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Characterization of Plant Growth-Promoting Traits of Free-Living Diazotrophic Bacteria and Their Inoculation Effects on Growth and Nitrogen Uptake of Crop Plants

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The search for diverse plant growth-promoting (PGP) diazotrophic bacteria is gaining momentum as efforts are made to exploit them as biofertilizers for various economically important crops. In the present study, 17 diazotrophic strains belonging to eight different genera isolated from rice paddy fields were screened for multiple PGP traits and evaluated for their inoculation effects on canola and rice plants. All of the strains tested positive for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and production of indole 3-acetic acid (IAA) and ammonia (NH_3). Additionally, four of the strains were able to solubilize phosphorus (P), five tested positive for zinc (Zn) solubilization and sulfur (S) oxidation, and eight strains produced siderophores. Based on the presence of multiple PGP traits, 10 strains were selected for inoculation studies. Treatment with *Herbaspirillum* sp. RFNB26 resulted in maximum root length (54.3%), seedling vigor, and dry biomass in canola, whereas *Paenibacillus* sp. RFNB4 exhibited the lowest activity under gnotobiotic conditions. However, under pot culture conditions, *Paenibacillus* sp. RFNB4 significantly increased plant height and dry biomass production by 42.3% and 29.5%, respectively. Canola plants and rhizosphere soils inoculated with *Bacillus* sp. RFNB6 exhibited significantly higher nitrogenase activity. In greenhouse experiments, *Serratia* sp. RFNB18 increased rice plant height by 35.1%, *Xanthomonas* sp. RFNB24 enhanced biomass production by 84.6%, and rice rhizosphere soils inoculated with *Herbaspirillum* sp. RFNB26 exhibited the highest nitrogenase activity. Our findings indicate that most of the selected strains possess multiple PGP properties that significantly improve the growth parameters of the two plants when tested under controlled conditions.

Keywords: Free-living diazotroph, nitrogen fixation, *nifH*, PGP, ACC deaminase, rice

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Plant growth-promoting bacteria (PGPB) [6] are associated with most plant species and are commonly found in many environments. A number of free-living soil bacteria are considered to be PGPB. However, it is neither a single genus or species of bacteria nor a single trait that augments plant growth promotion; rather, it is a consortium of bacteria that possess several PGP properties [30]. For instance, apart from its ability to convert atmospheric dinitrogen (N_2) into NH_3 that can be used by plants, *Azospirillum* sp. also possesses an array of other PGP traits, such as nutrient solubilization and uptake and enhanced stress resistance [13]. In this regard, N_2-fixing bacteria belonging to the genera *Azospirillum*, *Herbaspirillum*, *Burkholderia*, and *Pseudomonas* appear to be frequent colonizers of important crop plants and have been extensively studied [4, 14, 20, 29].

Thus, there are several mechanisms by which different PGPB may promote growth of crop plants: they may synthesize various phytohormones such as indole 3-acetic acid (IAA), produce siderophores that can provide iron to plants, solubilize minerals such as P, and synthesize enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase that can modulate plant growth and development [25, 31]. A particular PGPB strain may enhance plant growth and development using any one or more of these mechanisms [16]. Inoculation of plants with PGPB not only increases plant growth but also improves total NPK uptake [39, 46].

The beneficial effects of diazotrophic PGPB have been reported in many crops, including horticultural crops, oilseed crops such as canola [23], and cereal crops such as rice [17, 29]. Typically, bacteria that directly promote plant growth are selected from a large number of soil bacteria by testing each strain, either in growth pouches or in soil, a process that requires growing a large number of plants for each strain that is being assessed.

Mahadevappa and Shenoy [26] reported that free-living heterotrophic N_2-fixers are a potentially important source of N_2-fixation in rice fields, and many researchers have addressed the beneficial effects of N_2-fixing systems on rice growth using different strains under greenhouse and field conditions [28, 41]. It is the need of the hour to identify additional free-living diazotrophic bacteria with the potential for use as inocula in non-legume plants for sustainable agricultural production [9]. Therefore, in the present study, we screened a diverse group of strains for multiple PGP traits and evaluated their inoculation effects on canola under gnotobiotic growth pouch and pot culture conditions in a growth chamber and on rice under greenhouse conditions.

Materials and Methods

Bacterial Strains, Media, and Culture Conditions

The strains used in the present study are listed in Table 1. A total of 32 strains were previously isolated from paddy fields under long-term fertilization using four different N-free selective media (*i.e.*, NFMM [32], LGI-P [35], BAz [15], and JNFb [21]) as described by Islam *et al.* (unpublished). Seventeen strains having appreciably higher nitrogenase activity were selected for this study. The 16S rRNA sequences of the respective bacteria were deposited in the GenBank database. Pure cultures of the strains were maintained in nutrient broth containing 50% glycerol at −80°C.

Determination of Plant Growth-Promoting Traits

Nitrogenase activity, IAA production, and ACC deaminase activity. Strains were screened for nitrogenase activity by the acetylene reduction activity (ARA) assay and for the presence of the *nifH* gene by PCR amplification of a 390-bp fragment as per Ueda *et al.* [42]. Ability to produce IAA was determined according to the method of Yim *et al.* [47] with little modification. One-hundred µl aliquots of strains grown in JNFb broth were transferred to 25 ml of LB medium containing 500 µg/ml of L-tryptophan (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) and grown at 28°C for 4 days. Reactions consisted of 2 ml of cell-free suspension to which 100 µl of 10 mM orthophosphoric acid and 4 ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added, followed by incubation at room temperature for 25 min. The presence of IAA in the culture supernatant was determined spectrophotometrically (UV-1601, Shimadzu) at 530 nm. Pure IAA (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) was used as the standard and uninoculated media served as the control. ACC deaminase activity was determined by growing the cells in minimal medium with 3 mM ACC as the sole N source. Production of α-ketobutyrate as a result of enzymatic cleavage of ACC by ACC deaminase was measured at 540 nm, as per Penrose and Glick [31] and compared with a standard curve of α-ketobutyrate (Sigma-Aldrich Co., St. Louis, MO, U.S.A.).

Mineral solubilization, sulfur oxidation, and siderophore production. The mineral P- and Zn-solubilizing ability of the strains was determined on Pikovskaya's agar medium [33] amended with 0.5% tricalcium phosphate [$Ca_3(PO_4)_2$] and 0.12% zinc oxide (ZnO) as inorganic P and Zn sources, respectively. Bacteria having solubilization potential were identified by the appearance of a clear halo around colonies against an opaque background. S-oxidizing potential was examined in a mineral salts–thiosulfate medium, in which a change in color from purple to yellow is an indication of S oxidation due to the production of sulfuric acid from thiosulfate [1]. Siderophores were detected by the formation of orange halos around bacterial colonies on Chrome Azural S (CAS) agar plates after incubation for 24 h at room temperature [38]. The presence of catechol-type and hydroxamate-type siderophores was determined by Arnow's assay and Atkin's assay, respectively [2, 3].

Ammonia production, hydrolytic activity and, cyanide production. Bacterial strains were tested for the production of NH_3 in peptone water. The accumulation of NH_3 was detected by adding Nessler's reagent (0.5 ml/tube). The appearance of a faint yellow color indicates production of a small amount of NH_3 whereas a deep yellow to brownish color indicates maximum NH_3 production. Cellulase and pectinase productions were determined as previously described [8]. The ability to produce hydrogen cyanide (HCN) was determined as per the method of Lorck [24].

Plant Inoculation Experiments

Gnotobiotic root elongation assay and pot experiment with canola. Seed treatment and gnotobiotic pouch assay with canola (*Brassica campestris*, Hungnong seeds; Seminis Korea Inc., Republic of Korea), an ethylene-sensitive plant, were performed according to Penrose and Glick [31]. Primary root length was measured on the fifth day of growth. Seedling vigor index (SVI) and dry biomass were also recorded.

For the pot culture experiment, canola seeds were surface sterilized and soaked in a bacterial suspension (10^8 CFU/ml) for 4 h. The seeds were then sown in plastic pots filled with Biosangto-Mix bed soil (Heung Nong Co., Ltd., Incheon, Gyeonggi-do, Republic of Korea) containing 65–70% cocoa peat, 15–20% peat moss, 8–10% perlite, and macronutrients (80–100 mg/l NH_4-N, 150–200 mg/l NO_3-N, 230–330 mg/l available P_2O_5, and 80–120 mg/l K_2O) at pH 5.5 to 6.5 with a moisture content of 50–60% and water holding capacity of 35–40%. The pots were kept in a growth chamber (DS 54 GLP; DASOL Scientific Co., Ltd., Korea) maintained at 20±1°C and 70% relative humidity with a light/dark cycle beginning with 12 h darkness followed by 12 h light. One ml of overnight culture (10^8 CFU/ml) was applied to the soil near the root zone on day 15. Assays were conducted on four replicates (five pots per replicate, two plants per pot) for each treatment. The plants were harvested after 30 days and growth parameters (plant height and plant biomass) were recorded. For determining the ARA in a rhizospheric sample, 1 g of soil from each treatment was placed in a 120-ml vial containing 40 ml of semisolid JNFb medium. Following incubation, the gas phase in the headspace was replaced with acetylene [10% (v/v)] and incubated again at 30°C for 24 h. Ethylene production was measured using a gas chromatograph (DS 6200; Donam Instruments Inc., Republic of Korea). The ARA in inoculated seedlings was determined by the method as described by Yim *et al.* [47].

Table 1. Plant growth-promoting characteristics of free-living diazotrophic bacteria isolated from paddy fields.

Strain	GenBank Accession No.	ARA[a]	IAA production[b]	Mineral solubilization		S oxidation	ACC deaminase[c]	Siderophore production	
				P	Zn			[Halo zone dia. (cm) in CAS]	Type
Paenibacillus sp. RFNB4	FJ266315	50.1±0.12 p	1.70±0.01 gh	–	+	+	130.0±1.25 g	–	–
Bacillus sp. RFNB6	FJ266317	57.8±0.69 o	1.29±0.11 i	–	–	–	120.3±0.47 i	–	–
Burkholderia sp. RFNB11	FJ266321	297.4±0.40 g	1.59±0.07 gh	–	–	–	307.4±1.50 a	2.0±0.23 a	Hydroxamate
Burkholderia sp. RFNB12	FJ266322	325.7±0.27 f	1.48±0.06 hi	–	–	–	252.0±1.17 b	1.9±0.15 a	Hydroxamate
Brevundimonas sp. RFNB15	FJ266325	290.4±0.39 h	3.08±0.10 c	–	–	–	126.0±0.75 h	–	–
Burkholderia sp. RFNB16	FJ266326	511.2±0.75 e	2.82±0.23 d	–	–	–	247.7±1.10 e	–	–
Serratia sp. RFNB17	FJ266327	115.4±0.64 k	2.29±0.17 f	++	++	++	112.1±0.49 j	1.5±0.06 b	Catechol
Serratia sp. RFNB18	FJ266328	70.7±0.29 n	2.45±0.20 ef	++	++	++	104.2±0.40 k	1.3±0.12 bcd	Catechol
Serratia sp. RFNB19	FJ266329	243.0±0.87 i	2.36±0.21 f	++	++	++	101.9±0.55 lk	1.4±0.06 bc	Catechol
Herbaspirillum sp. RFNB20	FJ266330	107.3±0.52 l	3.97±0.29 b	–	–	–	66.6±0.29 m	1.3±0.17 bcd	Hydroxamate
Sphingomonas sp. RFNB22	FJ266332	48.0±0.34 q	2.27±0.12 f	–	–	–	220.6±1.18 d	–	–
Xanthomonas sp. RFNB24	FJ266334	895.6±0.46 c	1.75±0.02 g	–	–	+	188.8±0.69 e	–	–
Herbaspirillum sp. RFNB26	FJ266336	2844.8±0.75 a	4.39±0.17 a	–	–	–	98.5±0.56 l	1.1±0.00 cd	Hydroxamate
Sphingomonas sp. RFNB28	FJ266338	87.8±0.48 m	2.88±0.14 cd	+	–	–	179.2±1.79 f	–	–
Herbaspirillum sp. RFNB30	FJ266340	620.4±0.40 d	4.56±0.32 a	–	–	–	105.2±0.87 k	1.0±0.12 d	Hydroxamate
Azorhizobium sp. RFNB31	FJ266341	1367.8±0.85 b	2.45±0.09 ef	–	–	–	186.8±2.77 e	–	–
Brevundimonas sp. RFNB32	FJ266342	195.6±0.29 j	2.69±0.10 de	–	+	–	68.9±0.46 m	–	–

[a]ARA is expressed as nmol ethylene/h/mg protein.
[b]The amount of IAA produced by the strains is expressed as μg/ml.
[c]ACC deaminase activity is expressed as nmol α-ketobutyrate/min/mg protein.
++=strongly positive, +=positive, –=negative. The ARA data were taken from Islam *et al.* (unpublished).
All of the strains were found to be positive for the *nifH* gene and NH_3 production, but negative for HCN and cellulase production. Strains RFNB16, RFNB17, RFNB18, RFNB19, RFNB20, RFNB22, and RFNB32 were positive for pectinase production.
Data are presented as mean±standard error (SE) of three replicates. In the same column, significant differences according to Fisher's protected LSD test at $P \leq 0.05$ level are indicated by different letters.

Greenhouse studies. Pure bacterial cultures were grown in nutrient broth at 28°C, centrifuged, and diluted to a final concentration of 10^8 CFU/ml in sterile distilled water. Rice (*Oryza sativa*, cv. Dong-Jin) seeds were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite (NaOCl) for 20 min, and then rinsed 5–6 times with sterile distilled water. The seeds were dried overnight in a laminar flow cabinet, and then immersed in a bacterial suspension for 5 h. Following imbibition, the excess suspension was drained and the seeds were carefully transferred to experimental pots filled with air-dried Biosangto-Mix bed soil and kept in the greenhouse (12 h photoperiod). The plants were irrigated with water twice a week. One ml of overnight culture (10^8 CFU/ml) was applied to the soil near the root zone on days 15 and 30. The crop was uprooted 45 days post-sowing, and growth parameters and nutrient uptake were recorded. The experiment was conducted on six replicates (three pots per replicate, two plants per pot) for each treatment and was completely randomized.

Total N content in rice plants was measured with a Kjeldahl Auto 1030 Analyzer (Tecator, Hoganas, Sweden) after digestion with sulfuric acid and potassium sulfate. The size of the viable bacterial population in rhizosphere and rhizoplane samples was determined according to Miles and Misra [27] using N-free JNFb medium for diazotrophs and nutrient agar for total heterotrophs. The ARA in rhizospheric soils was determined as described above and in inoculated rice roots according to Kim *et al.* [20].

Statistical Analyses

Data for each treatment were subjected to a variance analysis using SAS software (version 9.1; Cary, NC, U.S.A.). When analysis of variance showed significant treatment effects, Fisher's protected LSD test ($p \leq 0.05$) was applied to make comparisons between treatments.

Results

Assessment of Potential PGP Properties

Apart from N_2-fixation, the diazotrophic bacterial strains of all genera used in the present study possess different traits related to PGP, which are described in Table 1. The selected strains had appreciably high nitrogenase activity, ranging from 48.0 to 2844.8 nmol ethylene/h/mg of protein, and PCR amplification confirmed the presence of the *nifH* gene (data not shown). Likewise, all strains produced IAA, ranging from 1.29 μg/ml in *Bacillus* sp. RFNB6 to 4.56 μg/ml in *Herbaspirillum* sp. RFNB30. ACC deaminase activity varied from 66.6 nmol of α-ketobutyrate released/min/mg of protein in *Herbaspirillum* sp. RFNB20 to 307.4 in *Burkholderia* sp. RFNB11. The *Serratia* strains, RFNB17, RFNB18, and RFNB19, were found to strongly solubilize both P and Zn. *Sphingomonas* sp. RFNB28 poorly solubilized inorganic P, whereas *Paenibacillus* sp. RFNB4 and *Brevundimonas* sp. RFNB32 poorly solubilized inorganic Zn. *Paenibacillus* sp. RFNB4, the three *Serratia* strains, and *Xanthomonas* sp. RFNB24 were proved to be S oxidizers. The two *Burkholderia* strains (RFNB11 and EFNB12), the three *Serratia* strains, and the three *Herbaspirillum* strains (RFNB20, RFNB26, and RFNB30) tested positive for siderophore production in CAS medium. Whereas the *Serratia* strains produced catechol, the other two genera produced the hydroxamate type of siderophore. Furthermore, all the strains tested positive for NH_3 production, but were negative for cellulose and HCN production. However, strains RFNB16, RFNB17, RFNB18, RFNB19, RFNB20, RFNB22, and RFNB32 were found to produce pectinase.

Plant Inoculation Experiments

Growth of canola under gnotobiotic and pot culture conditions. The inoculation effect of diazotrophic bacteria on root growth, SVI, and dry biomass production in canola under gnotobiotic conditions is shown in Table 2. After five days of growth in gnotobiotic pouches, canola roots originating from seeds treated with *Brevundimonas* sp. RFNB15, *Serratia* strains RFNB18 and RFNB19,

Table 2. Influence of diazotrophic bacterial inoculation on early growth stage of canola under gnotobiotic conditions.

Treatment	Root length (cm)	Seedling vigor index (SVI)	Dry biomass (mg)
Paenibacillus sp. RFNB4	6.33±0.17 g	762.0±3.44 j	116±1.15 abcd
Bacillus sp. RFNB6	7.61±0.06 cde	1,097.3±9.28 d	123±4.62 ab
Burkholderia sp. RFNB11	7.25±0.12 def	999.1±8.72 h	111±1.73 cd
Brevundimonas sp. RFNB15	7.78±0.64 cd	1,039.7±7.51 f	122±4.04 ab
Serratia sp. RFNB18	8.02±0.35 c	1,025.5±3.46 g	119±0.58 abc
Serratia sp. RFNB19	7.80±0.23 cd	1,152.0±4.62 c	124±3.46 ab
Sphingomonas sp. RFNB22	7.04±0.09 efg	1,021.2±1.65 g	116±5.20 abcd
Xanthomonas sp. RFNB24	6.86±0.06 fg	1,075.0±2.97 e	118±1.15 abc
Herbaspirillum sp. RFNB26	10.86±0.40 a	1,368.0±5.77 a	125±2.31 a
Azorhizobium sp. RFNB31	9.21±0.58 b	1,259.3±4.04 b	108±2.89 d
Control	7.04±0.35 efg	963.0±1.22 i	115±1.73 bcd

Data are presented as mean±standard error (SE) of three replicates. Seeds failing to germinate after 2 days were marked and the roots originating from them were not measured. Mean root length of 5-day-old canola seedlings was assessed by means of the root elongation assay from measurements of 50 seedlings (5 seeds/growth pouch; 10 growth pouches/treatment). The seedling vigor index (SVI) was calculated using the formula: SVI=% of germination×seedling length (root length+shoot length). In the same column, significant differences according to Fisher's protected LSD test at $p \leq 0.05$ level are indicated by different letters.

Table 3. Effect of inoculation with diazotrophic PGPB on canola plants after 30 days of growth under pot culture conditions.

Treatment	Plant height (cm)	Plant biomass (mg/plant)	
		Fresh weight	Dry weight
Paenibacillus sp. RFNB4	16.14±0.59 a	151.5±1.67 a	14.5±0.35 a
Bacillus sp. RFNB6	13.32±1.17 bc	141.4±0.52 b	11.6±0.81 c
Burkholderia sp. RFNB11	13.74±0.87 abc	131.9±1.27 c	15.1±0.64 a
Brevundimonas sp. RFNB15	15.20±1.17 ab	104.7±2.19 d	9.8±0.17 d
Serratia sp. RFNB18	11.32±0.46 c	97.1±2.60 e	13.4±0.29 b
Serratia sp. RFNB19	11.76±1.02 c	92.5±0.81 f	11.4±11.4 c
Sphingomonas sp. RFNB22	13.70±0.75 abc	92.0±0.75 f	14.9±0.29 a
Xanthomonas sp. RFNB24	12.52±0.61 c	96.4±3.12 e	8.9±0.06 e
Herbaspirillum sp. RFNB26	13.80±1.10 abc	143.6±2.60 b	11.6±0.12 c
Azorhizobium sp. RFNB31	13.30±0.81 bc	142.9±0.92 b	11.3±0.23 c
Control	11.34±0.64 c	93.6±1.27 f	11.2±0.64 c

Data are presented as mean±standard error (SE) of three replicates. Letters indicate a statistically significant difference between treatments according to Fisher's protected LSD test at $p \leq 0.05$. Mean separation within a column followed by the same letters do not differ significantly.

Herbaspirillum sp. RFNB26, and *Azorhizobium* sp. RFNB31 showed significant increases in length compared with the control. Inoculation with *Herbaspirillum* sp. RFNB26 resulted in the longest root length (10.86 cm) and SVI (1368.0), whereas treatment with *Paenibacillus* sp. RFNB4 gave the shortest root length (6.33 cm) and SVI (762.0). A significant amount of dry biomass production (125 mg) was also observed in *Herbaspirillum* sp. RFNB26-treated seedlings. Under pot culture conditions, inoculation of canola with *Paenibacillus* sp. RFNB4 proved beneficial for producing maximum plant height (16.14 cm) and fresh weight (151.5 mg/plant) (Table 3). On the other hand, plants inoculated with *Brevundimonas* sp. RFNB15 and *Xanthomonas* sp. RFNB24 produced significantly less dry biomass compared with the control. *Bacillus* sp. RFNB6-inoculated canola plants and their rhizosphere soils exhibited significantly higher nitrogenase activity compared with the control, amounting to 18.27 nmol ethylene/d/plant and 94.50 nmol ethylene/h/g of dry soil, respectively (Fig. 1A).

Greenhouse experiments with rice. On the basis of multiple PGP properties, 10 diazotrophic strains from diverse genera were selected for *in vivo* experiments to evaluate their inoculation effects in rice under greenhouse conditions. The results indicate that bacterial inoculation had a considerable impact on growth and N uptake in rice (Table 4). Inoculation with *Paenibacillus* sp. RFNB4, *Brevundimonas* sp. RFNB15, *Serratia* sp. RFNB18 and RFNB19, and *Xanthomonas* sp. RFNB24 resulted in a significant increase in shoot length (13.4%, 22.5%, 38.7%, 32.6%, and 35.3%, respectively). However, in the case of root length, only plants treated with *Serratia* sp. RFNB18 exhibited a significant increase (27.9%) compared with uninoculated plants. Maximum production of shoot and root biomass was recorded for plants treated with RFNB24 and was 26.83 and 29.17 mg/plant, respectively. With the exception of *Serratia* sp. RFNB19 and *Xanthomonas* sp. RFNB24, all of the strains increased total N content of plants compared with the control; treatment with *Herbaspirillum*

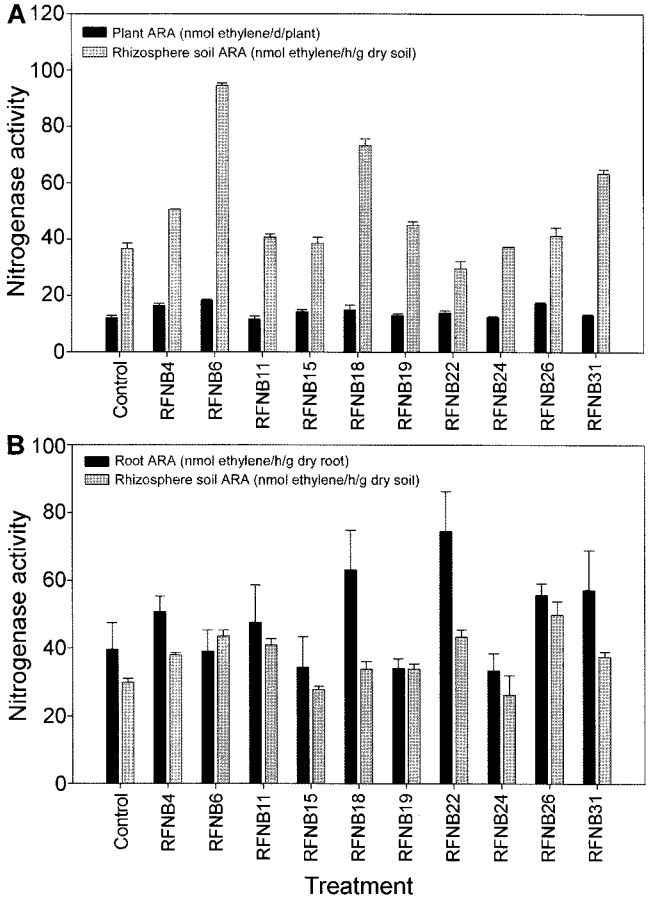

Fig. 1. Nitrogenase activity of diazotrophic strains in association with (**A**) canola plants and their rhizospheric soils after 30 days of growth in a growth chamber, and (**B**) roots and rhizospheric soils of rice plants grown under greenhouse conditions for a period of 45 days.

Data are presented as mean±standard error (SE) of three replicates.

Table 4. Evaluation of the inoculation effect of diazotrophic PGPB on growth parameters and population dynamics in samples from 45-day-old rice plants grown under greenhouse conditions.

Treatment	Plant height (cm)		Dry biomass (mg/plant)		Total N (%)	Diazotrophic population[a]	
	Shoot length	Root length	Shoot weight	Root weight		Rhizosphere	Rhizoplane
Paenibacillus sp. RFNB4	17.56±0.49 cd	8.55±0.67 abc	17.17±0.33 d	14.00±1.50 ef	3.25±0.02 e	6.88±0.08 cd	6.23±0.03 de
Bacillus sp. RFNB6	16.81±0.46 de	8.77±0.48 abc	18.75±1.58 cd	18.25±2.58 cd	3.26±0.03 e	7.03±0.10 b	6.13±0.02 ef
Burkholderia sp. RFNB11	17.28±0.52 cde	8.63±0.19 abc	17.08±0.75 d	14.25±3.75 ef	3.48±0.06 c	7.19±0.13 a	6.31±0.06 d
Brevundimonas sp. RFNB15	18.98±0.68 bc	9.53±0.67 ab	21.67±0.83 bc	20.09±4.08 bc	3.27±0.05 e	7.00±0.18 bc	6.04±0.13 f
Serratia sp. RFNB18	21.48±0.72 a	9.98±0.18 a	26.59±1.58 a	20.67±3.00 bc	3.38±0.02 d	6.84±0.04 d	6.31±0.03 d
Serratia sp. RFNB19	20.54±0.44 ab	8.91±0.09 abc	24.00±0.17 ab	22.67±1.16 b	3.21±0.00 e	7.02±0.11 b	6.07±0.07 f
Sphingomonas sp. RFNB22	16.42±0.10 de	7.24±0.04 c	17.42±0.25 cd	12.92±1.91 f	3.40±0.06 cd	6.79±0.18 d	6.60±0.04 b
Xanthomonas sp. RFNB24	20.96±1.26 a	7.96±0.07 bc	26.83±3.00 a	29.17±2.66 a	3.22±0.01 e	6.83±0.06 d	6.45±0.08 c
Herbaspirillum sp. RFNB26	15.62±0.75 e	7.99±0.59 bc	17.09±1.58 d	16.17±1.33 de	3.87±0.07 a	7.01±0.14 b	6.79±0.11 a
Azorhizobium sp. RFNB31	16.76±0.14 de	7.98±0.53 bc	19.09±0.58 cd	15.75±2.75 def	3.75±0.12 b	7.13±0.11 ab	6.69±0.03 ab
Control	15.49±0.14 e	7.80±1.33 bc	16.50±0.83 d	13.84±3.66 ef	3.22±0.03 e	6.80±0.10 d	6.31±0.08 d

[a]Data were estimated after 5 days of growth in JNFb media, and the result expressed as log CFU/g dry soil and log CFU/g sample for rhizosphere and rhizoplane, respectively.
Data are presented as mean±standard error (SE) of three replicates. Letters indicate a statistically significant difference between treatments according to Fisher's protected LSD test at $p \leq 0.05$. Mean separation within a column followed by the same letters do not differ significantly.

sp. RFNB26 resulted in the highest concentration (3.87%), followed by *Azorhizobium* sp. RFNB31.

The size of the diazotrophic population in rhizoplane and rhizosphere soils following treatment with the different strains is shown in Table 4. The number of diazotrophs was higher in rhizosphere soil samples than in the rhizoplane. Treatment with all strains, with the exception of *Sphingomonas* sp. RFNB22, resulted in an increase in the rhizosphere diazotrophic population compared with the uninoculated control. In the rhizoplane, the maximum number of bacteria (6.79 log CFU/g of sample) was found in *Herbaspirillum* sp. RFNB26-inoculated plants, followed by RFNB31-treated plants. The total heterotrophic bacteria population following the different treatments ranged from 7.67 to 8.22 log CFU/g of dry soil in the rhizosphere, and 6.13 to 6.84 log CFU/g of sample in the rhizoplane (data not shown). Nitrogenase activity in plant roots and rhizospheric soils was also affected by inoculation with diazotrophic strains (Fig. 1B). Roots of *Sphingomonas* sp. RFNB22-inoculated rice plants showed significantly higher nitrogenase activity (74.58 nmol ethylene/h/g of dry root) than the control, followed by roots of *Serratia* sp. RFNB18-treated plants. In rhizospheric soils, maximum nitrogenase activity (49.91 nmol ethylene/h/g of dry soil) was measured in the *Herbaspirillum* sp. RFNB26 treatment and was significantly higher compared with the control.

DISCUSSION

The search for a diverse group of diazotrophs with useful PGP traits from various crop sources paves the way for the reduction of costs associated with the use of N fertilizers as well as minimizes the risk of pollution from continuous application of chemical fertilizers. In particular, the use of strains with multiple PGP properties would help to increase crop productivity on a sustainable basis. Toward this end, we examined the PGP potential of diazotrophs isolated from paddy fields and evaluated their inoculation effects on the growth of canola and rice plants under controlled conditions.

All of the selected strains possess significant nitrogenase activity, and amplification of the *nifH* gene confirmed their ability to fix N_2. Among these strains, *Herbaspirillum* RFNB26 produced the maximum amount of IAA and tested positive for siderophore production as well as ACC deaminase activity. The presence of multiple PGP traits in *Herbaspirillum* sp. has not been previously noted; however, members of the genus have been documented to be potential PGPB in both rice and sugarcane plants [17, 30]. The production of IAA and related compounds has been demonstrated in many diazotrophs, including *Acetobacter diazotrophicus*, *Azospirillum* sp., *Azotobacter* sp., and *Paenibacillus polymyxa* [13]. In our study, all *Herbaspirillum* strains showed relatively high IAA production compared with other strains. In support of this result, it is reported that *Azospirillum* and *Herbaspirillum* are auxin producers as well as N_2-fixers.

The present study confirms the wide distribution of ACC deaminase activity in diverse diazotrophic bacterial genera, in accordance with previous studies [7, 12]. Furthermore, the *Burkholderia* strains evaluated in the present study have higher ACC deaminase activity than the other strains (Table 1). The distribution of ACC deaminase activity is common among *Burkholderia* species. Bacteria containing ACC deaminase bind to roots and/or seed coats and stimulate root elongation by lowering the ethylene level in plants [22]. Although all of the strains we evaluated produce ACC deaminase, a few of them significantly enhanced root length and other growth parameters in canola under both gnotobiotic and pot culture conditions, indicating that ACC deaminase activity alone was not responsible for root elongation and plant growth [34].

Phosphorus and Zn are two important plant nutrients, and the beneficial role of PGPB in maintaining adequate levels of these mineral nutrients in crop production has been previously reported [36, 37]. In our study, only 23.5% and 29.4% of the strains exhibited P and Zn solubilization, respectively. To find a relatively low number of P solubilizers among the tested strains is not surprising, since Hameeda *et al.* [18], while attempting to improve the growth of maize in India, also found that only 5 of the 207 isolates they evaluated possessed the ability to solubilize P. A positive correlation between the potential for P and Zn solubilization has been reported [45], and was seen in the three *Serratia* strains that we tested. In addition, five strains were also found to oxidize S, which would enhance the production of sulfates and make them available for plant growth [44]. Bacterial siderophores are an important class of compounds that enhance plant growth and protect plant health by binding to available iron (Fe^{3+}) in soils [40, 43]. In this study, we found that 47.1% of the diazotrophic strains produce siderophores; however, these strains are restricted to *Serratia*, *Burkholderia*, and *Herbaspirillum*. All the strains tested positive for NH_3 production, but were negative for HCN and cellulose production. The absence of HCN production in these strains could indicate that this ability is not widely distributed among diverse free-living diazotrophic bacteria in paddy fields.

Biofertilizers increase crop growth by a combination of mechanisms, which include biological nitrogen fixation (BNF), phytohormone production, increasing the availability of soil nutrients, and disease control [10]. In the present study, gnotobiotic assays were conducted to test the inoculation effect of diazotrophic bacteria on root elongation in canola. Seeds treated with diverse diazotrophic strains showed a considerable increase in root length as well as SVI and dry biomass compared with the control. *Herbaspirillum*

sp. RFNB26 significantly increased dry biomass production (Table 2). In a similar study conducted under gnotobiotic conditions, Baldani *et al.* [5] reported that the inoculation of rice with *Herbaspirillum seropedicae* enhanced plant dry weight by 71.5%. In addition, we detected nitrogenase activity in canola plants following inoculation with diazotrophic bacteria in the pot culture experiment (Fig. 1A). Similar results were reported by Yim *et al.* [47] in diazotrophic bacteria isolated from Chinese cabbage. Furthermore, we detected a significant amount of nitrogenase activity in rhizosphere soils of canola treated with diazotrophic bacteria. However, there was no clear correlation between the ARA of pure strains and their performance in inoculated canola plants or in rhizospheric soils.

Under greenhouse conditions, inoculation of rice with free-living diazotrophic bacteria remarkably increased plant height and dry biomass production compared with the control. However, the nitrogenase activity of pure cultures of the strains did not correlate much with their performance in inoculated rice plants. For instance, *Sphingomonas* sp. RFNB22 had the lowest ARA in pure culture but showed the maximum activity in association with roots. This result implies that the ability of diazotrophs to fix N_2 under pure culture conditions and in association with plants is not always the same, a conclusion that is supported by the findings of Kim *et al.* [20]. Furthermore, significantly higher diazotrophic populations were observed only in rhizoplane and rhizosphere samples treated with either *Herbaspirillum* sp. RFNB26 or *Azorhizobium* sp. RFNB31. Large diazotrophic populations were detected in uninoculated plant samples, indicating that colonization by indigenous bacteria had occurred as well. Most of the tested strains enhanced nitrogenase activity in rhizoplane and rhizosphere soils, implying that the inoculants had a positive effect on rice growth. However, indigenous diazotrophic populations could have contributed to the promotion of plant growth [29]. Inoculation with diazotrophic bacteria increased the level of N accumulation in 45-day-old rice seedlings, which reached 20.2% in the treatment with *Herbaspirillum* strain RFNB26 (Table 4). This result signifies the efficient transfer of N to plants by the inoculated diazotrophs.

Perhaps as a result of the varied ability of diazotrophic strains to exhibit their PGP potential under different environmental conditions, we did not find a correlation between the results obtained with canola under growth chamber conditions and those obtained for rice under greenhouse conditions. Nonetheless, the selected diazotrophic strains were effective in promoting growth of the two plants under both conditions, perhaps because of the ability to stimulate plant growth directly by any one of the PGP mechanisms or a combination of them.

Studies predict that diazotrophic bacteria help in maintaining soil fertility and N input in disturbed ecosystems [11, 19]. The diazotrophic strains used in the present study, which were able to produce IAA and siderophores, synthesize ACC deaminase, oxidize S, and solubilize P and Zn, could be used to promote growth of oil-seed crops such as canola as well as cereal crops such as rice under controlled conditions. The present compilation of specific PGP traits of diverse diazotrophs suggests that these particular organisms can promote plant growth by more than one mechanism and that these traits could be better exploited if a diverse group of free-living diazotrophic PGPB are selected for use. However, any practical application of these results should be preceded by further evaluation under field conditions. Besides exploring the potential for BNF and other promising PGP functions carried out by free-living diazotrophs, it is also important to ensure that the bacteria are well adapted to environmental conditions before they are utilized as inoculant strains. Free-living diazotrophs could be very useful in the formulation of new microbial inocula and could be applied most profitably to economically important non-legume crops.

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