)	Halozone diameter (cm)	Solubilisation index
BP1	*Bacillus megaterium*	0.4	0.6	2.5
BP2	*Raoultella ornithinolytica*	0.7	1.2	2.7
BP3	*Klebsiella oxytoca*	0.6	1.2	3
BP4	*Klebsiella oxytoca*	0.6	1.0	2.67
BP5	*Klebsiella oxytoca*	0.6	1.0	2.67
BP6	*Acinetobacter calcoaceticus*	0.1	1.0	11
BP7	*Klebsiella oxytoca*	0.1	1.1	12
BP8	*Acinetobacter oleivorans*	0.7	1.1	2.57
BP9	*Arthrobacter mysorens*	0.4	0.5	2.25
BP10	*Kosakonia cloacae*	0.6	1.2	3
BP11	*Bacillus anthracis*	0.3	0.5	2.67
BP12	*Klebsiella oxytoca*	0.6	0.8	2.33
BP13	*Kosakonia ludwigii*	1.4	1.5	2.07
BP14	*Acinetobacter oleivorans*	0.3	1.0	4.33
BP15	*Klebsiella oxytoca*	0.2	0.5	3.5
BP16	*Acinetobacter calcoaceticus*	0.2	1.0	6
BP17	*Acinetobacter calcoaceticus*	0.2	1.0	6
BP18	*Acinetobacter calcoaceticus*	0.1	0.9	10
BP19	*Kosakonia ludwigii*	0.4	1.0	3.5
RP1	*Klebsiella oxytoca*	0.9	1.3	2.44
RP2	*Klebsiella oxytoca*	0.3	0.6	3
RP3	*Raoultella ornithinolytica*	0.8	1.0	2.25
RP4	*Bacillus megaterium*	0.8	1.1	2.38
RP5	*Bacillus pumilus*	1.0	1.1	2.1
RP6	*Klebsiella oxytoca*	0.4	0.6	2.5
RP7	*Burkholderia ferrariae*	0.7	1.2	2.71
RP8	*Klebsiella oxytoca*	0.1	0.3	4

*Some of the isolates were designated the same names by 16S rDNA sequence since the initial selection was done based on colony morphology.

Table 3. Plant growth promoting activity of the bacterial isolates.

Isolate	Closest species assigned by 16SrDNA analysis	ACC deaminase activity	Catalase test	Hydrogen cyanide test	Ammonia test	Protease test
BP1	*Bacillus megaterium*	+	+	-	+	-
BP2	*Raoultella ornithinolytica*	+	+	-	+	+
BP3	*Klebsiella oxytoca*	+	+	-	+	-
BP4	*Klebsiella oxytoca*	+	+	+	+	+
BP5	*Klebsiella oxytoca*	+	+	+	+	+
BP6	*Acinetobacter calcoaceticus*	+	+	-	+	+
BP7	*Klebsiella oxytoca*	+	+	+	+	+
BP8	*Acinetobacter oleivorans*	+	+	-	+	+
BP9	*Arthrobacter mysorens*	-	+	+	+	+
BP10	*Kosakonia cloacae*	+	+	+	+	+
BP11	*Bacillus anthracis*	+	+	-	+	+
BP12	*Klebsiella oxytoca*	+	+	-	+	+
BP13	*Kosakonia ludwigii*	+	+	+	+	-
BP14	*Acinetobacter oleivorans*	+	+	+	+	+
BP15	*Klebsiella oxytoca*	+	+	+	+	+

Table 3. Contd

BP16	*Acinetobacter calcoaceticus*	+	+	+	+	+
BP17	*Acinetobacter calcoaceticus*	+	+	-	+	+
BP18	*Acinetobacter calcoaceticus*	+	+	-	+	+
BP19	*Kosakonia ludwigii*	+	+	+	+	+
RP1	*Klebsiella oxytoca*	+	+	-	+	+
RP2	*Klebsiella oxytoca*	-	-	+	+	+
RP3	*Raoultella ornithinolytica*	+	+	+	+	+
RP4	*Bacillus megaterium*	+	+	+	+	+
RP5	*Bacillus pumilus*	+	+	+	+	+
RP6	*Klebsiella oxytoca*	-	+	+	+	+
RP7	*Burkholderia ferrariae*	-	+	-	+	-
RP8	*Klebsiella oxytoca*	+	+	+	+	+
Total (+)		23 (85%)	26 (96%)	16 (59%)	27 (100%)	23 (85%)

- Stands for negative test result and + stands for positive test result.

positive (Table 3). Only *K. oxytoca* isolate RP2 was catalase negative but some other *K. oxytoca* were verified to be catalase positive. Of the 27 isolates, 23(85%) of the isolates showed proteolytic activity. The four isolates that were negative for protease activity were identified as closest to: *Bacillus megaterium. K. oxytoca, K. ludwigii* and *B. ferrariae*.

HCN and ammonia production

It was observed that 59% of the isolates produced hydrogen cyanide. The 11 isolates did not have HCN production activity included isolates of: *B. megaterium, Raoultella ornithinolytica, K. oxytoca, Bacillus anthracis, A. calcoaceticus and B. ferrariae.* Again, some isolates of *Klebsiella* were observed to be negative for HCN (Table 3). All the isolates tested positive for ammonia production (Table 3).

Molecular identification of bacterial isolates using 16S rRNA gene sequence

A total of 27 16S rRNA gene amplicons were sequenced. The sequences were inspected by eye and edited using BioEdit, and then using the BLAST on the NCBI genbank at nucleotide level. The identity (percentages of similarity) of the isolates closest to the known species in the rDNA database is presented in Table 4. Some of the isolates were repeated because the initial selection was based on colony morphology observation, which was not specific. When considering the proportion of the isolates, *K. oxytoca* isolates had the highest frequency (37%) followed by the *Acinetobacter oleivorans* (15%) (Table 4). The least abundant species were *R. ornithinolytica, A. calcoaceticus, A. mysorens, Kosakonia cloacae, B. anthracis, Bacillus pumilus* and *B. ferrariae* at 3.7% each (Table 4). Some of the well-known PGP genera such as *Burkholderia* and *Kosakonia* were isolated.

DISCUSSION

In this paper, microbial analysis of the *T. esculentum* rhizosphere and also bulk soil revealed a considerable diversity of bacterial community with plant growth promoting characteristics. The study is cautious to note that this is only a portion of the actual diversity present as it is excluding the unculturable bacteria as well as those that could be cultured with different complex and enrichment media or altered atmospheric conditions. In this study, 27 strains were identified using colony morphology and characterized to species level by 16S rDNA sequencing. This paper serves as basis for future prospects and acts as a point of reference for future research in understanding plant –microbe interactions of this non-nodule forming marama bean. Regarding ACC deaminase activity, 23 isolates showed ACC deaminase activity. The observation is consistent with the report of Glick et al. (1998) and Glick (2005) who demonstrated that ACC deaminase activity enhances seed emergence, promotes root elongation, lowers ethylene levels and enhance plant growth a characteristic that could be of benefit to marama bean evolutionarily. In this study, 23 isolates showed proteases activity. Protease is fungal cell wall degrading enzymes, which can offer protection of marama bean against mycological pathogens that may encroach its proximity. In this study it was observed that *K. oxytoca, A. calcoaceticus, K. cloacae, K. ludwigii, R. ornithinolytica, B. megaterium and B. pumilus* showed more than one plant growth promoting characteristic. They solubilized insoluble phosphates, displayed ACC deaminase activity, produced ammonia, hydrogen cyanide, catalase and protease. With these results, PGP bacteria possessing simultaneously both phosphate

Table 4. Molecular identification of isolates to closest strain in the NCBI genbank based on sequence similarity.

Isolate	Strain identification*	NCBI Accession number	Percentage similarity (%)
BP1	*Bacillus megaterium*	NR_043401.1	99
BP2	*Raoultella ornithinolytica*	NR_102983.1	99
BP3	*Klebsiella oxytoca*	NR_041749.1	99
BP4	*Klebsiella oxytoca*	NR_041749.1	99
BP5	*Klebsiella oxytoca*	NR_041749.1	99
BP6	*Acinetobacter calcoaceticus*	NR_042387.1	99
BP7	*Klebsiella oxytoca*	NR_041749.1	99
BP8	*Acinetobacter oleivorans*	NR_102814.1	100
BP9	*Arthrobacter mysorens*	NR_025613.1	99
BP10	*Kosakonia cloacae*	NR_028912.1	99
BP11	*Bacillus anthracis*	NR_074453.1	100
BP12	*Klebsiella oxytoca*	NR_041749.1	99
BP13	*Kosakonia ludwigii*	NR_042349.1	99
BP14	*Acinetobacter oleivorans*	NR_102814.1	99
BP15	*Klebsiella oxytoca*	NR_041749.1	99
BP16	*Acinetobacter calcoaceticus*	NR_042387.1	99
BP17	*Acinetobacter calcoaceticus*	NR_042387.1	99
B18	*Acinetobacter calcoaceticus*	NR_042387.1	99
B19	*Kosakonia ludwigii*	NR_042349.1	99
RP1	*Klebsiella oxytoca*	NR_041749.1	99
RP2	*Klebsiella oxytoca*	NR_102982.1	99
RP3	*Raoultella ornithinolytica*	NR_102983.1	99
RP4	*Bacillus megaterium*	NR_074290.1	100
RP5	*Bacillus pumilus*	NR_074977.1	100
RP6	*Klebsiella oxytoca*	NR_041749.1	99
RP7	*Burkholderia ferrariae*	NR_043890.1	99
RP8	*Klebsiella oxytoca*	NR_102982.1	98

*Species identified based on best score on % similarity to the 16S rDNA sequence.

solubilizing plus ACC deaminase activities may improve phosphate nutrition and plant growth more effectively than those carrying either one of these two growth promoting traits. It is easy to imagine that using bacteria with at least dual plant growth promoting activities as biofertiliser inoculants will be a desirable and sustainable option to replace eco-unfriendly chemical fertilizers. The catalase activity that was observed in the isolated bacteria can be interpreted to be of evolutionary and beneficial value to the *T. esculentum*-bacteria association. Bacterial strains with catalase activity are highly resistant to environmental, mechanical and chemical stress (Glick et al., 1998). The environment where *T. esculentum* grows is stressful with high day's temperatures of above 37°C, low pH and poor nutrient content in the soil (Chimwamurombe, 2010).

Of all the species isolated, 59% were positive for HCN production, including the *Kosakonia* species and some *K. oxytoca* isolates. Hydrogen cyanide production by bacteria has been reported to have an important role in the biological control of pathogens and as an inducer of plant resistance (Voisard, 1989; Bulgarelli et al., 2013). These bacteria may be playing a role in protection of marama bean since very low disease incidence has been observed in its natural habitat. All the isolates were able to produce ammonia in plate assays. This was an interesting observation since the production of ammonia is an important feature of plant growth promoting bacteria that influences plant growth by making nitrogen (N) available to the plant. This is of particular importance to marama bean that has high protein containing seeds yet it grows in N-poor soils of the Kalahari sandy ecological zone (Dakora et al., 1999).

In general, the mechanisms of action by which plant growth promoting bacteria enhance and promote plant growth are not clearly known and understood, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilisation and promotion of the mineral nutrient uptake are usually believed to be

involved in plant growth promotion (Glick, 1995; Bulgarelli et al., 2013; Ali et al., 2014). Many papers have been published related to the screening of plant growth promoting bacteria from crop plants such as rice, maize and sugar cane but rare from marama bean. It was observed in this study that two bacterial isolates showed the highest solubilisation index of more than 11. Isolates BP6 (*A. calcoaceticus*), and BP7 (*K. cloacae*) showed the highest phosphate solubilising activity. In previous studies, it has been reported that phosphate solubilizing bacteria enhanced the growth and yield of inoculated plants by giving higher yields up to 20% in case of maize and lettuce (Chabot et al., 1993). Similarly, Gaind and Gaur (2004) found that the use of rock phosphate, coupled with phosphate solubilizing bacteria, produced results comparable to superphosphate + phosphate solubilizing bacterial inoculants. Thus, phosphate biofertilisers in the form of microorganisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization. PGP bacteria have been reported to produce significant increases in growth and yield of agriculturally important crops in direct response to inoculation (Amara and Dahdoh, 1997; Grönemeyer et al., 2012). Furthermore, inoculation with rhizobacteria containing ACC-deaminase activity has been shown to alter the endogenous levels of ethylene, which subsequently leads to changes in plant growth. The bacterium actually prevents ethylene caused inhibition of root elongation. ACC accumulation is stimulated by several stress factors. If the ethylene concentration remains high after germination, root elongation is inhibited (Bulgarelli et al., 2013). The inhibitory effect of ethylene on plant root elongation can be reduced by the activity of ACC-deaminase; an enzyme produced by some soil microorganisms (Bulgarelli et al., 2013; Ali et al., 2014).

It was anticipated that most of the bacterial genera were isolated from both the bulk soil could also be isolated from the root rhizosphere parts of the experimental plants. However, it was strikingly remarkable to observe that two of the bacterial isolates (*B. ferrariae* and *B. pumilus*) were only retrieved from the rhizosphere. The explanation for this observation remains to be investigated. Likewise, *B. anthracis, A. mysorens, K. cloacae, and A. oleivorans* were only isolated from the bulk soil samples. Similarly, the explanation for this striking observation still needs further investigation.

Conclusion

We conclude that in the *T. esculentum* (marama bean) rhizosphere, there are numerous plant associated bacteria, some of which have potential to be used as plant growth promoting inoculants for arid agro ecological zones since they displayed phytobeneficial traits under *in vitro* conditions. Furthermore, with the exception of well-known PGP bacterial genera like *Rhizobium, Bradyrhizobium, Azospirillum* and *Herbaspirillum*, several novel putative PGP species were detected in our screening procedure. We recommend that the identified isolates may have potential as plant beneficial inoculants for sandy, nutrient deficient agro-ecologies like the Kalahari areas where application of chemical fertilisers will be an economically demanding endeavour for the communal farmers. Similar studies on edible but neglected plants regarding presence of beneficial microbial communities should be enhanced as climate change effects begin to manifest in susceptible areas like the Kalahari region.

Conflict of interests

The authors did not declare any conflict of interest.

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