

# Diversity of Culturable Plant Growth-Promoting Bacterial Endophytes Associated with Sugarcane Roots and Their Effect of Growth by Co-Inoculation of Diazotrophs and Actinomycetes

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Received: 20 December 2015 / Accepted: 15 March 2016 / Published online: 31 May 2016  
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**Abstract** Application of environmentally friendly agents to reduce the use of chemicals and to enhance growth of plants is an ultimate goal of sustainable agriculture. The use of plant growth-promoting endophytes has become of great interest as a way to enhance plant growth and additionally protect plants from phytopathogens. In this study, 135 isolates of endophytic bacteria including actinomycetes were isolated from roots of commercial sugarcane plants cultivated in Thailand and were characterized for plant growth-promoting (PGP) traits. Based on morphological and 16S rRNA sequence analysis, the endophytes were distributed into 14 genera of which the most dominant species belong to *Bacillus*, *Enterobacter*, *Microbispora*, and *Streptomyces*. Two strains of endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21; and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected based on their PGP traits including 1-aminocyclopropane-1-decarboxylate deaminase, indole-3-acetic acid, nitrogen fixation, phosphate solubilization, and siderophore production for evaluation of sugarcane growth enhancement by individual and co-inoculation. Sixty days after co-inoculation by

endophytic diazotrophs and actinomycetes, the growth parameters of sugarcane plants were significantly greater than that of individual and un-inoculated plants. The results indicated that these endophytes have high potential as PGP agents that could be applied to promote sugarcane growth and could be developed as active added value biofertilizers in the future.

**Keywords** Actinomycete · Diazotroph · Endophyte · Diversity · Plant growth promotion · Sugarcane

## Introduction

Sugarcane (*Saccharum officinarum* L.) is a tropical plant belonging to the grass family, Gramineae, as well as maize, rice, sorghum, and wheat. Sugarcane is the world's largest crop and is economically important in Thailand, which ranks fourth among sugarcane producers on the world market, after Brazil, India, and China (Food and Agriculture Organization of the United Nations 2015). Although sugar is the primary product from sugarcane, ethanol has become a new high-value product because of the worldwide interest in replacing gasoline with biofuel.

For several decades, boosting crop yields by chemical fertilizers and various nutrients and pesticides has dominated agronomy (Tilman 1998). Now, the use of environmentally friendly strategies for reducing the use of chemicals to enhance growth of sugarcane plants is an ultimate goal of sustainable agriculture. One of the alternative ways to promote plant growth and protect plants from diseases is the application of bacterial endophytes. These are free-living bacteria that colonize the inside of the plants and can affect plant growth by direct and indirect mechanisms (Gupta and others 2000; Glick 2012). They

**Electronic supplementary material** The online version of this article (doi:10.1007/s00344-016-9604-3) contains supplementary material, which is available to authorized users.

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are termed plant growth-promoting endophytes (PGPE) (Taulé and others 2011) with various PGP traits including solubilization of rock phosphates, production of siderophores to scavenge iron, production of phytohormones, fixation of nitrogen, secretion of 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce ethylene levels in plants, and production of specialized metabolites to protect plants from phytopathogens (Gupta and others 2000; Mendes and others 2007; Glick 2012). A range of PGPE including endophytic actinomycetes significantly increase vegetative growth and grain yield of several crops, including jatropha, maize, rice, sorghum, sugarcane, and wheat (Olivares and others 1997; Yanni and others 2001; Govindarajan and others 2007; Luna and others 2010; Rungin and others 2012; de Jesus Santos and others 2014; Alves and others 2015; Qin and others 2015) by increasing shoot and root lengths as well as biomass through the secretion of plant growth-promoting substances, increasing the availability of nutrients, or promoting growth by combined modes of action (Vessey 2003). Nevertheless, the application of single bacterial strains resulted in inconsistencies in the field (Bashan and Holguin 1997). Therefore, co-inoculation of compatible bacteria has been found to more significantly enhance the growth of plants than individual inoculation. For example, co-inoculation of mixtures of diazotrophic bacteria gave better growth of rice and sugarcane (Govindarajan and others 2007); and co-culture of diazotrophs and fungal endophytes revealed better growth and yield of chickpea (Verma and others 2014).

This study aimed to select powerful endophytic PGP diazotrophs and actinomycetes isolated from sugarcane plants in Thailand. The diversity of culturable root-associated endophytic bacteria and actinomycetes was systematically analyzed and their PGP traits and plant growth-promoting effects on sugarcane plants were thoroughly evaluated after individual and co-culture inoculation.

## Materials and Methods

### Isolation of Culturable Endophytic Bacteria and Actinomycetes from Sugarcane

Five-month-old healthy sugarcane plant cultivars LK92-11 and KK3, commercial canes mainly planted in Thailand for sugar industry, were obtained from Kud Chock and Nongkonthai sugarcane plantations, Phu Khiao, Chaiyaphum, Thailand through Mitr Phol Sugarcane Research Center. The plant samples were vigorously shaken to loosen attached soil from the roots and air-dried at room temperature (RT) overnight, then kept at 4 °C till use.

To isolate root-associated endophytic bacteria, sugarcane roots were surface sterilized using the method of Rachniyom and others (2015). The roots were first rinsed under running tap water for 2–3 min to remove soil particles, and then immersed with shaking in sterilized 0.1 % (v/v) Tween 20 for 5 min, followed by 70 % ethanol for 5 min, 1 % sodium hypochlorite solution for 10 min, 10 % (w/v) sodium hydrogen carbonate solution for 10 min, and finally washed three times with sterile distilled water.

Endophytic bacteria were isolated according to the method for isolation of nitrogen-fixing bacteria described by Cavalcante and Dobereiner (1988). The roots were crushed in 5 % sucrose solution and ten-fold serial dilutions were made. A 100 µl sample of each dilution was dropped into semisolid LGI medium (Baldani and others 2014) and incubated at 28 °C for 7–10 days. A veil-like pellicle near the surface of the medium appeared and was streaked onto LGI agar and nutrient agar (NA) supplemented with 50 µg ml<sup>-1</sup> nystatin and 50 µg ml<sup>-1</sup> cycloheximide and incubated at 28 °C for 3–7 days. The final rinsing water was also spread on both media to confirm surface sterilization. Colonies of bacteria were picked and purified on LGI agar and NA. The pure cultures were classified by Gram staining and morphological observation under the light microscope. Bacteria were stored in 40 % glycerol solution at –80 °C.

For isolation of endophytic actinomycetes, the surface-sterilized roots were macerated in ¼ Ringer's solution (0.9 % NaCl, 0.042 % KCl, 0.048 % CaCl<sub>2</sub>, and 0.02 % NaHCO<sub>3</sub>). The root materials and the liquid solution were, respectively, placed and spread onto starch casein agar (SCA) (Küster and Williams 1964) and water agar (WA) supplemented with 50 µg ml<sup>-1</sup> nalidixic acid, 100 µg ml<sup>-1</sup> ampicillin, 50 µg ml<sup>-1</sup> nystatin, and 50 µg ml<sup>-1</sup> cycloheximide and incubated at 28 °C for 2–4 weeks. The final rinsing water was also spread on both media to confirm surface sterilization. Colonies of endophytic actinomycetes were picked and purified on mannitol soya (MS) agar (Hobbs and others 1989). The pure cultures were stored in 20 % glycerol solution at –80 °C.

### Identification of Endophytic Bacteria

All the bacterial endophytes were grouped based on their colony morphology on agar plates. Endophytic bacteria were grown on NA and Tryptic Soy Agar (TSA) media at 28 °C for 2 days. Endophytic actinomycetes were grown on International *Streptomyces* Project (Shirling and Gottlieb 1966) Media No. 2, 3, and 4 at 28 °C for 14 days. The representatives of endophytic bacteria and actinomycetes were selected based on morphological characteristics for 16S rRNA gene sequencing and analysis. Genomic DNA of bacteria was prepared by a standard protocol (Green and

Sambrook 2012). The 16S rRNA gene was amplified using universal primers and the PCR protocol of Lane (1991). For actinomycetes, genomic DNA was prepared according to Kieser and others (2000). 16S rRNA genes were amplified using primers and the PCR protocol of Rachniyom and others (2015). PCR products were purified using Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's protocol and subjected to DNA sequencing at Macrogen Inc. (Korea). All sequences obtained from the 16S rRNA genes were edited manually using BioEdit (Hall 1999), then compared with type strains using the EzTaxon-e server (Kim and others 2012a). The sequences of the isolates and the most similar species were aligned using CLUSTAL X program version 2.0 (Larkin and others 2007). Phylogenetic tree was constructed using neighbor-joining algorithm (Saitou and Nei 1987) in MEGA software version 6.0 (Tamura and others 2013). The topology of tree was evaluated with bootstrap analysis based on 1000 resamplings (Felsenstein 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura 1980).

## Characterization of Plant Growth-Promoting Traits

### *Indole-3-Acetic Acid (IAA) Production*

IAA production was determined by a colorimetric method (Pilet and Chollet 1970). Endophytes were inoculated into glucose-beef extract broth supplemented with 10 mM L-tryptophan and incubated at 28 °C for 7 days in the dark. The culture was then centrifuged and 1 ml of the supernatant was transferred into a vial containing 2 ml of Salkowski's reagent (Pilet and Chollet 1970). The mixture was left at RT for 30 min in the dark. Development of a pink-red color indicated IAA production.

### *Siderophore Production*

An overnight-NB culture of bacteria ( $10^7$  CFU ml<sup>-1</sup>) or an agar plug of the 14-day growth of actinomycetes on MS agar was, respectively, dropped or placed on chrome azurol S medium (Schwyn and Neilands 1987) and incubated at 28 °C for 7 days for non-actinomycete bacteria and 14 days for actinomycetes. An orange halo indicated siderophore production.

### *Phosphate Solubilization*

Phosphate solubilization was detected by the modified method of Rodríguez and Fraga (1999). An overnight-NB culture of bacteria ( $10^7$  CFU ml<sup>-1</sup>) or an agar plug of 14-day growth of actinomycetes on MS agar was, respectively, dropped or placed on Pikovskaya's agar

(Pikovskaya 1948) containing 0.5 % (w/v) Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> and incubated at 28 °C for 7 days for non-actinomycete bacteria and 14 days for actinomycetes. Formation of a clear zone indicated solubilizing ability.

### *1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity*

ACC deaminase activity was determined by the method of Glick and others (1995) using N-free medium (Nfb) (Dobereiner and Day 1976) for bacteria and minimal medium (MM) (Kieser and others 2000) for actinomycetes containing 0.3 m mol l<sup>-1</sup> ACC (Sigma, USA) as a sole nitrogen source. MM with 0.1 % (w/v) NH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub> was used as a positive control and cultivation without ACC was used as a negative control. After incubation at 28 °C for 7 days for non-actinomycete bacteria and 14 days for actinomycetes, colony growth on Nfb or MM with addition of ACC indicated ACC deaminase activity.

### *Nitrogen Fixation and nifH Gene Amplification*

Nitrogenase activity was determined by the acetylene reduction assay (ARA) according to Hardy and others (1973). An overnight-NB culture of bacteria ( $10^7$  CFU ml<sup>-1</sup>) was washed three times with distilled water to remove NB before transferring to 5 ml of semisolid Nfb medium and incubated at 28 °C for 2 days. Next, 10 % (v/v) of gas in the headspace of the test tube was replaced by acetylene and left at RT for 1 h. Gas was collected from the headspace and analyzed for ethylene by gas chromatography (Hewlett Packard HP Series II, Japan). The nitrogen-fixing *Azospirillum brasilense* TS13 (Meunchang and others 2004) was used as a positive control and an uninoculated tube served as a negative control. All of the bacterial isolates and *Azospirillum brasilense* TS13 as positive control were subjected to *nifH* gene amplification using primers *nifH*-F (5'-AAAGGNGGNATCGGNA ANTCCACCAC-3') and *nifH*-R (5'-TTGTTNGCNGCN TACATNGCCATCAT-3') and the PCR protocol described by Rösch and others (2002).

### *Evaluation for Antimicrobial Activity*

To evaluate antimicrobial activity, seven test microbes, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 259233, *Aspergillus niger* ATCC 6275, *Colletotrichum falcatum* DOAC 1655, and *Fusarium moniliforme* DOAC 1224, were used. Antibacterial activity was evaluated by an overlay method (Anand and others 2006). An overnight-NB culture of bacteria ( $10^7$  CFU ml<sup>-1</sup>) or a 14-day-old MS agar plug of actinomycetes was,

respectively, dropped or placed on NA plates and incubated for 24 h. The assay plates were overlaid with test bacteria ( $10^7$  CFU ml<sup>-1</sup>). Appearance of an inhibition zone indicated positive activity.

Antifungal activity was evaluated by dual-culture assay (Fokkema 1976). An overnight-NB culture of bacteria ( $10^7$  CFU ml<sup>-1</sup>) was spotted on potato dextrose agar and incubated for 24 h. Actinomycetes were streaked on one-third of the area of PDA and allowed to grow for 7 days. An agar plug of the test fungus was prepared using a sterile cork borer (diameter 8 mm), placed opposite the bacterial colony and incubated at RT for 7 days. Inhibition of fungal growth was compared to the growth of the fungal control.

### Evaluation of Plant Growth Promotion by Individual and Co-Inoculation

Endophytic bacteria and actinomycetes were selected based upon their PGP traits to evaluate sugarcane growth enhancement by individual and co-culture inoculation. To abnegate the effect of antagonistic activity among them, the selected isolates were tested for antibacterial activity against each other, and only the strains showing no activity were used to inoculate sugarcane. Tissue cultured sugarcane plants of cultivar LK92-11 were obtained from the Plant Tissue Culture Unit at Central Laboratory and Greenhouse Complex, Kasetsart University (Kamphaengsaen Campus). Sugarcane plantlets were rinsed with sterile distilled water to remove remaining agar from the roots. The height of the plants was normalized to 6.5 cm by truncating shoots and roots. Plants were then inoculated with bacteria either individual or co-culture ( $10^7$  CFU ml<sup>-1</sup> of each strain) by the root dip method (Musson and others 1995) in a tissue culture bottle containing Murashige and Skoog (MS0) medium (Murashige and Skoog 1962) and immersed at RT for 24 h. Inoculated sugarcane plants were transferred to cleaned mini-pot (6 × 5.5 cm) containing sterilized hydroponic materials (perlite:vermiculite, 3:1). The experiment was set up in completely randomized design (CRD) using five replicate mini-pots per treatment and each treatment was replicated twice. Plants were maintained in the greenhouse and supplied with sterilized sucrose-free MS0 broth twice a day for 60 days. The plants were then harvested and examined for root and shoot lengths, and root and shoot fresh/dry weights. Plants were surface sterilized and the inoculated endophytes were re-isolated according to the procedure described above. Plant growth parameters were statistically analyzed by one-way ANOVA and Tukey's multiple range tests (TMRT) using SPSS (version 16.0) at  $p = 0.05$  to determine the efficacy of un-inoculated, individual, and co-culture inoculation to promote growth of sugarcane plants.

## Results

### Distribution of Sugarcane Root-Associated Bacterial Endophytes

One hundred and thirty-five culturable root-associated bacterial isolates were obtained from sugarcane. They comprised 52 isolates of endophytic bacteria and 83 isolates of actinomycetes. According to the preliminary dereplication by morphological criteria and cultural characteristics, 19 and 11 isolates of endophytic bacteria and actinomycetes were assigned to the genus level using 16S rRNA sequence analysis, respectively. Sequence similarity of 16S rRNA genes of these endophytes (ranging from 1282 to 1513 bp) to the closest type strains from the EzTaxon-e server was 98.4–100 % (Table 1). All of the representative strains displayed considerable diversity and were distributed under 11 families of *Bacillaceae*, *Enterobacteriaceae*, *Micrococcaceae*, *Micromonosporaceae*, *Moraxellaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Streptomycetaceae*, *Streptosporangiaceae*, and *Thermomonosporaceae* comprised 10 genera of bacteria and 4 genera of actinomycetes (Fig. 1). *Streptomyces* was the most frequently isolated genus ( $n = 47$ , 34.81 %) followed by *Microbispora* ( $n = 29$ , 21.48 %), *Enterobacter* ( $n = 23$ , 17.04 %), *Bacillus* ( $n = 14$ , 10.37 %), *Actinomadura* ( $n = 5$ , 3.70 %), *Pantoea* ( $n = 4$ , 2.96 %), *Acinetobacter* ( $n = 2$ , 1.48 %), *Kosakonia* ( $n = 2$ , 1.48 %), *Micromonospora* ( $n = 2$ , 1.48 %), *Paenibacillus* ( $n = 2$ , 1.48 %), *Staphylococcus* ( $n = 2$ , 1.48 %), *Lysinibacillus* ( $n = 1$ , 0.74 %), *Micrococcus* ( $n = 1$ , 0.74 %), and *Pseudomonas* ( $n = 1$ , 0.74 %). 16S rRNA gene sequence similarity values of four strains, *Actinomadura* sp. GKU 822, *Microbispora* sp. GKU 823, GKU 898, and *Pantoea* sp. EN-29 with closest type strains were lower than 99 % (Table 1) and therefore probably represent new species.

### Plant Growth-Promoting Traits of Sugarcane Endophytes

The plant growth-promoting (PGP) traits, including antagonistic activity, were examined from 135 strains of sugarcane endophytes (Table 2). Among them, three strains of actinomycetes, *Streptomyces* sp. GKU 833, GKU 879, and GKU 895, and two strains of bacteria, *Bacillus* sp. EN-27 and *Enterobacter* sp. EN-21, showed the highest activity of PGP traits. The highest frequency of PGP trait possessed by both endophytic bacteria and actinomycetes in this study was siderophore production, followed by IAA production and phosphate solubilization (Table 2). Most of the siderophore-producing species belong to the genera

**Table 1** Colony morphology and 16S rRNA sequence similarity of plant growth-promoting bacterial endophytes from sugarcane to the closest type strains of validly described species

Isolates ( <i>n</i> )	Colony morphology <sup>a</sup>	Closest type strains		Accession no.
		Species	Similarity (%)	
<i>Actinomycetes</i>				
GKU 822 (5)	Dv, Dv	<i>Actinomadura rayongensis</i> RY35-68 <sup>T</sup>	98.7	KF638418
GKU 824 (9)	Syb, Syb	<i>Microbispora amethystogenes</i> JCM3021 <sup>T</sup>	99.1	KP637152
GKU 898 (8)	So, Mo	<i>Microbispora amethystogenes</i> JCM3021 <sup>T</sup>	98.6	KP702933
GKU 823 (12)	Myp, Myp	<i>Microbispora hainanensis</i> 211020 <sup>T</sup>	98.8	KR560040
GKU 885 (2)	Gy, Gy	<i>Micromonospora carbonacea</i> DSM 43815 <sup>T</sup>	99.6	KP637151
GKU 869 (3)	Lgo, Lgo	<i>Streptomyces angustmycinicus</i> NBRC 3934 <sup>T</sup>	99.9	KP702932
GKU 895 (23)	Lgrb, Mr	<i>Streptomyces canus</i> NRRLB-1989 <sup>T</sup>	99.4	KP637153
GKU 867 (2)	Gg, Ly	<i>Streptomyces lannensis</i> TA4-8 <sup>T</sup>	99.9	KP702931
GKU 866 (5)	Lgg, Dgy	<i>Streptomyces tendae</i> ATCC 19812 <sup>T</sup>	99.1	KP637154
GKU 810 (2)	Pg, Lgyb	<i>Streptomyces somaliensis</i> NBRC 12916 <sup>T</sup>	99.9	KP640619
GKU 883 (12)	Pg, Bg	<i>Streptomyces coeruleus</i> ISP 5146 <sup>T</sup>	99.5	KP765677
<i>Bacteria</i>				
EN-5 (1)	C, E, Co, Cr, –ve	<i>Acinetobacter nosocomialis</i> NIPH 2119 <sup>T</sup>	100.0	KP702924
EN-41 (1)	C, E, P, W, –ve	<i>Acinetobacter oleivorans</i> DR1 <sup>T</sup>	100.0	KP702928
EN-24 (2)	I, L, Um, Cr, +ve	<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> FZB42 <sup>T</sup>	100.0	KP702917
EN-25 (7)	C, E, Co, Cr, +ve	<i>Bacillus aryabhatai</i> B8W22 <sup>T</sup>	99.9	KP702925
EN-27 (2)	I, Cu, Co, Cr, +ve	<i>Bacillus safensis</i> FO-36b <sup>T</sup>	100.0	KP702915
EN-14 (1)	C, U, Co, Cr, +ve	<i>Bacillus aerophilus</i> 28K <sup>T</sup>	100.0	KP702929
EN-7 (2)	C, U, R, Cr, +ve	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> KCTC 13429 <sup>T</sup>	99.7	KP702936
EN-21 (20)	C, E, Co, O, –ve	<i>Enterobacter asburiae</i> JCM6051 <sup>T</sup>	99.0	KP765678
EN-23 (3)	C, E, Co, W, –ve	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> LMG2683 <sup>T</sup>	100.0	KP765679
EN-17 (1)	C, U, Co, Cr, –ve	<i>Kosakonia cowanii</i> CIP 107300 <sup>T</sup>	99.8	KP702937
EN-20 (1)	C, E, Co, W, –ve	<i>Kosakonia oryzae</i> Ola 51 <sup>T</sup>	99.7	KP702913
EN-9 (1)	I, E, Co, Cr, +ve	<i>Lysinibacillus macroides</i> LMG 18474 <sup>T</sup>	100.0	KP702920
EN-31 (1)	C, E, Co, Y, +ve	<i>Micrococcus yunnanensis</i> YIM65004 <sup>T</sup>	99.9	KP702921
EN-26 (2)	C, E, Co, O, +ve	<i>Paenibacillus illinoisensis</i> NRRL NRS-1356 <sup>T</sup>	99.8	KP702927
EN-29 (2)	I, E, R, Cr, –ve	<i>Pantoea cypripedii</i> ATCC29267 <sup>T</sup>	98.4	KP702918
EN-4 (1)	I, E, R, Cr, –ve	<i>Pantoea dispersa</i> LMG 2603 <sup>T</sup>	99.8	KP702916
EN-37 (1)	I, E, R, W, –ve	<i>Pantoea dispersa</i> LMG 2603 <sup>T</sup>	99.3	KP702922
EN-34 (1)	C, E, Co, Cr, –ve	<i>Pseudomonas guariconensis</i> PCAVU11 <sup>T</sup>	99.6	KP702919
EN-28 (2)	C, E, Co, Y, +ve	<i>Staphylococcus cohnii</i> subsp. <i>cohnii</i> ATCC 29974 <sup>T</sup>	100.0	KP702923

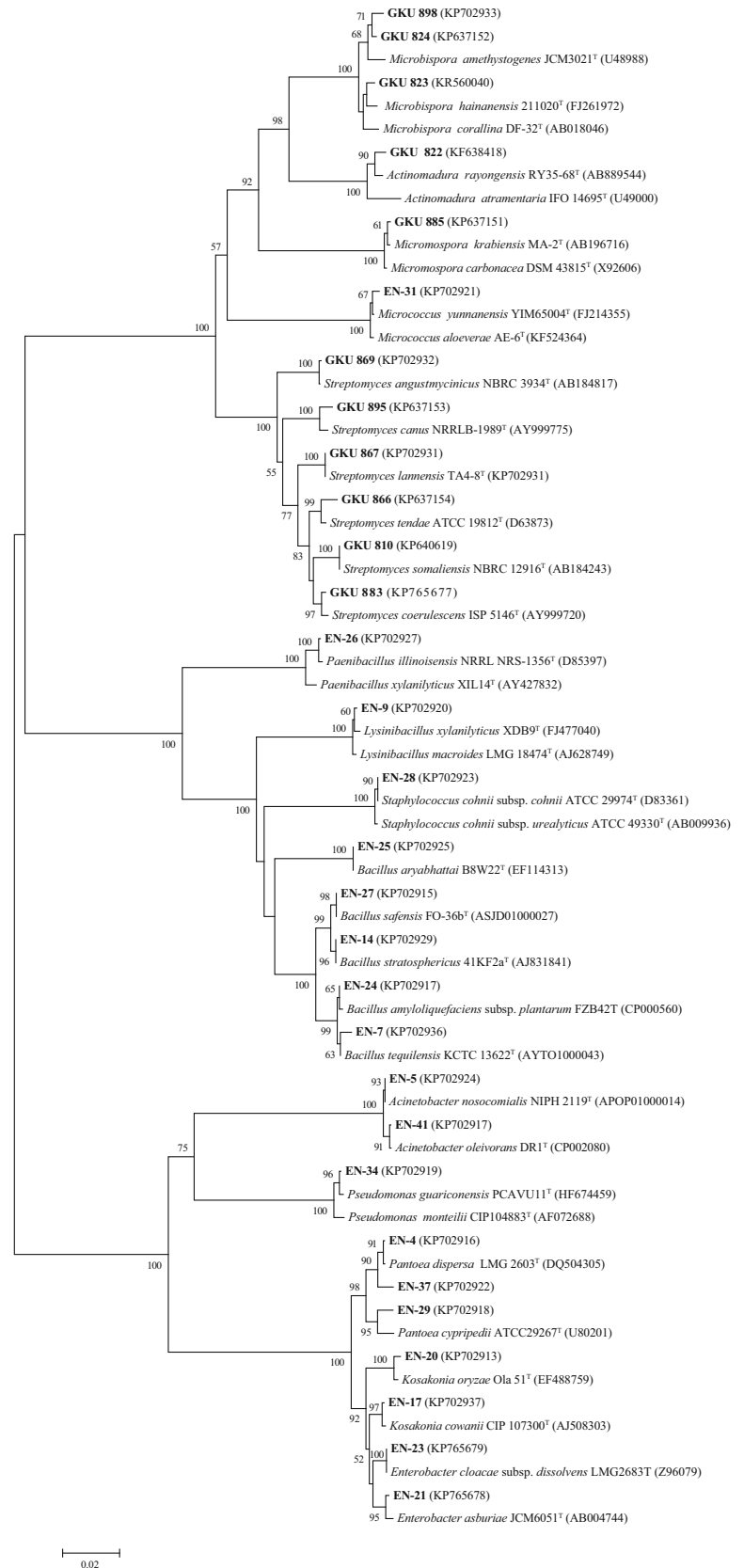
<sup>a</sup> Color of aerial and substrate mycelia of endophytic actinomycetes on ISP2 medium. *Bg* blackish green, *Dgy* dark grayish yellow, *Dv* dark violet, *Gg* greenish gray, *Gy* grayish yellow, *Lgg* light greenish gray, *Lgo* light grayish olive, *Lgrb* light grayish reddish brown, *Lgyb* light grayish yellowish brown, *Ly* light yellow, *Mo* moderate orange, *Mr* moderate reddish, *Myp* moderate yellowish pink, *Pg* paul green, *So* strong orange, *Syb* strong yellowish brown. Colony morphology of endophytic bacteria on NA medium. Form of colony (C/I; circular/irregular), margin of colony (E/Cu/U/L; entire/curled/undulate/lobate), elevation (Co/Um/R/P; convex/umbonate/raised/pulvinate), color (Cr/Y/W/O; cream/yellow/white/off-white), Gram's staining (–ve/+ve; Gram negative/Gram positive). *n* number of isolates

*Streptomyces*, *Microbispora*, and *Bacillus*; while IAA producing dominant species were *Streptomyces*, *Microbispora*, and *Enterobacter*. Members of the genera *Streptomyces* and *Microbispora* were also the majority species capable of phosphate solubilization. However, the highest activity of phosphate solubilization was found in *Pantoea* sp. EN-29 and EN-39 (data not shown). Besides, ACC

deaminase-production was mainly detected in species belonging to *Streptomyces* and *Bacillus* (Table 2).

To evaluate nitrogenase activity, endophytic bacteria were screened using the ARA method. Only six out of the 52 strains belonging to *Bacillus*, *Enterobacter*, *Lysinibacillus*, and *Pantoea* could reduce acetylene gas, ranging between 14.59 and 25.39 nmol C<sub>2</sub>H<sub>4</sub> h<sup>–1</sup> (10<sup>7</sup> cells)<sup>–1</sup>.

**Fig. 1** Neighbor-joining tree based on 16S rRNA gene sequences showing relationships between the representative endophytic bacteria and actinomycetes isolated from sugarcane roots and the nearest type strains. Number of branch points indicates bootstrap value based on 1000 replications; only values above 50 % are shown. Bar, 0.01 substitutions per nucleotide position



**Table 2** Number of positive isolates of sugarcane endophytic bacteria and actinomycetes with plant growth-promoting traits

Genus (n)	Plant growth-promoting traits <sup>a</sup> (%)						Antagonistic activity (%)					
	IAA	SD	P	ACC	ARA	BC	EC	PA	SA	AN	CF	FM
<i>Actinomycetes</i>												
<i>Actinomadura</i> (5)	2 (40)	4 (80)	–	–	ND	–	–	–	–	2 (40)	2 (40)	3 (60)
<i>Microbispora</i> (29)	14 (48.3)	20 (68.9)	19 (65.5)	–	ND	1 (3.4)	–	–	–	4 (13.8)	2 (6.9)	17 (58.6)
<i>Micromonospora</i> (2)	–	2 (100)	–	–	ND	–	–	–	–	2 (100)	1 (50)	2 (100)
<i>Streptomyces</i> (47)	26 (55.3)	33 (70.2)	18 (38.3)	11 (23.4)	ND	12 (25.5)	1 (2.1)	–	4 (8.5)	21 (44.6)	22 (46.8)	23 (48.9)
Total (83)	42 (50.6)	59 (71.0)	37 (44.6)	11 (13.2)	–	13 (15.7)	1 (1.2)	–	4 (4.8)	29 (34.9)	27 (32.5)	45 (54.2)
<i>Bacteria</i>												
<i>Acinetobacter</i> (2)	–	–	–	1 (50)	–	–	–	–	–	–	–	–
<i>Bacillus</i> (14)	3 (21.4)	10 (71.4)	3 (21.4)	7 (50)	2 (14.3)	5 (35.7)	6 (42.8)	–	1 (7.1)	4 (28.6)	8 (57.1)	8 (57.1)
<i>Enterobacter</i> (23)	11 (47.8)	9 (39.1)	2 (8.7)	3 (13)	2 (8.7)	–	–	–	–	–	4 (17.4)	5 (21.7)
<i>Kosakonia</i> (2)	1 (50)	–	–	–	–	–	–	–	–	–	–	–
<i>Lysinibacillus</i> (1)	–	–	–	1 (100)	1 (100)	–	–	–	–	–	–	–
<i>Micrococcus</i> (1)	1 (100)	–	–	–	–	–	–	–	–	–	–	–
<i>Paenibacillus</i> (2)	1 (50)	2 (100)	1 (50)	–	–	1 (50)	–	–	–	–	2 (100)	2 (100)
<i>Pantoea</i> (4)	2 (50)	–	3 (75)	–	1 (100)	–	–	–	–	–	–	3 (75)
<i>Pseudomonas</i> (1)	1 (100)	–	–	–	–	–	–	–	–	–	–	–
<i>Staphylococcus</i> (2)	–	–	–	1 (50)	–	–	–	–	–	–	1 (50)	1 (50)
Total (52)	20 (38.5)	21 (40.4)	9 (17.3)	13 (25)	6 (11.5)	6 (11.5)	6 (11.5)	–	1 (1.9)	4 (7.7)	15 (28.8)	19 (36.5)

<sup>a</sup> IAA indole-3-acetic acid, SD siderophore, P phosphate solubilization, ACC 1-aminocyclopropane-1-carboxylate deaminase, ARA acetylene reduction assay, BC *B. cereus* ATCC 11778, EC *E. coli* ATCC 8739, PA *Ps. aeruginosa* ATCC 15442, SA *Staph. aureus* ATCC 259233, AN *A. niger* ATCC 6275, CF *C. falcatum* DOAC 1655, FM *F. moniliforme* DOAC 1224, n number of strains, ND not determined, – none

**Table 3** Identification and PGP traits of selected sugarcane diazotrophic bacteria and endophytic actinomycetes

Strains	Accession no.	Closest type strains	Similarity (%)	Plant growth-promoting traits <sup>a</sup>						Antagonistic activities			
				IAA	SD	P	ACC	ARA <sup>b</sup>	nifH	BC	AN	CF	FM
EN-21	KP765678	<i>Enterobacter asburiae</i> JCM6051 <sup>T</sup>	99.00	+	+	–	+	25.39	+ <sup>c</sup>	–	–	–	–
EN-24	KP702917	<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> FZB42 <sup>T</sup>	99.52	–	+	–	–	20.11	–	+	+	+	+
GKU 823	KR560040	<i>Microbispora hainanensis</i> 211020 <sup>T</sup>	98.77	+	+	+	–	ND	ND	–	+	–	–
GKU 895	KP637153	<i>Streptomyces canus</i> NRRL B-1989 <sup>T</sup>	99.36	+	+	+	+	ND	ND	+	–	+	+

<sup>a</sup> IAA indole-3-acetic acid, SD siderophore, P phosphate solubilization, ACC 1-aminocyclopropane-1-carboxylate deaminase, ARA acetylene reduction assay, nifH dinitrogenase reductase encoding gene, BC *B. cereus* ATCC 11778, AN *A. niger* ATCC 6275, CF *C. falcatum* DOAC 1655, FM *F. moniliforme* DOAC 1224, + positive activity, – negative activity, ND not determined

<sup>b</sup> ARA values represent activity expressed as nmol C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> (10<sup>-7</sup> cells)<sup>-1</sup>

<sup>c</sup> Accession No. KT275189

The highest level of nitrogenase activity was found in *Enterobacter* sp. EN-21 (25.39) and *Bacillus* sp. EN-24 (20.11). To evaluate nitrogen fixation ability, nifH gene amplification was performed. Ten nifH-positive strains (19.23 % of the total) were obtained. There were five strains, namely *Bacillus* sp. EN-27, *Enterobacter* sp. EN-21, *Enterobacter* sp. EN-30, *Lysinibacillus* sp. EN-9, and *Pantoea* sp. EN-39 that exhibited both nitrogenase activity and a nifH gene (Fig. S1). However, the nifH gene was absent in an ARA-positive endophyte, *Bacillus* sp. EN-24.

Antagonistic potential tests conducted against seven test strains of bacteria and fungi revealed that the most susceptible fungi that interacted with the endophytes were *Fusarium moniliforme*, followed by *Aspergillus niger* and *Colletotrichum falcatum*, while the most susceptible bacterium was *Bacillus cereus* (Table 2). Surprisingly, none of the endophytes inhibited *Pseudomonas aeruginosa*. Six strains of endophytes, *Bacillus* sp. EN-8, *Bacillus* sp. EN-15, *Bacillus* sp. EN-24, *Actinomadura* sp. GKU 870, *Streptomyces* sp. GKU 833, and *Streptomyces* sp. GKU 878, effectively inhibited growth of *A. niger* and the pathogenic fungi, *F. moniliforme* and *C. falcatum*, causing red rot disease in sugarcane (Alexander and Viswanathan 2002). *B. cereus* and *Escherichia coli* were the test bacteria most frequently inhibited by the endophytic bacteria as well as actinomycetes (Table 2).

### Sugarcane Plant Growth Enhancement

Based on PGP traits, two strains of endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21, and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected to evaluate

growth enhancement of sugarcane in pot experiments (Table 3). Antagonistic test between each strain was performed and reviewed no effect to each other; therefore, they were suitable for co-culture inoculation. Nine treatments included (T1) *Enterobacter* sp. EN-21, (T2) *Bacillus* sp. EN-24, (T3) *Microbispora* sp. GKU 823, (T4) *Streptomyces* sp. GKU 895, (T5) EN-21 + EN-24, (T6) GKU 823 + GKU 895, (T7) GKU 823 + EN-21 + EN-24, (T8) GKU 895 + EN-21 + EN-24, (T9) GKU 823 + GKU 895 + EN-21 + EN-24 and un-inoculated control were conducted to investigate individual and co-culture inoculation. Two-month-old sugarcane plants treated by either individual or co-inoculation gave statistically significantly ( $p \leq 0.05$ ) greater root and shoot lengths, and root and shoot biomass than un-inoculated plants (Table 4; Fig. 2). Either individual endophytic diazotroph or actinomycete inoculated plants (T1, T2, T3, and T4) gave measurable increments in root length (51–89 %), shoot fresh weight (67–106 %), and shoot dry weight (76–146 %) but remarkably increased in root fresh weight (147–278 %; Table 4). The increase of root fresh weight was due to increased lateral root production (data not shown). These parameters were not significantly different when two strains of diazotrophs (T5) and actinomycetes (T6) were inoculated except shoot fresh (126 %) and dry weight (180 %) that T6 gave notably increased. The percentage increase in shoot length of individual and co-inoculated sugarcane were higher than that of the un-inoculated controls, especially T4, T5, T7, and T9, which were significantly (44–59 %) greater; while significant increase in root length was noticed in T7, T8, and T9 (125–128 %; Table 4). The most significant enhancement of growth appeared in T7 and T8 in which each endophytic



**Table 4** Effect of individual and co-inoculation of endophytic diazotrophs and actinomycetes on growth of sugarcane plants at 60 days after inoculation

Treatments	Root				Shoot				Root re-isolation (CFU g FW <sup>-1</sup> )	
	Length (cm)	FW (g)	DW (g)	Length (cm)	FW (g)	DW (g)	Bacteria	Actinomycetes		
Un-inoculated control	6.25 ± 0.96 <sup>b</sup>	1.04 ± 0.02 <sup>f</sup>	0.13 ± 0.02 <sup>e</sup>	30.58 ± 4.66 <sup>b</sup>	2.65 ± 0.08 <sup>d</sup>	0.30 ± 0.01 <sup>e</sup>	–	–		
T1: <i>Enterobacter</i> sp. EN-21	9.45 ± 1.54 <sup>ab</sup>	3.24 ± 0.10 <sup>de</sup>	0.25 ± 0.03 <sup>cd</sup>	42.62 ± 6.64 <sup>ab</sup>	4.88 ± 0.16 <sup>abc</sup>	0.70 ± 0.03 <sup>bc</sup>	5.27 ± 0.35 × 10 <sup>4</sup>	–		
T2: <i>Bacillus</i> sp. EN-24	11.43 ± 1.31 <sup>ab</sup>	3.93 ± 0.13 <sup>abcd</sup>	0.37 ± 0.01 <sup>ab</sup>	42.66 ± 3.48 <sup>ab</sup>	5.07 ± 0.22 <sup>abc</sup>	0.74 ± 0.04 <sup>abc</sup>	8.22 ± 0.17 × 10 <sup>4</sup>	–		
T3: <i>Microbispora</i> sp. GKU 823	9.82 ± 1.53 <sup>ab</sup>	2.57 ± 0.05 <sup>e</sup>	0.16 ± 0.02 <sup>e</sup>	39.82 ± 2.22 <sup>ab</sup>	4.43 ± 0.10 <sup>c</sup>	0.53 ± 0.03 <sup>d</sup>	–	9.47 ± 0.07 × 10 <sup>3</sup>		
T4: <i>Streptomyces</i> sp. GKU 895	11.85 ± 3.83 <sup>ab</sup>	3.34 ± 0.05 <sup>cde</sup>	0.26 ± 0.03 <sup>cd</sup>	43.98 ± 7.82 <sup>a</sup>	5.48 ± 0.12 <sup>abc</sup>	0.72 ± 0.05 <sup>bc</sup>	–	2.05 ± 0.50 × 10 <sup>4</sup>		
T5: EN-21 + EN-24	12.05 ± 1.87 <sup>ab</sup>	3.79 ± 0.14 <sup>abcd</sup>	0.22 ± 0.05 <sup>de</sup>	45.74 ± 6.30 <sup>a</sup>	4.62 ± 0.11 <sup>bc</sup>	0.63 ± 0.03 <sup>cd</sup>	8.27 ± 0.70 × 10 <sup>4</sup>	–		
T6: GKU 823 + GKU 895	11.49 ± 3.48 <sup>ab</sup>	4.24 ± 0.08 <sup>abc</sup>	0.33 ± 0.05 <sup>abc</sup>	40.64 ± 5.84 <sup>ab</sup>	5.99 ± 0.08 <sup>a</sup>	0.84 ± 0.09 <sup>a</sup>	–	9.22 ± 0.10 × 10 <sup>3</sup>		
T7: GKU 823 + EN-21 + EN-24	14.25 ± 3.71 <sup>a</sup>	4.70 ± 0.10 <sup>a</sup>	0.36 ± 0.09 <sup>ab</sup>	48.22 ± 6.56 <sup>a</sup>	5.73 ± 0.07 <sup>ab</sup>	0.74 ± 0.07 <sup>ab</sup>	6.67 ± 0.81 × 10 <sup>3</sup>	5.31 ± 0.15 × 10 <sup>3</sup>		
T8: GKU 895 + EN-21 + EN-24	14.30 ± 5.24 <sup>a</sup>	4.33 ± 0.05 <sup>ab</sup>	0.40 ± 0.04 <sup>a</sup>	41.92 ± 4.60 <sup>ab</sup>	5.98 ± 0.05 <sup>a</sup>	0.78 ± 0.06 <sup>ab</sup>	4.72 ± 0.65 × 10 <sup>3</sup>	4.35 ± 0.73 × 10 <sup>3</sup>		
T9: GKU 823 + GKU 895 + EN-21 + EN-24	14.12 ± 2.95 <sup>a</sup>	3.46 ± 0.10 <sup>bcd</sup>	0.28 ± 0.03 <sup>bcd</sup>	48.64 ± 2.21 <sup>a</sup>	5.29 ± 0.07 <sup>abc</sup>	0.73 ± 0.02 <sup>bc</sup>	1.49 ± 0.11 × 10 <sup>3</sup>	3.01 ± 0.85 × 10 <sup>3</sup>		

Data are means ± standard deviations (SD) of five replicates. Means designated with same letters do not differ significantly ( $p \leq 0.05$ ) according to the Tukey's multiple range test

FW fresh weight, DW dried weight

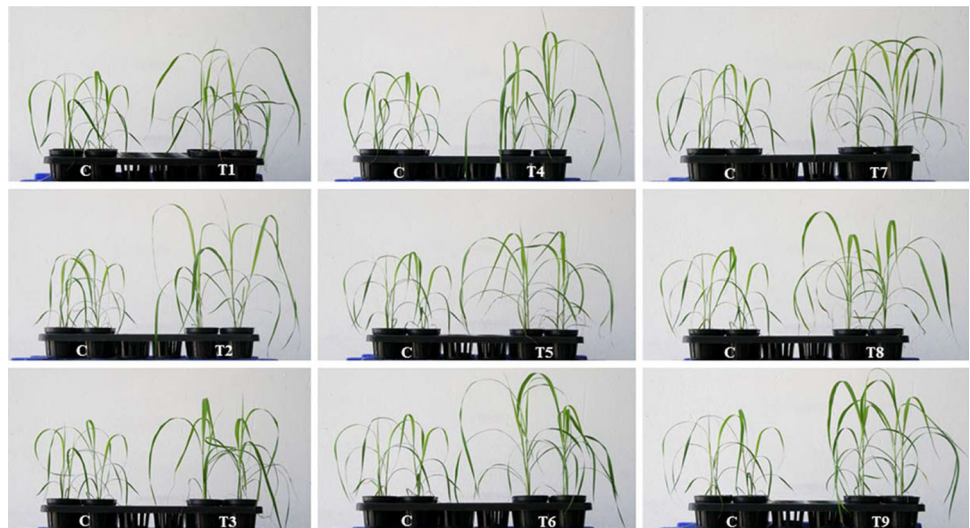
actinomycete was co-inoculated with both *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24. T7 and T8 caused drastic increases of 128–129 % in root length, 316–352 % in root fresh weight, 177–208 % in root dry weight, 37–58 % in shoot length, 116–126 % in shoot fresh weight, and 146–160 % in shoot dry weight over those of un-inoculated treatments. The results suggested that the enhancement of growth in sugarcane was due to multiple growth-promoting traits from the mixture of the bacterial endophytes (Table 3). Our results indicated that co-inoculation of either endophytic *Microbispora* or *Streptomyces* combined with the mixture of *Enterobacter* and *Bacillus* gave higher growth of sugarcane than individual inoculation.

Remarkably, when four strains of endophytes were co-inoculated onto sugarcane plants (T9), some growth parameters such as root and shoot fresh/dried weights were significantly lower than those of T7 and T8 and some (root fresh/dried weight) were even lower than single-strain inoculation (Table 4). Re-isolation experiments of the endophytes from two-month-old sugarcane plants were then performed and the identity of isolates was proved by examination of colony morphology and 16S rRNA sequencing. Inoculated endophytic diazotrophs and actinomycetes abundantly appeared in roots but less in stems in every treatment (data not shown). About 10<sup>4</sup> CFU g fresh weight<sup>-1</sup> were found in sugarcane roots inoculated with individual (T1, T2, T3, T4) and combinations of two bacterial strains (T5 and T6; Table 4). However, the viable cell numbers of the endophytes decreased 10 times when more than two strains of bacteria were co-inoculated (T7, T8, and T9); particularly T9 gave the lowest. This might be explained by a reduction of plant biomass in T9 (Table 4) due to the low population of the endophytes. In addition, this re-isolation experiment confirmed that *Enterobacter* sp. EN-21, *Bacillus* sp. EN-24, *Microbispora* sp. GKU 823, and *Streptomyces* sp. GKU 895 are true endophytes of sugarcane.

## Discussion

Plant growth-promoting endophytes (PGPE) are heterogeneous groups of bacteria that beneficial to host plants (Gaiero and others 2013). Several PGPE can encourage plant growth through the release of phytohormones, increment of nutrients, and protection from phytopathogens. In this work, culture-dependent approach was used to isolate culturable root-associated bacteria including actinomycetes from sugarcane cultivated in Thailand. The sugarcane roots used for isolation in this study were successfully surface disinfected in which no colonies were detected from the final rinses on any medium agar used;

**Fig. 2** Effects of individual and co-inoculation of endophytic bacteria and actinomycetes on growth promotion of sugarcane plants at 60 days after inoculation. T1, *Enterobacter* sp. EN-21; T2, *Bacillus* sp. EN-24; T3, *Microbispora* sp. GKU 823; T4, *Streptomyces* sp. GKU 895; T5, EN-21 + EN-24; T6, GKU 823 + GKU 895; T7, GKU 823 + EN-21 + EN-24; T8, GKU 895 + EN-21 + EN-24; T9, GKU 823 + GKU 895 + EN-21 + EN-24 and C, un-inoculated control



thus, the isolated bacteria were true endophytes. From the isolation procedures used in this work, 38.52 % of endophytic bacteria and 61.58 % of actinomycetes were obtained. Fourteen genera were systematically identified and the most dominant species were *Bacillus*, *Enterobacter*, *Microbispora*, and *Streptomyces*.

Diazotrophs appeared to be the most numerous bacteria so far isolated from sugarcane including *Azospirillum* spp., *Burkholderia* spp., *Enterobacter* spp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., *Klebsiella* spp., *Pantoea* spp., and *Pseudomonas* spp. (Asis and others 2000; Suman and others 2001; Perin and others 2006; Govindarajan and others 2007; Mendes and others 2007; Castro-González and others 2011; Taulé and others 2011; Lin and others 2012). Surprisingly, the well-known sugarcane diazotrophic species, including *Azospirillum* spp., *Burkholderia* spp., *G. diazotrophicus*, and *Herbaspirillum* spp. (Govindarajan and others 2007; Taulé and others 2011), were not found in this work. A plausible explanation for their notable absence could be that the composition of the endophytic bacterial community depends to some degree on a specificity of particular bacterial genotypes for particular sugarcane cultivars, different geographic origins, and level of fertilizers used (Reis-Junior and others 2000; Taulé and others 2011). Moreover, the sugarcane root-associated nitrogen-fixing bacteria in this work were isolated without using the enrichment method of reseeded in the N-free medium (Castro-González and others 2011), the amount of diazotrophic bacteria could, therefore, be affected and resulted in less population. The majority of endophytic bacteria isolated in this study were mainly found as members of the genera *Bacillus* and *Enterobacter*. The results were in agreement with several reports of which *Bacillus* and *Enterobacter* were dominant sugarcane

endophytes (Velazquez and others 2008; Magnani and others 2010; Taulé and others 2011).

Although endophytic actinomycetes have been isolated from several different kinds of plants including a variety of medicinal and crop plants (Coombs and Franco 2003; Tian and others 2007; Zhao and others 2011), this is the first report to describe the diversity of endophytic actinomycetes from sugarcane. The dominance of *Streptomyces* spp. in the culturable diversity of root-associated endophytes in this report has been noticed in several previous studies from various plant species, that is, wheat, rice, and herbaceous and medicinal plants (Coombs and Franco 2003; Tian and others 2007; Zhao and others 2011; Kim and others 2012; Li and others 2012). However, we found different genera of non-streptomycetes (*Actinomadura*, *Microbispora*, and *Micromonospora*) associated with roots of sugarcane (Fig. 1). The presence of *Actinomadura*, *Microbispora*, and *Micromonospora* was in agreement with previous reports that they are common genera found in root tissues of plants (Qin and others 2011; Mingma and others 2014). Although *Streptomyces* spp. was predominantly associated with sugarcane roots, we observed a large number of colonies of *Microbispora* spp. (21.48 %). The results suggested that *Microbispora* spp. also largely occupied in root tissues of sugarcane. This work indicated that sugarcane are rich bio-resources for diversity of endophytic bacteria including diazotrophs and actinomycetes.

PGP traits were examined in sugarcane endophytes and revealed that strains belong to genera *Streptomyces*, *Bacillus*, and *Enterobacter* showed the highest activity of PGP traits. *Bacillus* and *Enterobacter* have been previously reported to promote the growth of maize, sugarcane, and tobacco (Klopper and others 2004; Lin and others 2012; Naveed and others 2014). The highest frequency of PGP

trait possessed by sugarcane endophytes in this study was siderophore production, followed by IAA production and phosphate solubilization. It has been suggested that siderophores are involved in both plant growth and health protection by chelating iron (Jaber and others 2002; Rungin and others 2012; Radzki and others 2013). Recent work of Rungin and others (2012) also confirmed that a siderophore-producing endophytic *Streptomyces* increased root/shoot lengths and biomass of rice and mung bean compared to the deficient mutant. It was demonstrated that bacterial endophytes carrying IAA trait could increase root elongation and could affect the development of lateral roots, which improves the plant's nutrient uptake from the rhizosphere (Idris and others 2007; Goudjal and others 2013). A recent report showed that *Microbispora* spp. and *Streptomyces* spp. exhibited a high variability in IAA production and resulted in significant promotion of shoot length of mandarin seedlings (Shutsrirung and others 2013). Members of PGPE in the genera *Bacillus*, *Enterobacter*, *Pseudomonas*, *Micromonospora*, and *Streptomyces* are known as phosphate solubilizers (Hamdali and others 2008; Bashan and others 2013) with the ability to convert insoluble compounds of phosphorus into available phosphates that enhance nutrient availability to plants (Son and others 2006). However, the highest activity of phosphate solubilization in this work was found in *Pantoea* spp. Recently, phosphate-solubilizing *Pantoea* has been reported as a plant growth promoter which increased height and dry weight of *Lotus tenuis* cv. Pampa INTA (Castagno and others 2011). PGPE harboring ACC deaminase in sugarcane could facilitate plant growth by conversion of ACC to ammonia and  $\alpha$ -ketobutyrate, which bacteria can consume and consequently lower the ethylene level in plants (Glick 2005).

Most of the endophytic bacteria isolated from sugarcane have been reported as diazotrophs, with effects on plant growth promotion (Govindarajan and others 2007; Lin and others 2012). In this work, only 11.53 % out of sugarcane endophytic bacteria showed nitrogenase activity belonging to *Bacillus*, *Enterobacter*, *Lysinibacillus*, and *Pantoea* (Table 2). Although the standard protocol for isolation of diazotrophic bacteria was employed (Cavalcante and Dobreiner 1988), fewer ARA positives were often obtained from plants such as rye grass (Habibi and others 2014) and rice (Rangjaroen and others 2015). Our ranges of ARA activity were in accordance with previous reports of *Enterobacter* spp. and *Bacillus* spp. (Habibi and others 2014). When *nifH* gene amplification was investigated to evaluate nitrogen fixation ability of ARA-positive endophytes, 19.23 % of the total was detected (Table 2). There were only five strains which exhibited both nitrogenase activity and a *nifH* gene (Fig. S1). Our results were in agreement with Yim and others (2009) in which the *nifH*

gene could not be amplified from all ARA positives. Although, ARA is a common method to detect nitrogenase activity of microbial cultures and has been widely used to consequently identify nitrogen-fixing bacteria (Hardy and others 1973), it was suggested that such activity varies with growth stage, culture condition, and media composition. Hence, amplification of the *nifH* gene, encoding dinitrogenase reductase, was additionally used to perform the possible nitrogen-fixing ability. Although the *nifH* gene is the most widely used marker gene for identification of nitrogen-fixing bacteria, nucleotide sequences of *nifH* genes were diverse in many microorganisms (Waugh and others 1995; Zehr and others 2003). In this experiment, degenerate primers were used for amplification of *nifH* gene (Rösch and others 2002), nevertheless, it was yet absent in an ARA-positive *Bacillus* sp. EN-24 (Fig. S1). The absence of the *nifH* gene could therefore be explained by the variability of this gene (Waugh and others 1995; Zehr and others 2003). It is suggested that the other nitrogenase genes, *nifD* and *nifK*, could be used to confirm the nitrogen-fixing ability since *nifHDK* genes are known to encode the components of the nitrogenase enzyme complex (Howard and Rees 1996).

Some recent reports have indicated that the root nodule-associated *Micromonospora* has the possible ability to fix atmospheric nitrogen (Trujillo and others 2010; Lorena and others 2012), but endophytic *Micromonospora* from medicago plants apparently had no functional nitrogenase and consequently no nitrogen fixation occurred (Martinez and others 2014). Because nitrogen fixation in endophytic actinomycetes has not yet been definitively explained and there is very little information about it, ARA assays were not performed on actinomycetes in this work.

Antagonistic tests revealed susceptible bacteria and fungi including *F. moniliforme* and *C. falcatum* causing red rot disease in sugarcane (Alexander and Viswanathan 2002) that interacted with the endophytes. *B. cereus* and *E. coli* were most frequently inhibited (Table 2), which was consistent with a previous report (Hassan and others 2010). The results of this study indicated that endophytic bacteria and actinomycetes from sugarcane carried multiple traits of PGP and are therefore considered to be safe agents to apply for plant growth enhancement and to control phytopathogens in sugarcane plantations.

Based on PGP traits, two endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21, and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected to evaluate growth enhancement of sugarcane plants by individual and co-inoculation. Two-month-old sugarcane plants treated by either individual or co-inoculation gave significantly greater biomass and lengths of root and shoot than uninoculated control (Table 4; Fig. 2). The increase of root

fresh weight was due to increased lateral root production, which might be the effect of plant hormone production by the endophytes (Idris and others 2007; Goudjal and others 2013). The most significant enhancement of growth appeared in the treatment that each endophytic actinomycete was co-inoculated with both *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24. Because some *Enterobacter* species are known to be human pathogens, *Enterobacter* sp. EN-21 should be further investigated concerning human health if it is to be applied at the field level. It has been demonstrated that several diazotrophic bacteria, including *Bacillus* and *Enterobacter*, could promote growth of sugarcane (Lin and others 2012) as well as jatropha, maize, rice, sorghum, sugarcane, and wheat (Olivares and others 1997; Yanni and others 2001; Govindarajan and others 2007; Luna and others 2010; Rungin and others 2012; de Jesus Santos and others 2014; Alves and others 2015; Qin and others 2015). Furthermore, co-inoculation of five diazotrophs (*Azospirillum*, *Burkholderia*, *G. diazotrophicus*, *Klebsiella*, and *Pseudomonas*) was reported to give better growth of rice (Govindarajan and others 2007); while, co-culture of diazotrophs and a fungal endophyte, *Trichoderma*, revealed better growth and yield of chickpea (Verma and others 2014). It was suggested that the combination of phosphate-solubilizing, nitrogen-fixing, and phytohormone producing bacteria could provide a more balanced nutrition for plants and stimulate more growth of plants. For example, the combination of a phosphate-solubilizing *B. megaterium*, nitrogen-fixing *B. subtilis*, and *Rhizobium leguminosarum* *bv. phaseoli* caused a significant enhancement of seed yield and an uptake of macronutrients and micronutrients elements in common bean (Elkoca and others 2010). Mixed inoculation of *Rhizobium*, a phosphate-solubilizing *B. megaterium* subsp. *phospaticum* strain-PB, and a biocontrol fungus *Trichoderma* sp. was reported to increase seed germination, nutrient uptake, plant height, number of branches, nodulation, pea yield, and total biomass of chickpea compared to either individual inoculation or an un-inoculated control (Rudresh and others 2005). Therefore, the plant growth-promoting properties of endophytic bacteria and actinomycetes suggest that these bacteria merit further investigation for potentially safe and environmentally friendly biofertilizers which can help us limit the use of chemical fertilizers in agriculture.

**Acknowledgments** WK was awarded a Ph.D. scholarship by the Royal Golden Jubilee of the Thailand Research Fund (RGJ-TRF). The authors thank the Soil Microbiology Research Group, Department of Agriculture for their support and for providing facilities for ARA assay. This work was supported by Thailand Research Fund under Grant No. BRG5880004; Faculty of Science, Kasetsart University under Grant No. RFG 1-11 and Mitr Phol Sugarcane Research Center.

## Compliance with Ethical Standards

**Conflict of interest** None.

## References

- Alexander K, Viswanathan R (2002) Diseases of sugarcane in India and its rapid diagnosis. In: Singh SB, Rao GP, Eswaramoorthy S (eds) Sugarcane crop management. Sci Tech Publishing, USA, pp 10–51
- Alves G, Videira S, Urquiaga S, Reis V (2015) Differential plant growth promotion and nitrogen fixation in two genotypes of maize by several *Herbaspirillum* inoculants. Plant Soil 387:307–321
- Anand TP, Bhat AW, Shouche YS, Roy U, Siddharth J, Sarma SP (2006) Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East India. Microbiol Res 161:252–262
- Asis CA, Kubota M, Ohta H, Arima Y, Chebotar VK, K-i Tsuchiya, Akao S (2000) Isolation and partial characterization of endophytic diazotrophs associated with Japanese sugarcane cultivar. Soil Sci Plant Nutr 46:759–765
- Baldani J, Reis V, Videira S, Boddey L, Baldani V (2014) The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. Plant Soil 384:413–431
- Bashan Y, Holguin G (1997) Azospirillum-plant relationships: environmental and physiological advances (1990–1996). Can J Microbiol 43:103–121
- Bashan Y, Kamnev A, de-Bashan L (2013) Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. Biol Fert Soils 49:465–479
- Castagno LN, Estrella MJ, Sannazzaro AI, Grassano AE, Ruiz OA (2011) Phosphate-solubilization mechanism and *in vitro* plant growth promotion activity mediated by *Pantoea eucalypti* isolated from *Lotus tenuis* rhizosphere in the Salado River Basin (Argentina). J Appl Microbiol 110:1151–1165
- Castro-González R, Martínez-Aguilar L, Ramírez-Trujillo A, Estrada-de los Santos P, Caballero-Mellado J (2011) High diversity of culturable *Burkholderia* species associated with sugarcane. Plant Soil 345:155–169
- Cavalcante VA, Dobereiner J (1988) A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant Soil 108:23–31
- Coombs JT, Franco CMM (2003) Isolation and identification of actinobacteria from surface-sterilized wheat roots. Appl Environ Microbiol 69:5603–5608
- de Jesus Santos A, Martins C, Santos P, Corrêa É, Barbosa H, Sandoval A, Oliveira L, de Souza J, Soares A (2014) Diazotrophic bacteria associated with sisal (*Agave sisalana* Perrine ex Engelm): potential for plant growth promotion. Plant Soil 385:37–48
- Dobereiner J, Day JM (1976) Associative symbiosis in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. In: Newton W, Nyman C (eds) Proceeding of The first international symposium on nitrogen fixation. Washington State University Press, Washington, pp 518–538
- Elkoca E, Turan M, Donmez MF (2010) Effects of single, dual and triple inoculation with *Bacillus subtilis*, *Bacillus megaterium* and *Rhizobium leguminosarum* *bv. phaseoli* on nodulation, nutrient

- uptake, yield and yield parameters of common bean (*Phaseolus vulgaris* L. cv. 'Elkoca-05'). *J Plant Nutr* 33:2104–2119
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Fokkema NJ (1976) Antagonism between fungal saprophytes and pathogens on aerial plant surfaces. In: Dickinson CH, Preece TF (eds) *Microbiology of aerial plant surfaces*. Academic Press, London, pp 487–505
- Food and Agriculture Organization of the United Nations (2015) *Crop production*. Accessed 24 Oct 2015
- Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE (2013) Inside the root microbiome: bacterial root endophytes and plant growth promotion. *Am J Bot* 100:1738–1750
- Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiol Lett* 251:1–7
- Glick BR (2012) Plant-growth promoting bacteria: mechanisms and applications. *Scientifica (Cairo)* 2012:963401
- Glick BR, Karaturović D, Newell P (1995) A novel procedure for rapid isolation of plant growth-promoting rhizobacteria. *Can J Microbiol* 41:533–536
- Goudjal Y, Toumatia O, Sabaou N, Barakate M, Mathieu F, Zitouni A (2013) Endophytic actinomycetes from spontaneous plants of Algerian Sahara: indole-3-acetic acid production and tomato plants growth promoting activity. *World J Microbiol Biotechnol* 29:1821–1829
- Govindarajan M, Kwon S-W, Weon H-Y (2007) Isolation, molecular characterization and growth-promoting activities of endophytic sugarcane diazotroph *Klebsiella* sp. GR9. *World J Microbiol Biotechnol* 23:997–1006
- Green MR, Sambrook J (2012) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, New York
- Gupta A, Gopal M, Tilak KV (2000) Mechanism of plant growth promotion by rhizobacteria. *Indian J Exp Biol* 38:856–862
- Habibi S, Djedidi S, Prongjunthuek K, Mortuza M, Ohkama-Ohtsu N, Sekimoto H, Yokoyama T (2014) Physiological and genetic characterization of rice nitrogen fixer PGPR isolated from rhizosphere soils of different crops. *Plant Soil* 379:51–66
- Hamdali H, Bouizgarne B, Hafidi M, Lebrihi A, Virolle MJ, Ouhdouch Y (2008) Screening for rock phosphate solubilizing actinomycetes from Moroccan phosphate mines. *Appl Soil Ecol* 38:12–19
- Hardy RWF, Burns RC, Holsten RD (1973) Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biol Biochem* 5:47–81
- Hassan MN, Afghan S, Hafeez FY (2010) Suppression of red rot caused by *Colletotrichum falcatum* on sugarcane plants using plant growth-promoting rhizobacteria. *Biocontrol* 55:531–542
- Hobbs G, Frazer C, Gardner DJ, Cullum J, Oliver S (1989) Dispersed growth of *Streptomyces* in liquid culture. *Appl Microbiol Biotechnol* 31:272–277
- Howard JB, Rees DC (1996) Structural basis of biological nitrogen fixation. *Chem Rev* 96:2965–2982
- Idris EE, Iglesias DJ, Talon M, Borriss R (2007) Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Mol Plant Microbe Interact* 20:619–626
- Jaber M, Harald K, Ömer E, Konrad M (2002) The central role of microbial activity for iron acquisition in maize and sunflower. *Biol Fertil Soils* 30:433–439
- Kieser T, Bibb M, Buttner M, Chater K, Hopwood D (2000) *Practical Streptomyces genetics*. The John Innes Foundation, Norwich
- Kim T-U, Cho S-H, Han J-H, Shin Y, Lee H, Kim S (2012) Diversity and physiological properties of root endophytic actinobacteria in native herbaceous plants of Korea. *J Microbiol* 50:50–57
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kloepper JW, Ryu C-M, Zhang S (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94:1259–1266
- Küster E, Williams ST (1964) Selection of media for isolation of streptomycetes. *Nature* 202:928–929
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons, New York, pp 115–175
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948
- Li J, Zhao G-Z, Huang H-Y, Qin S, Zhu W-Y, Zhao L-X, Xu L-H, Zhang S, Li W-J, Strobel G (2012) Isolation and characterization of culturable endophytic actinobacteria associated with *Artemisia annua* L. *Antonie Van Leeuwenhoek* 101:515–527
- Lin L, Li Z, Hu C, Zhang X, Chang S, Yang L, Li Y, An Q (2012) Plant growth-promoting nitrogen-fixing enterobacteria are in association with sugarcane plants growing in Guangxi, China. *Microbes Environ* 27:391–398
- Lorena C, Cathrin S, Pilar A, Trujillo ME (2012) Diversity of *Micromonospora* strains isolated from nitrogen fixing nodules and rhizosphere of *Pisum sativum* analyzed by multilocus sequence analysis. *Syst Appl Microbiol* 35:73–80
- Luna MF, Galar ML, Aprea J, Molinari ML, Boiardi JL (2010) Colonization of sorghum and wheat by seed inoculation with *Gluconacetobacter diazotrophicus*. *Biotechnol Lett* 32:1071–1076
- Magnani GS, Didonet CM, Cruz LM, Picheth CF, Pedrosa FO, Souza EM (2010) Diversity of endophytic bacteria in Brazilian sugarcane. *Genet Mol Res* 9:250–258
- Martinez HP, Olivares J, Delgado A, Bedmar E, Martinez Molina E (2014) Endophytic *Micromonospora* from *Medicago sativa* are apparently not able to fix atmospheric nitrogen. *Soil Biol Biochem* 74:201–203
- Mendes R, Pizzirani-Kleiner AA, Araujo WL, Raaijmakers JM (2007) Diversity of cultivated endophytic bacteria from sugarcane: genetic and biochemical characterization of *Burkholderia cepacia* complex isolates. *Appl Environ Microbiol* 73:7259–7267
- Meunchang S, Panichsakpatana S, Ando S, Yokoyama T (2004) Phylogenetic and physiological characterization of indigenous *Azospirillum* isolates in Thailand. *Soil Sci Plant Nutr* 50:413–421
- Mingma R, Pathom-aree W, Trakulnaleamsai S, Thamchaipenet A, Duangmal K (2014) Isolation of rhizospheric and roots endophytic actinomycetes from Leguminosae plant and their activities to inhibit soybean pathogen, *Xanthomonas campestris* pv. *glycine*. *World J Microbiol Biotechnol* 30:271–280
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Musson G, McInroy JA, Kloepper JW (1995) Development of delivery systems for introducing endophytic bacteria into cotton. *Biocontrol Sci Technol* 5:407–416
- Naveed M, Mitter B, Yousaf S, Pastar M, Afzal M, Sessitsch A (2014) The endophyte *Enterobacter* sp. FD17: a maize growth enhancer selected based on rigorous testing of plant beneficial traits and colonization characteristics. *Biol Fert Soils* 50:249–262
- Olivares FL, James EK, Baldani JJ, Döbereiner J (1997) Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. *New Phytol* 135:723–737
- Perin L, Martinez-Aguilar L, Castro-Gonzalez R, Estrada-de Los Santos P, Cabellos-Avelar T, Guedes HV, Reis VM, Caballero-Mellado J (2006) Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. *Appl Environ Microbiol* 72:3103–3110

- Pikovskaya RI (1948) Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Mikrobiologiya* 17:362–370
- Pilet PE, Chollet R (1970) Sur le dosage colorimétrique de l'acide indolylacétique. *C R Acad Sci Paris Ser D* 271:1675–1678
- Qin S, Xing K, Jiang J-H, Xu L-H, Li W-J (2011) Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. *Appl Microbiol Biotechnol* 89:457–473
- Qin S, Miao Q, Feng W-W, Wang Y, Zhu X, Xing K, Jiang J-H (2015) Biodiversity and plant growth promoting traits of culturable endophytic actinobacteria associated with *Jatropha curcas* L. growing in Panxi dry-hot valley soil. *Appl Soil Ecol* 93:47–55
- Rachniyom H, Matsumoto A, Indananda C, Duangmal K, Takahashi Y, Thamchaipenet A (2015) *Nonomuraea syzygii* sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels). *Int J Syst Evol Microbiol* 65:1234–1240
- Radzki W, Gutierrez Mañero FJ, Algar E, Lucas García JA, García-Villaraco A, Ramos Solano B (2013) Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. *Antonie Van Leeuwenhoek* 104:321–330
- Rangjaroen C, Rerkasem B, Teaumroong N, Noisangiam R, Lumyong S (2015) Promoting plant growth in a commercial rice cultivar by endophytic diazotrophic bacteria isolated from rice landraces. *Ann Microbiol* 65:253–266
- Reis-Junior F, Reis V, Silva L, Döbereiner J (2000) Levantamento e quantificação de bactérias diazotróficas em diferentes genótipos de cana-de-açúcar (*Saccharum* spp.). *Pesq Agro Bras* 35:985–994
- Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol Adv* 17:319–339
- Rösch C, Mergel A, Bothe H (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl Environ Microbiol* 68:3818–3829
- Rudresh DL, Shivaprakash MK, Prasad RD (2005) Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea (*Cicer arketinum* L.). *Appl Soil Ecol* 28:139–146
- Rungin S, Indananda C, Suttiviriya P, Kruasuwan W, Jaemsang R, Thamchaipenet A (2012) Plant growth enhancing effects by a siderophore-producing endophytic streptomycete isolated from a Thai jasmine rice plant (*Oryza sativa* L. cv. KDML105). *Antonie Van Leeuwenhoek* 102:463–472
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160:47–56
- Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* 16:313–340
- Shutsrirung A, Chromkaew Y, Pathom-Aree W, Choonluchanon S, Boonkerd N (2013) Diversity of endophytic actinomycetes in mandarin grown in northern Thailand, their phytohormone production potential and plant growth promoting activity. *Soil Sci Plant Nutr* 59:322–330
- Son HJ, Park GT, Cha MS, Heo MS (2006) Solubilization of insoluble inorganic phosphates by a novel salt and pH tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. *Bioresour Technol* 97:204–210
- Suman A, Shasany AK, Singh M, Shahi HN, Gaur A, Khanuja SPS (2001) Molecular assessment of diversity among endophytic diazotrophs isolated from subtropical Indian sugarcane. *World J Microbiol Biotechnol* 17:39–45
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729
- Taulé C, Mareque C, Barlocco C, Hackembruch F, Reis VM, Sicardi M, Battistoni F (2011) The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum* L.), and the identification and characterization of part of the associated diazotrophic bacterial community. *Plant Soil* 356:35–49
- Tian X, Cao L, Tan H, Han W, Chen M, Liu Y, Zhou S (2007) Diversity of cultivated and uncultivated actinobacterial endophytes in the stems and roots of rice. *Microb Ecol* 53:700–707
- Tilman D (1998) The greening of the green revolution. *Nature* 396:211–212
- Trujillo ME, Alonso-Vega P, Rodríguez R, Carro L, Cerda E, Alonso P, Martínez-Molina E (2010) The genus *Micromonospora* is widespread in legume root nodules: the example of *Lupinus angustifolius*. *ISME J* 4:1265–1281
- Velazquez E, Rojas M, Lorite MJ, Rivas R, Zurdo-Pineiro JL, Heydrich M, Bedmar EJ (2008) Genetic diversity of endophytic bacteria which could be found in the apoplastic sap of the medullary parenchyma of the stem of healthy sugarcane plants. *J Basic Microbiol* 48:118–124
- Verma JP, Yadav J, Tiwari KN, Jaiswal DK (2014) Evaluation of plant growth promoting activities of microbial strains and their effect on growth and yield of chickpea (*Cicer arietinum* L.) in India. *Soil Biol Biochem* 70:33–37
- Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* 255:571–586
- Waugh SI, Paulsen DM, Mylona PV, Maynard RH, Premakumar R, Bishop PE (1995) The genes encoding the delta subunits of dinitrogenases 2 and 3 are required for Mo-independent diazotrophic growth by *Azotobacter vinelandii*. *J Bacteriol* 177:1505–1510
- Yanni YG, Rizk RY, El-Fattah FKA, Squartini A, Corich V, Giacomini A, de Bruijn F, Rademaker J, Maya-Flores J, Ostrom P, Vega-Hernandez M, Hollingsworth RI, Martinez-Molina E, Mateos P, Velazquez E, Wopereis J, Triplett E, Umali-Garcia M, Anama JA, Rolfe BG, Ladha JK, Hill J, Mujoo R, Perry KN, Dazzo FB (2001) The beneficial plant growth-promoting association of *Rhizobium leguminosarum* bv. *trifolii* with rice roots. *Aust J Plant Physiol* 28:845–870
- Yim W-J, Poonguzhali S, Madhaiyan M, Palaniappan P, Siddikee MA, Sa T (2009) Characterization of plant-growth promoting diazotrophic bacteria isolated from field grown Chinese cabbage under different fertilization conditions. *J Microbiol* 47:147–155
- Zehr JP, Jenkins BD, Short SM, Steward GF (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environ Microbiol* 5:539–554
- Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J, Lindstrom K, Zhang L, Zhang X, Strobel GA (2011) The diversity and antimicrobial activity of endophytic actinomycetes isolated from medicinal plants in Panxi plateau, China. *Curr Microbiol* 62:182–190