Ann Microbiol (2015) 65:253–266
DOI 10.1007/s13213-014-0857-4

ORIGINAL ARTICLE

Promoting plant growth in a commercial rice cultivar by endophytic diazotrophic bacteria isolated from rice landraces

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Received: 23 September 2013 / Accepted: 28 February 2014 / Published online: 11 April 2014

Abstract Population density of endophytic diazotrophic bacteria (EDB) was highest in the rice landrace root tissues at nursery stage. Indole-3-acetic acid (IAA) production (0.85–16.66 μg mL^{-1}) was found in 21 strains tested. More than 80 % (18 isolates) of the isolates solubilized phosphate, while only 28.57 % (six isolates) of selected strains produced siderophore. Seventy-one percent of tested isolates produced ammonia. The effects of EDB isolated from rice landraces on seed and on the growth of the commercial jasmine rice cultivar Khao Dawk Mali 105 were evaluated in greenhouse. Inoculation of all EDB on rice seeds significantly increased nitrogen content in roots (P=0.05). The potentially useful isolates belonged to four different genera *Burkholderia*, *Klebsiella*, *Novosphingobium* and *Sphingomonas*. In vivo colonization of *Burkholderia* sp. SS5, *Klebsiella* sp. SS2, *Novosphingobium* sp. TR4 and *Sphingomonas* sp. PS5 was confirmed using the commercial rice cultivar Khao Dawk Mali 105 as a model host. The inoculated roots with ß-glucuronidase (GUS)-tagged bacteria exhibited a blue color, which was most intense at the tip of root hairs, root tips, germination point and leaf tips.

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Keywords Rice · Endophytic diazotrophic bacteria · Plant growth promoter · Symbiotic colonization

Introduction

Rice landraces mostly grown for household consumption in Thailand reveal potential for cultivation in natural farming systems (Rangjaroen et al. 2014). Previous studies reported some of their qualities, such as increased higher iron content in their rice grains (Pintasen et al. 2007), tolerance to soil acidity (Phattarakul 2008), and insect pest resistance (Oupkaew et al. 2011), in different sets of germplasms. Such qualities may be influenced by both genetic variation and cultivation condition. Although nitrogen fertilizer is generally required in rice production (Ladha and Reddy 2003; Ishii et al. 2011), demand for crops grown without chemical inputs is increasing. The rice landraces growing without nitrogen fertilizer are likely to use naturally available nitrogen, derived either from biological nitrogen fixation by free-living and plant-associated diazotrophs, or from microbial mineralization of soil nitrogen. There are a number of studies concerning the diazotrophic bacteria that live in association with the rice plant, in genera including *Pantoea*, *Bacillus*, and *Sphingomonas* in the seed (Mano et al. 2007), *Bacillus*, *Bukholderia*, *Herbaspirillum*, *Klebsiella*, *Methylobacterium*, and *Novosphingobium* in the roots (Verma et al. 2004; Mano and Morisaki 2008), and *Azospirillum* and *Herbaspirillum* in the stem (Koomnok et al. 2007; Mano and Morisaki 2008). For one part of their life cycle, these endophytic bacteria inhabit the internal tissue of plants without eliciting any pathogenic symptoms or negative effect on their host (Ryan et al. 2008; Reinhold-Hurek and Hurek 2011). Many reports have revealed that these diazotrophic bacteria have the potential to promote plant growth, through various mechanisms, such as solubilization of inorganic phosphate and mineralization of organic phosphate (Jha and Kumar 2007), siderophore

production (Loaces et al. 2011) and synthesis of plant growth regulators like indole acetic acid (Piromyou et al. 2011; Arun et al. 2012), abscisic acid, gibberellic acid, cytokinins (Feng et al. 2006), vitamins (Baldani et al. 2000) and production of ACC-deaminase to reduce the level of ethylene (Tittabutr et al. 2008; Jha et al. 2012). Some of them, such as *Xanthomonas oryzae* and *Magnaporthe oryzae* (Yasuda et al. 2009), and *Botrytis cinerea* (Compant et al. 2005), exhibited biological control function against plant pathogens. These are evidences to support how the plant–microbe interaction has become one of the main factors of plant health and crop production development in natural production.

There are also many reports of application of EDB as a biofertilizer in the enhancement of rice growth and yield. Inoculation of *Azospirillum amazonense* increased dry matter accumulation of rice grain (7 to 11.6 %), the number of panicles (3 to 18.6 %) and nitrogen accumulation at grain maturation (3.5 to 18.5 %) (Rodrigues et al. 2008). Moreover, *A. brasilense* inoculum was reported to increase yield of rice up to 76.0 % (Thakuria et al. 2004). Some of these bacteria have already been applied as biofertilizers and they are available to farmers (Beatty and Good 2011). However, aside from environmental factors, the plant genotype is a very important agronomical aspect that should be considered during selection of diazotrophic strains, since it may act as selective filter for the endophytic bacteria that associate with the plants (Araújo et al. 2013). Many researchers have observed that wild (Engelhard et al. 2000; Elbeltagy et al. 2001; Chaudhary et al. 2012) and cultivated rice cultivars (Elbeltagy et al. 2001; Koomnok et al. 2007) are associated with different bacteria, and have suggested that it may result from plant breeding for higher yielding crop varieties. Rice landraces are known to have high genetic variation compared to modern improved cultivars. There are few reports about the isolation of EDB from rice landraces cultivated without fertilizer for their application in cultivation of commercial rice varieties. Among all rice-traded types, Thai jasmine rice has achieved recognition for its high quality. Since 1988, export volume of jasmine rice from Thailand has been growing at the average rate of 100,000 tons per year (Rerkasem 2007). Especially, the premium jasmine rice cultivar Khao Dawk Mali 105 is the most famous because of its aroma and tender cooking properties (Sapsirisopa et al. 2009). This study aimed to evaluate the potential of EDB isolated from different highland rice landraces for plant growth promotion. The possibility to apply EDB as a bio-fertilizer for cultivation of the commercial rice cultivar Khao Dawk Mali 105 was examined both in vitro and in vivo. In addition, the *in planta* symbiotic colonizations of the EDB were investigated to understand their existence for sustaining rice production.

Materials and methods

Sampling conditions and plant materials

Six rice landraces including Bue Po Lo-1 (BP1), Bue Po Lo-2 (BP2), Bue Pra Taw-1 (BPT-1), Bue Pra Taw-2 (BPT-2), Bue Pra Do (BPD) and Bue Saw Mi (BSM) were collected from a highland village at Chom Thong District, Chiang Mai, Thailand (1,137–1,214 m above sea level). Their geographical location was determined to be latitude N18.38798-N18.39682 and longitude E098.50261-E098.51631. The farm was established in 2005, and aimed to cultivate a variety of crops for household consumption of villagers living in the area. The farming system mainly involved natural organic practices with little or no chemical input. The rice seeds were obtained from the farmer in April, while other rice plant tissues at nursery, vegetative and reproductive stages were prepared from ten individual rice plants taken from the fields in June, August and September. Selected plants were transferred in plastic bags kept in an ice-box, and subsequently processed within 12 h.

Isolation of endophytic diazotrophic bacteria from rice plant tissues

The fresh rice tissue samples were gently washed with tap water and rinsed with sterilized water several times. Leaf, stem and root were separated and cut into 2–3 cm pieces. Each tissue derived from ten plants at the same growth stage were pooled together and surface sterilized with 70 % (v/v) ethyl alcohol for 1 min, 2 % (v/v) NaOCl for 2 min, 95 % (*v/v*) ethyl alcohol for 30 s and 30 % (v/v) H_2O_2 for 1 min, respectively, followed by washing four times with sterilized distilled water; excess moisture was then removed with sterile absorbent papers. Five pieces of sterilized samples from each rice tissue type and growth stage were placed on nutrient agar to confirm surface sterilization.

N-free Rennie medium semisolid agar (Elbeltagy et al. 2001) was used as a medium in serial dilutions for selective isolation of EDB. The medium was incubated at 30 °C for 5 days. Population density was estimated by most probable number (MPN) (Roesch et al. 2006) using the freeware MPN calculator (Saengkerdsub et al. 2007). Positive tubes were then applied onto plates containing N-free agar (Döbereiner et al. 1972) and incubated at 30 °C for 3 days. Single colonies of bacteria were picked, kept growing as subsurface pellicles (Prakamhang et al. 2009) in N-free RMR semisolid agar, and grown at 30 °C for 5 days. The pathogenicity of the obtained bacteria was tested by induction of infection on *Nicotiana tabacum* leaf (Klement 1963). Briefly, each bacterial isolate (10^7 CFU mL^{-1}) was inoculated into the intercellular spaces of tobacco leaf by injection, and the presence of necrotic zones was estimated after 48 h compared to positive (sterilized water) and negative controls (*X. oryzae*).

Evaluation of plant growth promotion potential

Nitrogenase activity of diazotropic bacteria at free-living stage

The nitrogen-fixing activity of the bacterial isolates was determined by acetylene reduction assay (ARA). The bacterial isolates were grown in N-free RMR semisolid agar at 30 °C for 7 days in test tubes. Air was removed from the culture tubes. Acetylene gas was then injected into the culture tubes to replace the air, at a final concentration of 10 % (v/v). The cultures were then incubated at 30 °C for 24 h. The ethylene concentration in head space was measured using a gas chromatograph equipped with flame ionization detector and Porapak-N column (Koomnok et al. 2007).

Nitrogenase activity of diazotropic bacteria at symbiotic stage

Nitrogenase activity of the bacterial isolates associated with rice seedlings was evaluated using the ARA method, as previously mentioned. In order to prepare the rice seedlings, unhusked rice seed was sterilized for 3 min in 95 % ethanol, and the husk was removed by hand. The seed without husk was then surface sterilized by a sterilization solution (1 l of the sterilization solution contains 30 mL of 10 % (v/v) sodium hypochlorite, 1 g of Na_2CO_3, 30 g of NaCl and 1.5 g NaOH) (Hurek et al. 1994). The seed was washed three times with sterile distilled water and treated by shaking in 0.01 % (w/v) $HgCl_2$ solution at room temperature for 7 min, followed by washing with sterile distilled water (Sriskandarajah et al. 1993). Each seed was planted into a culture tube containing 5 mL of nitrogen-free rice growth medium (Elbeltagy et al. 2001), and inoculated with each bacterial isolate (approximately 10^8 CFU mL^{-1}). The rice was grown for 7 days, and acetylene was then injected into gas phase, followed by the measurement steps described above. The nitrogenase activity was compared with and without bacterial inoculation of the same rice cultivar, with five replicates for each treatment.

Production of indole-3-acetic acid (IAA)

The production of IAA was determined by a modified colorimetric method (Glickmann and Dessaux 1995). Selected isolates of the EDB (10^7 CFU mL^{-1}) were inoculated into JM medium supplemented with 10 mM NH_4Cl and 100 μg mL^{-1} DL-tryptophan (Hartmann et al. 1983) and incubated at 30 °C for 72 h. Bacterial cultures were centrifuged at 4 °C at 10,000×*g* for 15 min, and the supernatant was quantified colorimetrically at 530 nm using Salkowski's reagent (Gordon and Weber 1951) compared to a standard indole-3-acetic acid solution (Sigma, USA). All experiments were done in five replicates.

Production of ammonia

The selected isolates of EDB were tested for the production of ammonia in peptone water. The bacteria (10^7 CFU mL^{-1}) were grown in peptone water and incubated at 30 °C for 48 h. The culture broth was centrifuged at 4 °C at 10,000×*g* for 15 min. The supernatant was collected and mixed with 0.5 mL of Nessler's reagent (Hach company, Loveland, USA). Development of brown to yellow color was recorded as a positive test for ammonia production (Cappuccino and Sherman 2001). All experiments were done in five replicates.

Production of siderophore

Chrome azurol S (CAS) assay was used to detect siderophores produced by the selected isolates of EDB (Schwyn and Neilands 1987). The isolates were stabbed on CAS agar plates and incubated in the dark at 30 °C for 2 days. After incubation, the culture plates that changed in color from blue to purple or yellow were considered as positive for siderophore production. All experiments were done in five replicates.

Solubilization of phosphate

Phosphate solubilization by the selected EDB isolates was tested using modified Pikovskaya's agar plates (Chaiharn and Lumyong 2009). The isolates were spotted on the agar plates and incubated at 30 °C for 24 h. The presence of a clear zone around a bacterial colony was interpreted as a positive. Calculation of the phosphate solubilization index (SI) was carried out following the previously described method (Kumar and Narula 1999). These experiments were done in five replicates.

Assays for cellulolytic enzymes

Cellulase assay for the selected EDB was performed on modified nitrogen free agar (Döbereiner et al. 1972) supplemented with 0.25 % (w/v) carboxymethyl cellulose instead of glucose and 0.5 % (w/v) tryptone (Verma et al. 2001). The bacteria were spotted on the agar plate and incubated at 30 °C for 48 h, then soaked with Congo red solution for 1 min and then rinsed with 1 M NaCl solution (Reinhold-Hurek et al. 1993). The screening for pectinase production was tested by spotting bacteria on the same nitrogen free agar that was supplemented with 0.5 % (w/v) tryptone and 0.5 % (w/v) citrus pectin instead of glucose. The culture plates were incubated at 30 °C for 48, then soaked with 2 % (w/v) hexadecyltrimethyl ammonium bromide (CTAB) solution for 30 min, and rinsed with 1 M NaCl solution to visualize the halo zone around the bacterial colonies (Mateos et al. 1992). Chitinase assay was carried out by spotting test of the bacteria onto chitin agar (Hsu and Lockwood 1975), while colloidal chitin component was

prepared following the protocol described by Chaiharn and Lumyong (2009). The culture plates were incubated at 30 °C for 72 h, and the presence of clear zone around inoculated colonies indicated positive chitinase activity.

Identification and classification of selected endophytic diazotrophic bacteria

The selected EDB were identified by phylogenetic analysis of the 16S rRNA gene. Total DNA of the bacteria was extracted using a modified potassium acetate method (Prakamhang et al. 2009) and used as a DNA template in PCR reactions. Amplification of the 16S rRNA gene was performed using a pair of universal primers, fD1 (5′-AGAGTTTGATCCTGGC TCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al. 1991). DNA sequencing was performed by MACROGEN (Korea). The DNA sequences were aligned and compared to sequences available in GenBank. Phylogenetic tree was constructed by neighbor-joining method using Mega 4 (Tamura et al. 2007), and confidence levels were estimated for 1,000 replicates by bootstrapping.

Tracking of *in planta* symbiotic colonization by endophytic diazotrophic bacteria

To obtain ß-glucuronidase (GUS)-marked strains, the plasmid pCAM120 (Wilson et al. 1995) was transferred into competent cells of selected EDB by electroporation. Conditions for the electroporation were 18 kv cm^{-1}, 100 Ω and 25 μF (Noisangiam et al. 2012). Blue colonies expressing GUS activity were selected in Luria-Bertani (LB) agar medium supplemented with streptomycin and gentamycin. Seeds of the commercial jasmine rice cultivar Khao Dawk Mali 105 were germinated on Petri dishes containing rice growth medium, and inoculated with the GUS-tagged bacteria. Rice seedlings were transferred into a test tube containing rice growth medium without nitrogen and grown at 25 °C under a light–dark cycle (16 h of light followed by 8 h of dark). Rice plant materials were sampled at various times, from 1 to 7 days after inoculation. Symbiotic colonization of the GUS-tagged bacteria within the rice plant tissues was examined by histochemical staining (Manassila et al. 2007).

Application of selected endophytic diazotrophic bacteria in commercial rice cultivar in vivo

The selected EDB for improving the growth of commercial jasmine rice cultivar Khao Dawk Mali 105 was evaluated in greenhouse. Seeds of the rice were surface sterilized as previously described. Five sterilized seeds were placed on nutrient agar to confirm the surface sterilization. The sterilized seeds were inoculated with each bacterial suspension (approximately 10^8 CFU mL^{-1}), transferred into seedbed and incubated for 7 days before transplantation into a plastic pot. The rice plants were grown hydroponically in nitrogen free solution (pH 5.5) (Elbeltagy et al. 2001). The pH of the nutrient solution was maintained by adding diluted NaOH or HCl daily, while the nutrient solution was changed twice weekly. Three replicate pots of five plants per pot were then transferred to a hydroponic cultivation system. Leaf nitrogen levels in rice were estimated weekly from 7 to 35 days after inoculation using a chlorophyll meter (Minolta SPAD502). Root and shoot lengths of inoculated plants were monitored at 7 and 35 days after inoculation. Thirty-five days after planting, shoot and root dry weight and % nitrogen were measured. The growth rate (GR) was calculated as followed, GR (cm day^{-1}) = $(W_2–W_1)/(T_2–T_1)$, where W_1 is the length of rice plant after 7 days from seedling emergence (T_1) and W_2 is the length of rice plant at the end of the experiment (T_2 : 35 days) (Medany et al. 2007).

Statistical analysis

Statistical analysis was carried out using the SPSS 16.0 computer program (SPSS, Chicago IL, USA), applying the Duncan's Multiple Range Test (DMRT) at significant level of $P=0.05$.

Results

Endophytic diazotrophic bacteria and their plant growth-promoting potentials

A total of 396 endophytic diazotrophic bacterial (EDB) strains were obtained from the different tissues (mature seeds, leaves, stems and roots) of different highland rice landraces, at different growth stages.

Subpellicel characteristics of isolated diazotrophic bacteria were observed in N-free semisolid medium inoculated with surface sterilized mature seeds, leaves, stems and root samples from six samples of different highland rice landraces. The population size of EDB in various parts and growth stages of these highland rice landraces ranged from 1.81±0.3 to 4.79±0.4 log MPN g^{-1} fresh tissue (Fig. 1). The highest population density of EDB from all rice samples was found in the root tissues at nursery stage, whereas the lowest population density was found in mature seeds from all rice cultivars. The EDB population density at all growth stages of the rice cultivars BP1, BP2, BPT2 and BPD was lower in leaves than in stems.

Out of 250 bacterial strains positive for ARA, 21 strains that were significantly positive ($P \leq 0.01$) were selected. As free-living bacteria, the selected strains showed nitrogenase activity ranging from 17.58 to 225.90 nmol ethylene h^{-1} mg

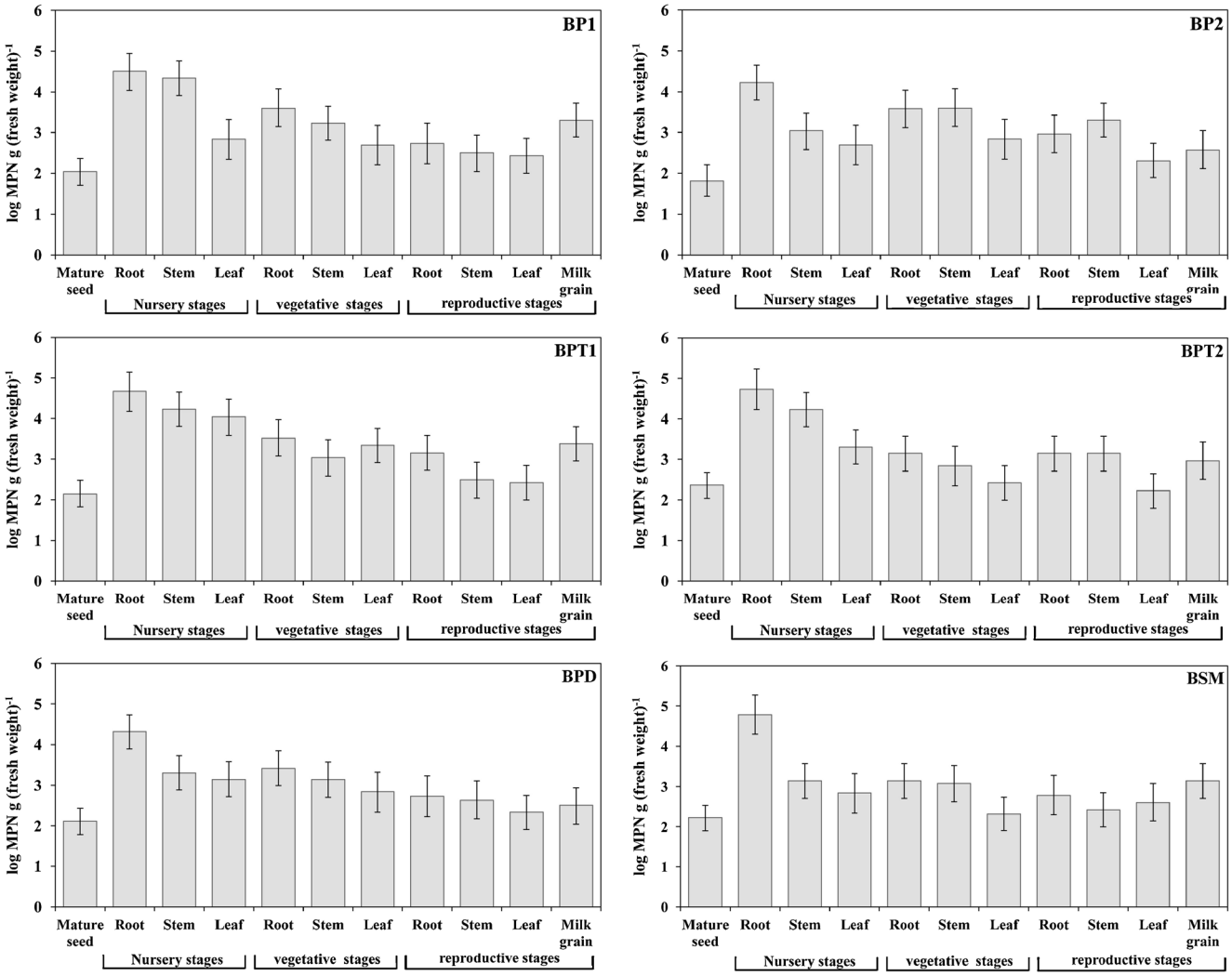

Fig. 1 Density of endophytic diazotrophic bacteria isolated from plant tissues of rice landraces. The rice cultivars include Bue Po Lo-1 (BP1), Bue Po Lo-2 (BP2), Bue Pra Taw-1 (BPT-1), Bue Pra Taw-2 (BPT-2), Bue Pra Do (BPD) and Bue Saw Mi (BSM). Each *bar* represents a most probable number±95 % confidence interval of five replicates

protein^{-1}. When endophytic, their nitrogenase activity ranged from 86.39 to 888.37 nmol ethylene h^{-1} g (dry weight)$^{-1}$ (Fig. 2).

The pathogenic activity of EDB was evaluated by inducing infection of the bacterium in the intercellular spaces of mature tobacco leaves and assessing the presence of necrotic zones. The results indicated the absence of hypersensitivity of tobacco plants to infection by any strain of EDB, while *X. oryzae* induced necrotic zones on the tobacco leaves 2 days after infection.

Nineteen selected EDB strains showed more than one trait of plant growth promotion. Out of 19 strains, four strains including RR3, RR5, SR6 and SS5 were positive for all tests of plant growth-promoting activities (Table 1). All selected EDB strains produced IAA in the presence of tryptophan, ranging from 0.85 to 16.66 μg mL^{-1}, with the significantly highest (P=0.05) IAA activity produced by bacteria strains PS7 and PS5 (16.67±0.42 and 16.16± 1.59 μg mL^{-1}, respectively). Among the selected bacterial strains, 15 (71.42 %) were able to produce ammonia in peptone water medium. Siderophores were detected in six strains (28.57 %). Five selected strains (PR3, PR4, RR3, RR5 and SR6) changed the medium from blue to a light yellow color, and only strain SS5 changed the medium to purple.

On the basis of the phosphate solubilization assay, 85.71 % of selected EDB were able to mobilize calcium phosphate. The strain TR5 showed the highest significant (P=0.05) solubilization index (2.24±0.10). Plant cell degrading enzymes activities were determined from the selected strains of EDB. Of these EDB, 95.24 % produced pectinase, while 85.71 % and 38.09 % of them showed activities of cellulase and chitinase, respectively. Seven (33.34 %) strains showed abilities to

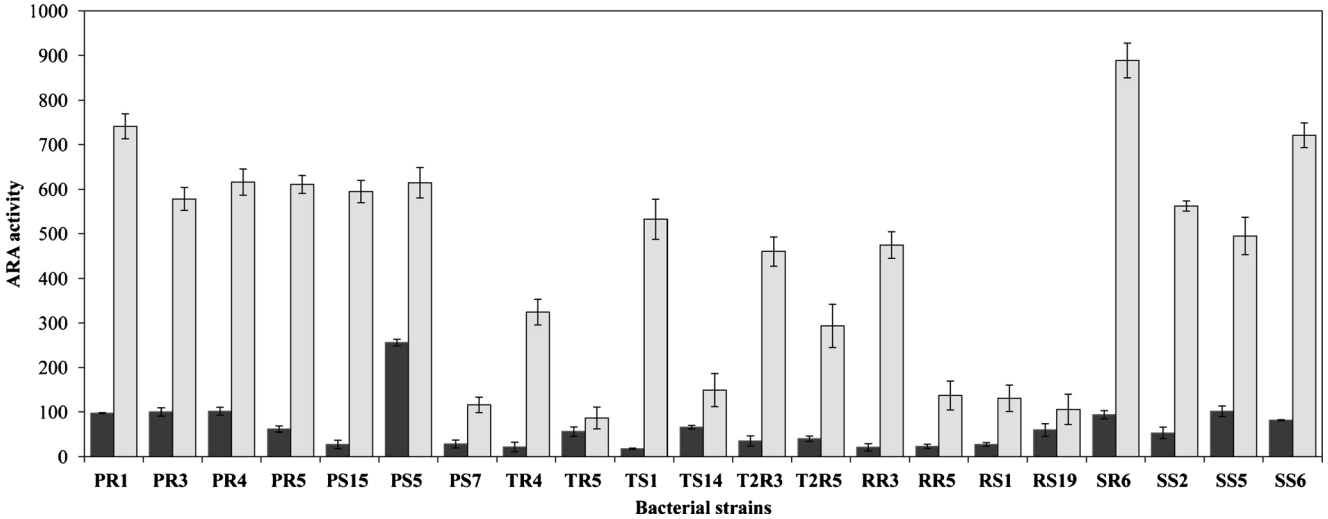

Fig. 2 Nitrogenase activity of diazotrophic bacteria under free-living (■, n mol C_2H_4 h^{-1} mg $protein^{-1}$) and symbiotic [□, n mol C_2H_4 h^{-1} g $(dry\ weight)^{-1}$] conditions, tested on the commercial rice cultivar Khao Dawk Mali 105 using an acetylene reduction assay. Each *bar* represents a means ± SD of five replicates

Table 1 Some characteristics of potential strains of endophytic diazotrophic bacteria

Strain[Φ]	Rice cultivar	Tissue type	Plant growth-promoting activities				Enzyme activities		
			IAA (ug mL^{-1})*	NH_3[Ξ]	Siderophore[Ψ]	SI[Φ]	Cel [♠]	Pec[♥]	Chi[♦]
PR1	Bue Po Lo 1	Root	6.47±0.34^{ef}	+	−	1.44±0.01^{cd}	+	+	+
PR3	Bue Po Lo 1	Root	0.85±0.03^{h}	−	+ (Yellow)	1.05±0.02^{ef}	+	+	+
PR4	Bue Po Lo 1	Root	2.12±0.23^{gh}	−	+ (Yellow)	1.18±0.05^{ef}	+	+	−
PR5	Bue Po Lo 1	Root	7.16±0.12^{ef}	+	−	1.71±0.08^{b}	+	+	+
PS15	Bue Po Lo 1	Mature seed	7.73±0.32^{de}	+	−	1.00±0.00^{f}	+	+	−
PS5	Bue Po Lo 2	Stem	16.16±1.59^{a}	+	−	1.26±0.05^{de}	+	+	−
PS7	Bue Po Lo 2	Stem	16.67±0.42^{a}	−	−	1.16±0.04^{ef}	+	+	+
TR4	Bue Pra Taw 1	Root	3.50±0.15^{g}	+	−	1.06±0.03^{ef}	+	+	+
TR5	Bue Pra Taw 1	Root	8.82±0.34^{d}	+	−	2.24±0.10^{a}	+	+	−
TS1	Bue Pra Taw 1	Mature seed	14.37±0.76^{b}	+	−	1.27±0.11^{de}	−	+	−
TS14	Bue Pra Taw 1	Mature seed	2.40±0.07^{g}	+	−	−	+	+	−
T2R3	Bue Pra Taw 2	Root	8.94±0.32^{d}	−	−	−	+	−	−
T2R5	Bue Pra Taw 2	Root	10.83±0.37^{c}	−	−	−	+	+	−
RR3	Bue Pra Do	Root	2.88±0.11^{g}	+	+ (Yellow)	1.12±0.03^{ef}	+	+	+
RR5	Bue Pra Do	Root	10.91±0.26^{c}	+	+ (Yellow)	1.17±0.00^{ef}	+	+	−
RS1	Bue Pra Do	Stem	11.57±0.39^{c}	+	−	1.08±0.03^{ef}	+	+	−
RS19	Bue Pra Do	Stem	11.29±0.28^{c}	−	−	1.17±0.00^{ef}	+	+	−
SR6	Bue Saw Mi	Root	6.50±0.16^{ef}	+	+ (Yellow)	1.44±0.12^{cd}	−	+	−
SS2	Bue Saw Mi	Stem	3.09±0.28^{g}	+	−	1.19±0.13^{ef}	−	+	+
SS5	Bue Saw Mi	Stem	5.75±0.19^{f}	+	+ (Purple)	1.53±0.09^{bc}	+	+	+
SS6	Bue Saw Mi	Stem	7.03±0.16^{ef}	+	−	1.24±0.03^{de}	+	+	−

[Φ] Endopytic diazotrophic bacterial strain; *IAA concentrations were estimated by a modified colorimetric method; [Ξ] Bacterial strains were tested for the production of ammonia in peptone water; [Ψ] Siderophore production by color change on CAS agar plates; [Φ]Solubilization index were estimated using modified Pikovskaya's agar plates; [♠] The activity of cellulase was determined by measuring the release of reducing sugars using carboxymethyl cellulose (CMC); [♥] The activity of pectinase was assayed on the indicator plates supplemented with citrus pectin; [♦] The activity of chitinase was assayed on the indicator plates supplemented with colloidal chitin

Value represents mean ± standard error (SE) and values followed by the same superscript letter ($^{a, b, c, d, e, f, g, h}$) in each column are not significantly different from each other as determined by DMRT ($p \leq 0.05$)

−, not detected; +, production

produce cellulase, pectinase and chitinase. Based on the differences in the multifunctions of promoting plant growth, ten strains (from 21 strains) were selected for further identification in greenhouse experiments.

Phylogeny of endophytic diazotrophic bacteria isolated from rice landraces

Phylogenetic analysis showed that the selected strains (ten strains) of EDB were clustered into three groups including α-β-proteobacteria and γ-proteobacteria (Fig. 3), which were *Burkholderia*, *Klebsiella*, *Novosphingobium* and *Sphingomonas*. Based on 16S rDNA sequence similarity, strains PR1, PR5 and SS5 were similar to *Burkholderia kururiensis* (≥ 99 % sequence similarity), strains TS1 and SS2 were similar to *Klebsiella* sp. (100 % and 99 % sequence similarity, respectively), strains PS5, SR6 and SS6 were similar to *Sphingomonas* sp. (99 % sequence similarity), strain TR4 was similar to *Novosphingobium* sp. (99 % sequence similarity), and strain RR3 was similar to *Novosphingobium nitrogenifigens* (100 % sequence similarity).

Symbiotic colonization in commercial rice cultivar by endophytic diazotrophic bacteria

Four strains, belonging to four genera, *Burkholderia* sp. SS5 (Fig. 4a–d), *Klebsiella* sp. SS2 (Fig. 4e–h), *Novosphingobium* sp. TR4 (Fig. 4i–l) and *Sphingomonas* sp. PS5 (Fig. 4m–p) marked with GUS were selected to use for investigating rice colonization. Two days after inoculation, all bacteria entered into rice radicles and coleoptiles (Fig. 4a, e, i and m), and they accumulated in the root hairs and lateral root junction (Fig. 4b, f j and n). Seven days after inoculation, the bacterial cells spread further through the plant tissue, migrating to root tissue (Fig. 4c, g, k and o) and leaf tissue (Fig. 4d, h, l and p). In the radicle, *Burkholderia* sp. SS5 (Fig. 4a), *Klebsiella* sp. SS2 (Fig. 4e) and *Sphingomonas* sp. PS5 (Fig. 4m) showed the same colonization pattern, while *Novosphingobium* sp. TR4 (Fig. 4i) showed less GUS staining at the root tip than other bacterial strains. In the root hairs zone, *Burkholderia* sp. SS5 (Fig. 4b) and *Sphingomonas* sp. PS5 (Fig. 4n) obviously showed higher GUS activity than *Klebsiella* sp. SS2 (Fig. 4f) and *Novosphingobium* sp. TR4 (Fig. 4j).

Greenhouse application of endophytic diazotrophic bacteria for commercial rice

Chlorophyll content in leaves was affected by the different EDB inoculated to seeds (Fig. 5). However, at some monitoring time, there was a significant (P=0.05) increase in chlorophyll content in leaves of rice plants after inoculation with each bacterium. Inoculation with any of the EDB strains tested resulted in significantly (P=0.05) higher chlorophyll content at the time of harvest. However, *Burkholderia* sp. SS5 significantly (P=0.05) increased the chlorophyll content at all stages, compared to non-inoculated plants.

Seven days after inoculation, *Novosphingobium* sp. TR4 significantly (*P=0.05*) enhanced the root and shoot length (14.39±0.30 and 7.05±0.04 cm, respectively; Table 2) when compared to non-inoculated plants. The longest roots were found in *Klebsiella* sp. SS2 inoculated plants (16.19± 0.24 cm). The other bacterial strains showed significantly longer roots at 35 days after inoculation. Inoculated plants showed a root growth rate (GR) ranging from 0.76 to 0.96 cm day^{-1}, depending on the bacterial strain. For all bacterial strains, root growth rate was significantly higher than that detected in non-inoculated plants, and the root:shoot ratio growth rate (GR), ranging from 2.93 to 5.02, was also higher.

Thirty five days after inoculation with *Novosphingobium* sp. TR4 and *Sphingomonas* sp. SS6, plant dry weight was significantly (P=0.05) higher compared to non-inoculated plants (Fig. 6). Rice seedlings inoculated with selected strains also contained significantly higher levels of nitrogen content in their roots compared to non-inoculated plants (Fig. 7).

Discussion

The EDB activity is the main factor in promoting the growth of plants by the cycling of nutrients (Widmer et al. 2006). Our results found that the population density of EDB was highest in roots, whereas the population size was lower in stems and leaves. In addition, the results are similar to EDB from the cultivated rice cultivar Khao Dawk Mali 105 (Prakamhang et al. 2009) and wild rice cultivars *Oryza rufipogon, O. nivara* and *O. granulata* (Koomnok et al. 2007). Rice roots harboring endophytes are the primary site of interaction between plants and microorganisms (Sessitsch et al. 2012). The root exudates produced by rice plants promote the interaction between endophytic bacteria and root tissues (Bacilio-Jiménez et al. 2003). Mano et al. (2007) suggested that the endophytic bacteria found in roots were not related to those in seeds and leaves of the *O. sativa* cultivar Kinuhikari. Nitrogen fixing ability could be detected in leaf consortia or aerial parts of rice tissues, which are exposed to the air and to O_2 produced by photosynthesis (Minamisawa et al. 2004; Prakamhang et al. 2009). In this study, the EDB population was maximal at the nursery stage, and decreased in all tissues with rice growth stage. Our results are in agreement with Mano et al. (2007), who reported that the total number of endophytic root bacteria was greatest in the rice roots at the early stage (Mano et al. 2007). However, Koomnok et al. (2007) reported that the number of diazotrophic bacteria in cultivated rice varieties Khao Dawk Mali 105, Kum Doi Saket and Bue Po Lo increased with aeing of the plants, to a maximum at the heading stage. This difference might be due to the variety of

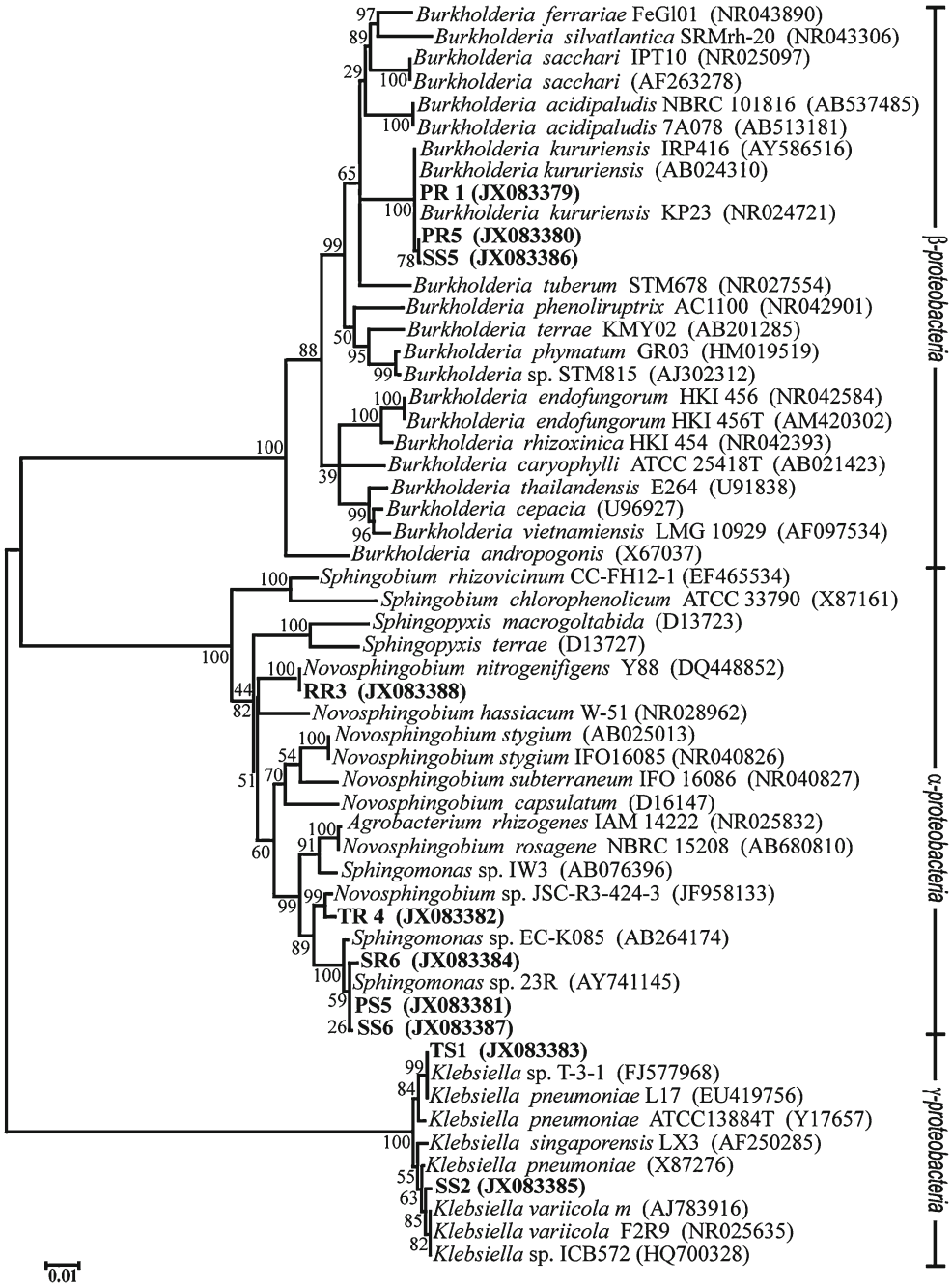

Fig. 3 Unrooted phylogenetic tree of nearly full-length 16S rRNA gene sequences showing the relationships among the endophytic diazotrophic bacteria isolated from highland rice landraces tissue and related genera. Bootstrap analysis was based on 1,000 replicates. Neighbor Joining tree and the scale bar present 0.01 nucleotide substitutions, where the accession numbers of the sequences are indicated in *parentheses*

rice, environmental parameters of rice cultivation, and plant genotype. The plant genotype affects the colonization and nutrient status of bacterial communities associated with the plant (Van Overbeek and Van Elsas 2008; Hardoim et al. 2011). According to Engelhard et al. (2000), the diazotrophic endophytic diversity was greater in modern rice cultivars than in wild rice.

The presence of diazotrophic bacteria in rice tissues was confirmed by acetylene reduction activity; inoculation with *Sphingomonas* sp. SR6 showed the highest activity (888.37 nmol C_2H_4 h^{-1} g dry $weight^{-1}$). Six strains of *Pseudomonas aeruginosa* grown on LGI media showed efficient reduction of acetylene, ranging from 23.34±1.8 to 28.91±3.5 nmol C_2H_4 h^{-1} mg $protein^{-1}$ (Gupta et al. 2013). Elbeltagy et al. (2001) found that the acetylene reduction activities of *O. officinalis* W0012 seedlings inoculated with diazotrophic *Herbaspirillum* sp. strain B501*gfp* showed ethylene production at a rate of 71.3±22.4 nmol h^{-1} g wet $weight^{-1}$. Nitrogen fixation was also detected in leaf consortia, which indicates the ability of EDB to fix nitrogen in plant leaves (Prakamhang et al. 2009). Nitrogen fixation activity in the endophytic stage did differ from nitrogen fixation in the free-living stage. One report on wheat root inoculated with *Azospirillum* spp. showed that the nitrogenase activity did not

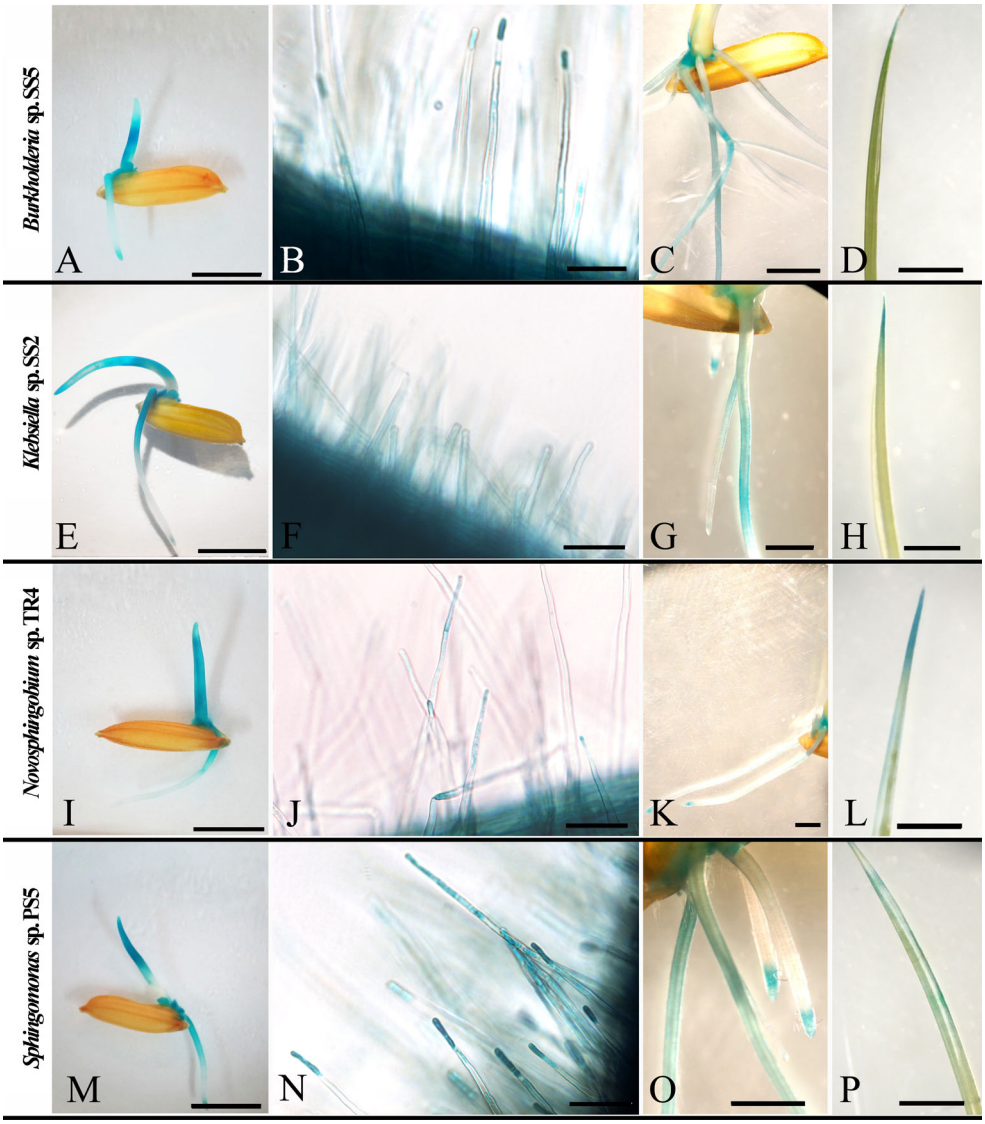

Fig. 4 The micrographs of Gus-tagged *Burkholderia* sp. SS5 (**a**–**d**), *Klebsiella* sp. SS2 (**e**–**h**), *Novosphingobium* sp. TR4 (**i**–**l**) and *Sphingomonas* sp. PS5 (**m**–**p**) during colonization on radical-coleoptiles (**a**, **e**, **i** and **m**; scale bar, 5 mm) and root hairs (**b**, **f**, **j** and **n**; scale bar, 20 μm) of rice 3 days after inoculation, and close-up view of bacterial infection of root tips (**c**, **g**, **k** and **o**); scale bar, 2 mm) and leaf tip (**d**, **h**, **l** and **p**; scale bar, 2 mm) at 7 days post inoculation

correlate well with nitrogen fixation in pure culture (Han and New 1998).

Multiple plant growth-promoting activities, such as production of IAA, ammonia, siderophore, and phosphate solubilization, varied according to the strain of EDB isolated from highland rice landraces (Table 1). Our results are similar to other findings for rhizobacteria, which exhibited more than two plant growth-promoting traits, including phytohormone production by the bacterium, enhancement of nutrient uptake (Souza et al. 2013), or prevention of the deleterious effects of one or more plant pathogen (Ahmad et al. 2008). The best-studied mechanisms of bacterial plant growth promotion include providing plants with resources/nutrients (Glick 2012). Some of the above-tested EDB stains were able to produce IAA, which may play a major role, as does plant growth-promoting phytohormone. The production may depend on species, but also depends on culture conditions, growth stage, and availability of substrate (Chaiharn and Lumyong 2011). IAA synthesized by bacteria may be involved at different levels in plant-bacterial interactions (Glick 2012).

Some strains of selected EDB are able to produce ammonia when grown on a nitrogen source. Production of ammonia was a common trait in free-living rhizospheric bacteria with multiple plant growth-promoting activities. Ammonia produced by bacteria accumulates and supplies nitrogen to host plants, promotes root and shoot elongation, and increases plant biomass (Rashid et al. 2012).

Siderophores are typically produced by bacteria, fungi, and monocotyledonous plants under iron-limited conditions (Loaces et al. 2011), making it antagonist against plant pathogens and inducing systemic resistance in plants (Ahmad et al. 2008). In our experiment, six isolates of EDB produced siderophores. The different color changes in the CAS agar to yellow or purple suggested the production of different types of siderophores, such as catecholates, hydroxamates and α-carboxylates, depending on the chemical nature of their

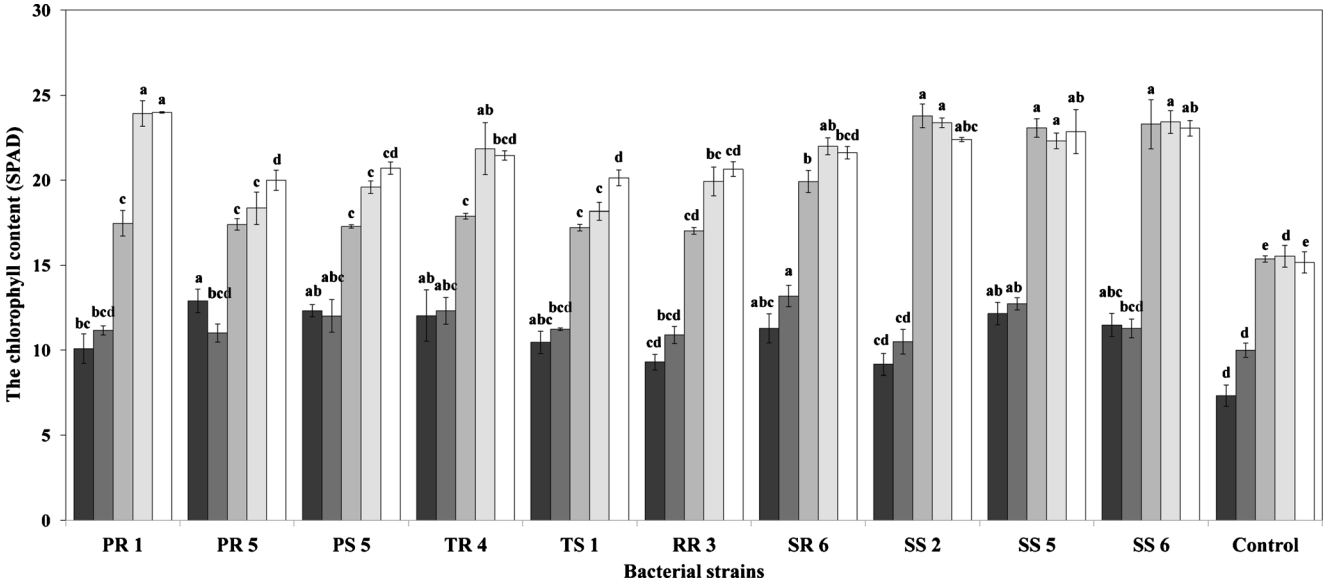

Fig. 5 The chlorophyll content of commercial rice cultivar Khao Dawk Mali 105 leaf inoculated with selected strains of endophytic diazotrophic bacteria grown in green house condition, detected by a SPAD meter after 7 (■), 14 (■), 21 (■), 28 (■) and 35 (■) days. Each *bar* represents a means ± standard error (SE) of three replicate; each replicate had five plants for each treatment. Means in each data followed by the same letter are not statistically different at the 0.05 level using Duncan's multiple range test (DMRT)

coordination sites with iron (Winkelmann 1990; Pérez-Miranda et al. 2007). The present study clearly revealed that more than 85 % of selected EDB could produce siderophores.

The ability to solubilize inorganic phosphate by transforming it into bioavailable forms is an important property of plant growth-promoting bacteria. Phosphorus is an important plant macronutrient that is required for growth and development. Therefore, the mobilization of this nutrient may enhance plant growth promotion and crop productivity. It is also involved in plant growth activity such as

Table 2 Effect of inoculation with selected isolates of endophytic diazotrophic bacteria from highland rice landraces on the length growth parameters of commercial jasmine rice cultivar Khao Dawk Mali 105 grown in greenhouse

Strain[Φ]	Days after inoculation						GR ratio[†]
	Root length (cm)[Ψ]			Shoot length (cm)[Ψ]			
	7	35	GR[¶]	7	35	GR	
PR1	13.59±0.25^{c}	40.59±0.68^{a}	0.96	5.78±0.05^{b}	11.15±0.22^{a}	0.19	5.02
PR5	11.37±0.16^{f}	34.46±1.17^{bc}	0.82	4.78±0.11^{cd}	11.25±0.52^{a}	0.27	3.40
PS5	12.28±0.13^{e}	38.39±1.51^{abc}	0.93	5.45±0.12^{b}	11.99±0.41^{a}	0.27	2.93
TR4	14.39±0.30^{b}	36.48±1.19^{abc}	0.79	7.05±0.04^{a}	12.53±0.55^{a}	0.23	3.57
TS1	13.69±0.49^{bc}	34.85±0.33^{bc}	0.76	4.51±0.29^{d}	11.17±0.36^{a}	0.25	3.14
RR3	12.43±0.24^{de}	33.83±0.90^{c}	0.76	4.49±0.29^{d}	11.11±0.70^{a}	0.23	3.99
SR6	13.16±0.29^{cd}	39.02±0.80^{ab}	0.92	5.51±0.11^{b}	12.11±0.41^{a}	0.21	4.69
SS2	16.19±0.24^{a}	38.10±2.72^{abc}	0.78	4.49±0.23^{d}	11.97±0.20^{a}	0.20	4.03
SS5	13.19±0.22^{cd}	35.69±2.27^{bc}	0.80	4.57±0.16^{d}	12.01±0.30^{a}	0.26	3.31
SS6	13.40±0.18^{c}	34.61±1.82^{bc}	0.76	5.26±0.15^{bc}	11.60±0.65^{a}	0.24	3.18
Control	13.31±0.14^{c}	21.71±0.48^{d}	0.30	4.57±0.21^{b}	11.44±0.28^{a}	0.23	2.91
% CV	4.44	6.91		8.14	6.20		

[Φ] Endopytic diazotrophic bacterial strain; [Ψ] Fresh root and shoot length was measured at 7 and 35 Days after inoculated by endopytic diazotrophic bacterial; [¶]GR, The growth rate; [†] GR ratio, the mean of GR root : shoot ratio

Values are means ± standard error (SE) of three experiments, each experiment had 10 plants for each treatment after 7 days growth and 5 plants for each treatment after 35 days growth. Means in a column followed by the same superscript letter ($^{a, b, c, d, e, f}$) are not statistically different from each other as determined by DMRT ($p \leq 0.05$). Coefficient of variation (%CV) is the percentage of the mean represented by the standard deviation

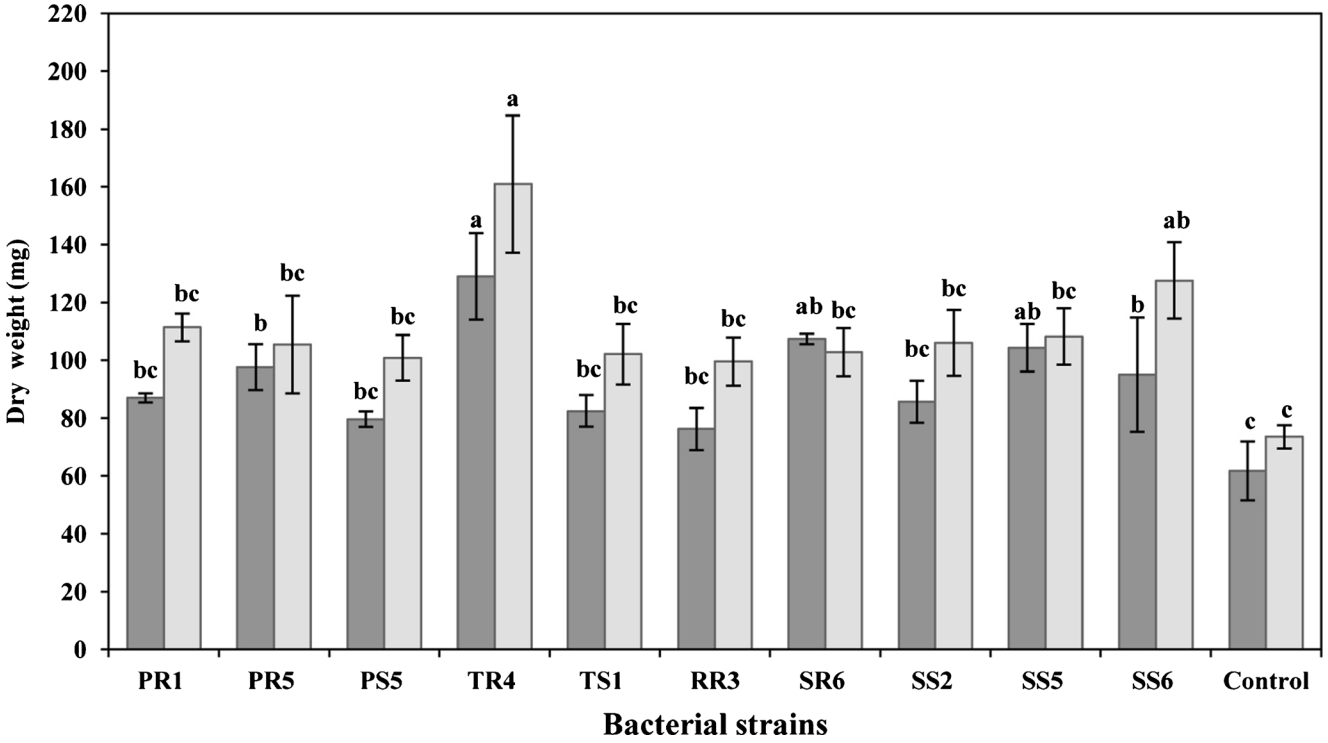

Fig. 6 Dry weight of the commercial rice cultivar Khao Dawk Mali 105 applied with selected isolates of endophytic diazotrophic bacteria, after 35 days of growth in green house experiment. Each *bar* represents a means ± standard error (SE) of three replicates; each replicate had five plants for each treatment. Means in each data set [root (■) and shoot (□)] followed by the same letter are not statistically different at the 0.05 level using Duncan's multiple range test (DMRT)

photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Laslo et al. 2012). All selected EDB strains were not phytopathogenic. In the present study, each EDB strain was inoculated into rice seeds cultivated under nitrogen-free hydroponic conditions, and rice growth promoted by EDBs was better compared to non-inoculated treatment. The promotion activity increased plant dry weight, percent nitrogen and chlorophyll content. Root

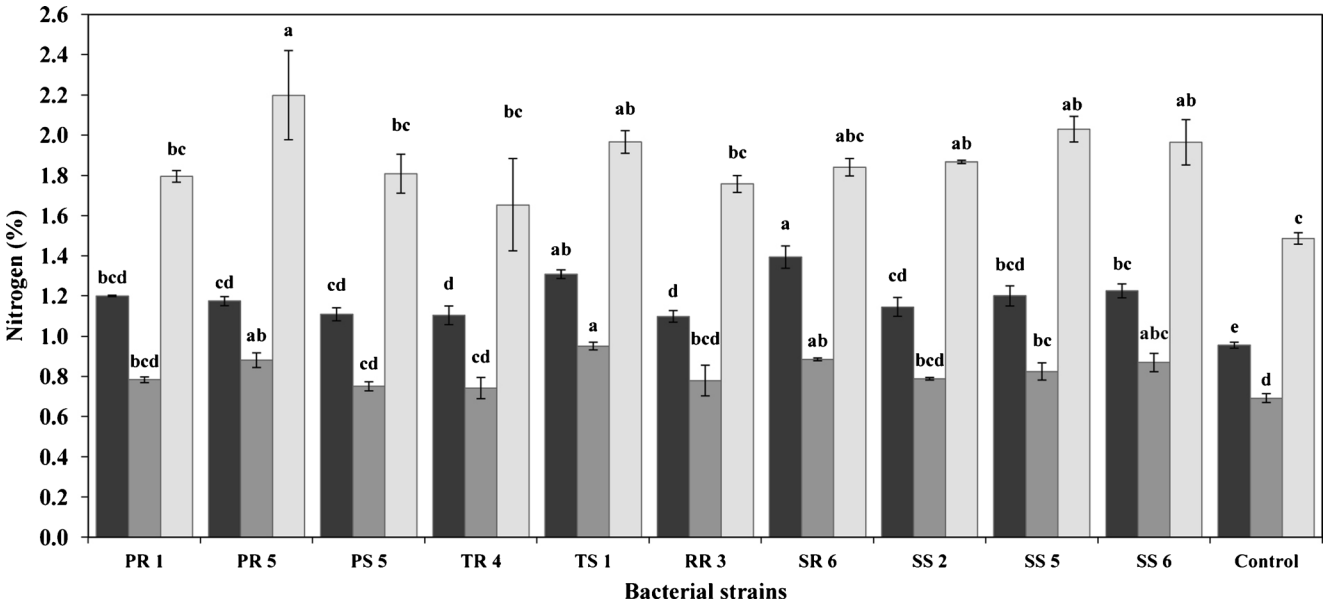

Fig. 7 Nitrogen in tissues of the commercial rice cultivar Khao Dawk Mali 105 receiving different selected isolates of endophytic diazotrophic bacteria, after 35 days of growth in green house experiment. Each *bar* represents a means ± standard error (SE) of three experiments; each experiments had five plants for each treatment. Means in each data set (■ root, ■ stem and □ leaf) followed by the same letter are not statistically different at the 0.05 level using Duncan's multiple range test (DMRT)

elongation effect was similar to that evidenced in a study on *Azospirillum* inoculated rice seedlings, where reduced length and root surface area were observed (Radwan et al. 2004; Rodrigues et al. 2008). This inhibition may be related to the production of auxin by bacteria in seedling roots (Rodrigues et al. 2008). Earlier studies reported that inoculating endophytic diazotrophs *Burkholderia* species to rice plants increased the biomass by 69 % and could fix 31 % of the total nitrogen captured by the plant (Baldani et al. 2000). *Azospirillum amazonense* inoculated on rice showed an increase of 7 to 11.6 % in rice grain dry matter and 3.5 to 18.5 % of nitrogen accumulation at grain maturation (Rodrigues et al. 2008). Moreover, rice inoculated with *A. brasilense* increased the maximum grain yield by up to 76.0 % (Thakuria et al. 2004). We consider that our selected EDB strains are likely to promote plant growth from multiple mechanisms, and can be applied as biofertilizer for promotion of cultivated plant growth.

Based on 16S rRNA sequence analysis, selected EDB were identified as members of four different genera: *Burkholderia*, *Klebsiella*, *Novosphingobium* and *Sphingomonas*. The results agreed to others investigators. The most common endophytic bacteria were isolated from plant tissue in the genera *Burkholderia*, *Enterobacter*, *Klebsiella*, *Phyllobacterium* and *Pseudomonas* (Mano and Morisaki 2008). While *Burkholderia* is commonly found in rice roots, *Sphingomonas* could be isolated from seeds and leaves (Mano and Morisaki 2008) and *Novosphingobium* could be isolated from rice roots (Sun et al. 2008).

In addition, colonization of rice was confirmed using GUS-marked *Burkholderia* sp. SS5, *Klebsiella* sp. SS2, *Novosphingobium* sp. TR4, and *Sphingomonas* sp. PS5. In inoculated roots staining was most intense at the tip of root hairs, root tip, germination point and leaf tip. This colonization pattern of rice tissues is probably due to the effect of cell wall-degrading enzymes such as cellulase, and/or pectinase properties of EDB. Similar results have been reported for rice inoculated with GUS-marked *Rheinheimera* strain VFR5-3 (Prakamhang et al. 2009), GUS-marked *Serratia marcescens* strain IRBG500 (Gyaneshwar et al. 2001), GFP-tagged *Herbaspirillum* sp. strain B501 (Elbeltagy et al. 2001; Zakria et al. 2007), and green fluorescent protein (GFP)-labeled *Bacillus megaterium* strain C4 (Liu et al. 2006). From our study on rice inoculation, it is clear that the root tips, the lateral roots junctions and the germination point were the three sites for the bacteria entry into rice tissues. It has been reported that many microorganisms enter into and persist in plants by primary colonization via the cracks formed in lateral root junctions (James 2000; Hardoim et al. 2008), and the root tip at the zone of elongation and differentiation (Reinhold-Hurek and Hurek 1998), and then quickly spread to the intercellular and intracellular spaces (Reinhold-Hurek and Hurek 1998; Chi et al. 2005). Cellulase and pectinase have also been suspected to play a role for internal colonization (Compant et al. 2005; Reinhold-Hurek and Hurek 2011). Finally, bacterial gene expression and regulation within a plant and the effect of co-inoculation of bacteria into rice under greenhouse and filed experiment should be further investigated.

Acknowledgments This research was supported by Office of the Higher Education Commission, Thailand under the National Research University Project and The Thailand Research Fund (Research-Team Promotion Grant RTA5580007). We thank The Graduated School of Chiang Mai University, Thailand for support, as well as The Model Farm Project, the Royal Initiatives of Her Majesty the Queen, Khun Tae Village, Thailand for assistance in sampling collection.

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