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Cultivable Endophytic Bacteria in Seeds of Dongxiang Wild Rice and Their Role in Plant-Growth Promotion

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Abstract: Dongxiang wild rice (*Oryza rufipogon* Griff.) germplasm is a precious resource for the improvement of agronomic traits in rice. Rice seeds also harbor a diverse endophytic bacterial community, and their interactions with their hosts and each other can influence plant growth and adaptability. Here, we investigated the community composition of cultivable endophytic bacteria obtained from the surface-sterilized seeds of Dongxiang wild rice and screened them for plant growth-promoting traits. Phylogenetic analysis of 16S rRNA gene sequences indicated that the 47 isolates were affiliated with five classes and 13 discrete genera, and *Bacillus* and *Microbacterium* predominated. Evaluations of plant growth promoting (PGP) traits showed that 45 endophytic bacteria isolates produced between 3.37 and 90.11 μg mL^{-1} of Indole-3-acetic acid (IAA), with the highest yield of 90.11 μg mL^{-1} (Fse28). Further, 37 of the isolates were able to solubilize mineral phosphate, while 28 other isolates had the ability of N_2-fixation, 17 isolates possessed 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity with the highest yield of 20.72 μmol mg^{-1} protein h^{-1} (Fse35), and 17 isolates were also able to produce siderophores. The two strains Fse28 and Fse35 had multiple PGP traits that significantly improved the agronomic traits (root length, shoot length, dry matter, and chlorophyll content) of cultivated rice seedlings. Our results illustrate the rich diversity of seed endophytic bacteria in Dongxiang wild rice and their potential for developing novel efficient bioinoculants to enhance soil fertility and favor seedling growth.

Keywords: Dongxiang wild rice seed; endophytic bacteria; plant growth-promoting trait; plant–microbe interaction

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1. Introduction

Plants can establish relationships with other members of their ecosystem, enjoying benefits to their growth and development while providing an ecological niche for thriving microorganisms [1,2]. Endophytes are microorganisms that inhabit the various tissues and organs of some or all stages of healthy plants and do not cause substantial damage to the host plant [3]. Researchers have successfully classified and reported more than 200 genera of endophytic bacteria from different plant tissues [4]. Endophytic bacteria sometimes provide multiple benefits to host plants, such as stimulation of plant growth by promoting biological nitrogen fixation, phosphate solubilization, phytohormone production (indole-3-acetic acid (IAA) and gibberellins), ACC deaminase activity, and enhancing antagonism against plant pathogens [5–8]. As a new agricultural resource, the study of endophytic bacteria and their interactions with plants has attracted much attention recently in the fields of plant science, agronomy, thermology, and ecology [9,10].

Seeds are as much an important plant organ as roots, leaves, and flowers, which have had to evolve in association with diverse microbial communities [11–13]. Previous studies

have reported that seed microbes may originate from the earliest stages of seed development on the parent plant and then are able to establish early in soil grown seedlings [14,15]. Evidence has also indicated that vertical or seed transmission can significantly contribute to the plant microbiome [16,17]. Indeed, endophytic seed microbes have been previously shown to have potential to promote plant growth or control plant diseases [11,18,19]. Some examples of these bacteria are *Bacillus, Enterococcus, Paenibacillus,* and *Methylobacterium*, which were isolated from *Eucalyptus* species seeds [20]. The common bacterial genera reported in seeds of different plant species are *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea*, *Acinetobacter*, *Bacillus*, and *Pseudomonas* [21]. Some seed endophytes are involved in plant growth promotion and can protect the host plant against pathogens. For example, Khalaf et al. [22] reported that *Bacillus* and *Paenibacillus* isolated from cucurbit seeds emit volatile organic compounds (VOCs) and secrete extracellular ribonucleases which could suppress fungal pathogens. Jeong et al. [23] observed that the colonization of seed endophytic bacterium *Kosakonia cowanii* GG1 into *Arabidopsis thaliana* resulted in the stimulation of plant growth under drought conditions. Xu et al. [24] found a seed-borne *Bacillus* strain that improved the root and shoot growth of tomato, probably through the production of ACC deaminase and nitrogen fixation. These results illustrate how seed endophytic bacteria may express functions beneficial to host plants and suggest they have potential for biotechnological applications.

Dongxiang wild rice (DXWR, *Oryza rufipogon* Griff.), the northernmost wild rice in Jiangxi Province, China and even the world (28°14′ N), is endemic to Dongxiang County with an annual average temperature of 17.7 °C and low temperature average in winter of −12.8 °C [25]. The ecological environment is surrounded by low hills, marshes, ditches, and ponds [26]. The presence in DXWR of genes related to high grain yield, disease and insect resistance, fertility restoration, as well as cold and drought tolerance have been extensively studied [27,28]. As an example, the NBS-LRR resistance gene of DXWR was cloned based on the conserved motif of NBS, and sequence analysis revealed that some amino acids were deleted [29]. Zhou et al. [30] found that, compared with cultivated rice, Dongxiang wild rice had a very high survival rate under salt stress, which was associated with large differences in its leaf and root transcriptomes. Microbes associated with plants may also play important roles in affecting significant plant properties. Previous studies have reported a number of endophytic bacteria (including *Phytobacter diazotrophicus)* with high nitrogen fixing enzyme activity and probiotic potential that were isolated from wild rice [31,32]. A study revealed a hitherto unreported endophytic bacterium from wild rice germplasm, *Microbacterium laevaniformans,* with high production of indole acetic acid and gibberellic acid [33]. Our earlier study described rhizobacteria [34], endophytic bacteria [35], fungi [36], and actinomycetes [37] from different tissues (root, stem and leaf) of DXWR, which showed high diversity. Zeng et al. [38] reported that *Pantoea agglomerans* T21 isolated from rhizosphere with multiple PGP traits could stimulate the growth of cultivated rice. However, the diversity and PGP effects of seed-colonizing endophytic bacteria from DXWR remain poorly understood. Therefore, assessing the community and beneficial functions of seed-colonizing endophytic bacteria will extend our knowledge of plant and microbial interactions.

In the present study, we investigated the community composition of cultivable endophytic bacteria obtained from the surface-sterilized seeds of Dongxiang wild rice using five isolation media, including nutrient agar (NA), 10-fold diluted nutrient agar (TDNA), Reasoner's 2A agar (R2A), Tryptone soy agar (TSA), and Baird-Parker agar (BPA). To further investigate the function of the cultivatable seed endophytic bacteria community, we measured nitrogen fixation, phosphorus solubilization, indole acetic acid (IAA) production, iron production carrier, and ACC deaminase activity of endophytic bacteria. We also screened for their plant growth promotion potential, inoculating them on domesticated rice seeds to evaluate their effect on seedling germination and growth. Our results show that DXWR seeds possess many cultivatable strains of bacterial endophytes that have potential for agricultural applications.

2. Materials and Methods

2.1. Isolation of Endophytic Bacteria

Healthy seeds of DXWR were collected from natural populations in a natural reserve located in Dongxiang County, Jiangxi Province, China (28°14′ N latitude and 116°30′ E longitude). All seeds were stored in an icebox and immediately brought to the laboratory. The tissues of the samples were screened for endophytic bacteria within 24 h.

Surface sterilization involved shaking seeds in autoclaved distilled water thrice followed by 5 min rinsing in sodium hypochlorite (5%), followed by draining of the bleach. Seeds were then rinsed with autoclaved distilled water, before being washed with 95% ethanol for 5 min. After draining the ethanol, the seeds were rinsed three times with autoclaved distilled water. To verify that the surface sterilization was adequate, 200 µL of the last wash were plated and cultured on five different types of agar media: nutrient agar (NA), 10-fold diluted nutrient agar (TDNA), Reasoner's 2A agar (R2A), Tryptone soy agar (TSA), and Baird-Parker agar (BPA) and these plates were incubated for 3 days at 30 °C.

Once seed surface sterility was confirmed, 15 seeds per gram were grounded gently in an autoclaved mortar using 0.5 mL of 50 mM Na_2HPO_4 buffer. The ground seed suspension (100 µL) was used for microbial culturing: 10-fold serial dilutions in 50 mM Na_2HPO_4 (10×, 100× and 1000×) were streaked on NA, TDNA, R2A, TSA, and BPA media followed by incubation for 1–7 days at 30 °C. Morphologically unique bacterial colonies from each plate were selected, streaked on fresh plates to purify, and finally cultured in LB broth (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.2) for glycerol stock and DNA extraction.

2.2. DNA Extraction, 16S rRNA Gene Amplification, Sequencing, and Strain Identification

Endophytic bacteria were cultured in LB broth at 37 °C with constant shaking for 24 h. For the taxonomic indentation of bacterial endophytes, their genomic DNA was extracted using an EZNA bacterial DNA kit (D3350-01, Omega, Norcross, GA, USA) following the manufacturer's instruction. The 16S rRNA gene was amplified using 27F (5′-AGAGTTTGATCCTG GCTCAG-3′) and 1492R (5′-TACGGCTACC TTGTTACGACTT-3′) universal primers [39]. Approximately 2 µL of total DNA (50 ng genomic DNA) was added to a PCR mixture containing 20 µL of rTaq PCR Mastermix (Takara Bio, Beijing, China), 2 µL of each primer (10 µM working stock), and H_2O to a final volume of 40 µL. Amplification was set at the following conditions in a DNA Thermal Cycler (S1000, BioRad, Hercules, CA, USA): denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min and final extension at 72 °C for 5 min. As a negative control, the template DNA was replaced by sterile double-distilled water. The PCR amplified products were resolved in 1.2% (*w*/*v*)) agarose gel and documented using the Gel documentation system (Gel Doc XR+, BioRad). Amplicons of about 1500 bp were selected and gel purified using a Gel Extraction Kit (Omega). The purification and sequencing of the PCR products was performed by Shanghai Invitrogen Company Ltd. (Shanghai, China).

The bacterial 16S rRNA sequences were matched against the nucleotide database using the Basic Local Alignment Search Tool of the US National Centre for Biotechnology Information (NCBI) for the final identification of the endophytic bacteria. Sequences were deposited in GenBank under accession numbers KJ733853~KJ733899. The most appropriate relative sequences were selected based on maximum identity and habitat in the NCBI database and imported into MEGA 5.0 [40]. To reveal the genetic distances between different bacterial strains intuitively, we constructed a rooted phylogenetic tree by using the neighbor-joining method combined with bootstrap analysis with 1000 replicates.

2.3. Assay for Plant Growth-Promoting Activities

Bacterial isolates were screened for N-fixation ability by observing the growth on nitrogen-free semisolid medium (BAz) [41]. Production of siderophore was estimated on chrome-azurol S-agar medium by observing the development of orange color around the bacterial colony [42]. To test the ability of bacteria to solubilize inorganic phosphate,

isolates were inoculated in duplicate to the NBRIP plate at 30 °C for 10 days [43]. The P content in the supernatant was tested by a spectrophotometrical method involving a 96-well microplate [44]. The production of IAA was determined by the colorimetric methodology described by Ribeiro and Cardoso [45] with some modifications. The ability of the isolates to produce ACC deaminase was also screened on minimal media containing ACC as their sole nitrogen source, as described by Penrose and Glick [46]. All the experiments were performed in triplicate and repeated three times.

2.4. Evaluation of Plant Growth Promotion in Rice Treated with Endophytic Bacteria

Based on the performance of PGPB in the experiments, two selected isolates, Fse28 and Fse35 were put through seed germination and seedling growth assays conducted in a greenhouse. Rice seeds were surface sterilized as previously described in this paper. 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}) or sterile water (uninoculated control) for 12 h, and then germinated on filter paper moistened with distilled water in a dark incubator at 30 °C. Evaluations of the length (cm) of the root and shoot were made after 7 days.

After seeds were germinated in the dark for 7 days, seedlings were transferred to pots (diameter, 15 cm; depth, 9 cm) that were filled with sterile silica sand. Hence, 10 seedlings were planted in each pot and three replicates were used for each treatment. The growth chamber experiment was conducted with a photoperiod cycle of 14 h light/10 h dark at 28 °C. Hoagland's nutrient solution (Hoagland's composition (mg L^{-1}): KNO_3 607, $Ca(NO_3)_2{\cdot}4H_2O$ 945.0, $MgSO_4{\cdot}7H_2O$ 493.0, $NH_4H_2PO_4$ 115.0, H_3BO_3 2.86, $MnCl_2{\cdot}4H_2O$ 2.13, $ZnSO_4{\cdot}7H_2O$ 0.22, $CuSO_4{\cdot}5H_2O$ 0.08, $H_2MoO{\cdot}4H_2O$ 0.02, $FeSO_4{\cdot}7H_2O$ 2.78, Na_2-EDTA 3.72) was watered every two days. After 10 and 20 days, plants were carefully removed from the pots and length data were recorded. The roots and shoots were separated and washed in distilled water, then dried and weighed.

For measuring chlorophyll content, 100 mg of finely chopped fresh leaves were placed in a capped measuring tube containing 25 mL of 80% acetone, and placed inside a refrigerator (4–8°C) for 28 h. The chlorophyll content was measured at 646.6 and 663.6 nm in a spectrophotometer and calculated using the equation of Porra [47].

2.5. Statistical Analysis

All results represent the means based on three or more independent replicates. Differences were compared with the one-way analysis of variance test and means were compared using Duncan's multiple range test, where $p < 0.05$ was considered to indicate a significant difference. Results were expressed as the mean ± SD.

3. Results

3.1. Diversity and Community Composition of Seed Cultivable Endophytic Bacteria

The surface disinfection of seeds was efficient at eliminating epiphytic bacteria because the seed imprint on nutrient agar yielded no bacterial growth. A total 165 endophytic bacteria were isolated from the seed of DXWR. Based on their morphological characteristics, 47 apparently distinct isolates were selected for further study. NA and TDNA mediums yielded in the highest number of isolates and highest diversity with eight different genera. The R2A medium obtained the next highest diversity with six different genera, while TSA and BPA medium obtained only five different genera each. *Bacillus* spp. Were recovered from all five medium. Meanwhile, the strains *Facklamia* spp., *Acinetobacter* spp., and *Burkholderia* spp. could be only isolated from NA. *Methylobacterium* spp. was only isolated from TDNA. *Micrococcus* spp. was only isolated from TSA (Table 1).

Table 1. The numbers and genera of endophytic bacteria recovered on the five different isolation media from DXWR seeds.

Isolation Medium	Numbers of Isolates	Genera
NA	14	*Microbacterium*, *Bacillus*, *Pseudomonas*, *Pantoea*, *Xanthomonas*, *Facklamia*, *Acinetobacter*, *Burkholderia*
DNA	13	*Methylobacterium*, *Pantoea*, *Curtobacterium*, *Microbacterium*, *Micrococcus*, *Bacillus*, *Xanthomonas*, *Sphingomonas*
BPA	6	*Bacillus*, *Microbacterium*, *Sphingomonas*, *Curtobacterium*, *Pseudomonas*
R_2A	6	*Bacillus*, *Microbacterium*, *Pseudomonas*, *Pantoea*, *Curtobacterium*, *Lysinibacillus*
TSA	8	*Bacillus*, *Microbacterium*, *Curtobacterium*, *Pantoea*, *Pseudomonas*

Analysis of 16S rDNA sequence from the 47 isolates indicated significant genetic diversity in to three different classes (α-, β- and γ-proteobacteria, Firmicutes and Actinobacteria) and 13 discrete genera (*Bacillus*, *Microbacterium*, *Curtobacterium*, *Pseudomonas*, *Pantoea*, *Xanthomonas*, *Sphingomonas*, *Methylobacterium*, *Burkholderia*, *Acinetobacter*, *Lysinibacillus*, *Facklamia and Micrococcus*) (Table 2, Figure 1). Members of the class γ-Proteobacteria were predominant in composition (n = 17, 36.2%), followed by Actinobacteria (n = 15, 31.9%), Firmicutes (n = 10, 21.3%), α-Proteobacteria (n = 4, 8.5%), and β-Proteobacteria (n = 1, 2.1%) (Figure 1a). Among 47 bacterial endophytes, *Bacillus* (n = 8, 17%) and *Microbacterium* (n = 8, 17%) were the predominant taxa, followed by *Curtobacterium* (n = 7, 14%), whereas six strains each of *Pseudomonas* and *Pantoea*, four strains of *Xanthomonas*, three strains of *Sphingomonas*, and one strain each of *Methylobacterium*, *Burkholderia*, *Acinetobacter*, *Lysinibacillus*, *Facklamia*, and *Micrococcus* were also identified (Figure 1b). The phylogenetic tree constructed based on 16S rDNA gene sequences for the 47 isolates are shown in Figure 2. The phylogenetic dendrogram indicates a division of the endophytic bacteria into thirteen distinct clades with different colors representing discrete genera (Figure 2). Overall, these results demonstrate the presence of abundant and diverse endophytic bacteria within seeds of DXWR.

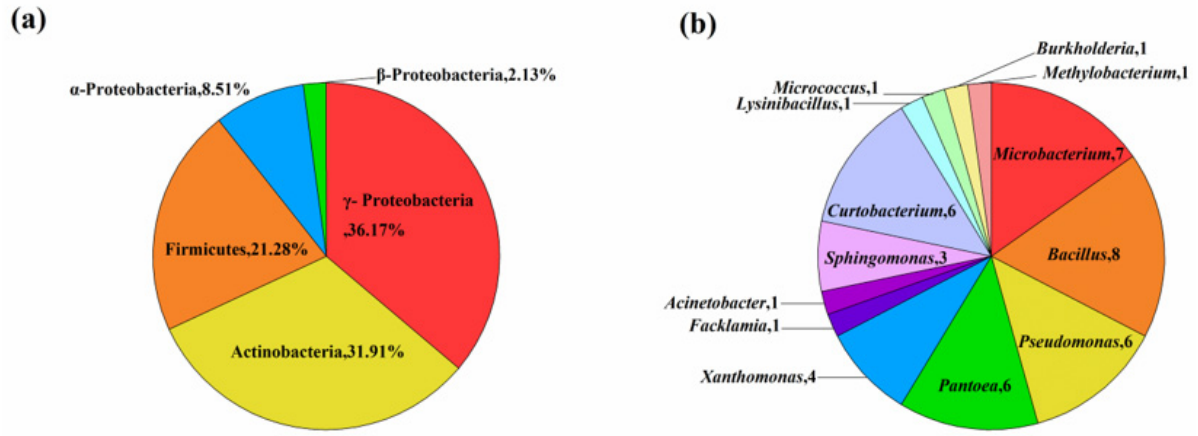

Figure 1. Taxonomic diversity of the obtained representative bacterial isolates. (**a**) Composition percentages of the isolates at the phylum level. (**b**) Numbers of isolates at the genus level.

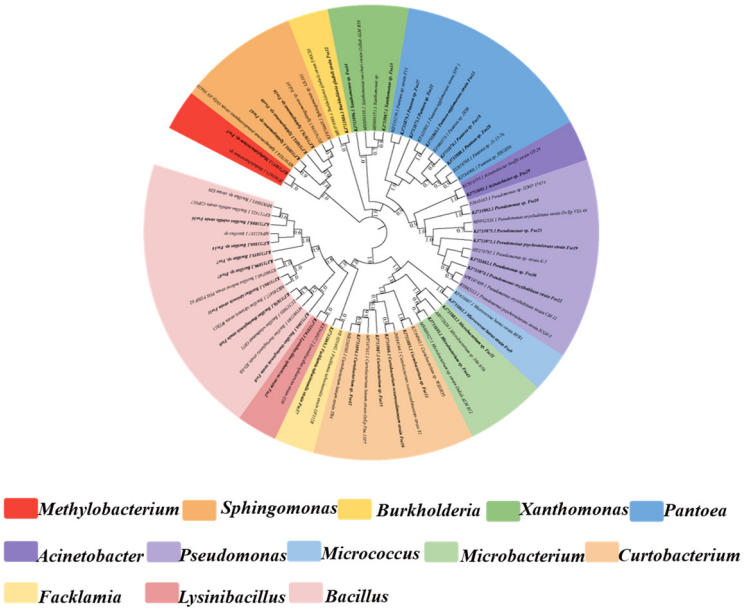

Figure 2. Phylogenetic tree of representative strains of each species of endophytic bacteria in DXWR and relative strains in GenBank database based on 16S rDNA sequences.

3.2. Screening for PGP Traits of Isolates from Oryza Rufipogon Griff. In Vitro

The potential of the isolates for plant growth promotion in this study was evaluated by screening for N_2 fixation, IAA production, ACC deaminase activity, siderophore production and mineral phosphate solubilization. The differential PGP traits identified in all of 47 isolates were summarized in Table 2. The numbers of endophytic bacteria isolated from seeds with different PGP traits is shown in Figure 3. Among the 47 isolates, a higher percentage of the isolates showed IAA production (95.74%), followed by phosphate solubilization (78.72%), nitrogen fixation (59.57%), siderophore production (36.17%), and ACC deaminase activity (36.17%), respectively. All the strains except for Fse11 and Fse47 possessed the ability to produce IAA at different efficiencies. The highest levels of IAA production (90.11 $\pm$ 5.90 μg mL^{-1}) were observed for *Pantoea* sp. strain Fse28. On the basis of the phosphate solubilization assay, 37 bacterial isolates were able to mobilize calcium phosphate, with *Methylobacterium* sp. strain Fse5 showing the highest activity of phosphate solubilization (576.34 $\pm$ 24.63 μg mL^{-1}). Seventeen strains produced ACC deaminase with activity ranging from 2.12 μmol mg^{-1} protein h^{-1} (*Curtobacterium* sp. strain Fse42) to 20.72 μmol mg^{-1} protein h^{-1} (*Curtobacterium* sp. strain Fse35). Further, 17 bacteria were positive for siderophore production with a higher proportion of *Bacilli* than *Actinobacteria*. Twenty-eight isolates grew in a N-free medium, indicative of their ability for N-fixation, however none of these were *Bacilli*. Looking for strains with multiple plant growth promoting traits, two strains (Fse28 and Fse35) were selected for further evaluation in seed germination and potted plant growth experiments.

Table 2. Summary of the endophytic bacteria isolated from DXWR seeds together with their respective strain codes, GenBank accession numbers, closest affiliations among the representative isolates in GenBank according to 16S rRNA sequence analysis, and PGP traits.

Strain Code	GenBank Number	Closest Relative from GenBank	Max. Identity	ACC Deaminase	N-Fixation	IAA (μg mL^{-1})	Siderophore	Phosphate Solubilisation (μg mL^{-1})
Fse5	KJ733857	*Methylobacterium* sp.	99	6.13 ± 0.27	–	33.72 ± 1.28	+	576.34 ± 24.63
Fse26	KJ733878	*Sphingomonas* sp.	99	-	+	22.86 ± 1.66	+	27.70 ± 6.15
Fse40	KJ733892	*Sphingomonas* sp.	99	-	+	22.45 ± 2.73	–	-
Fse41	KJ733893	*Sphingomonas* sp.	99	-	+	16.75 ± 1.76	–	-
Fse32	KJ733884	*Burkholderia gladioli*	100	16.91 ± 1.38	–	18.56 ± 0.69	+	339.07 ± 10.28
Fse10	KJ733862	*Pseudomonas* sp.	99	8.22 ± 0.68	+	36.76 ± 2.87	+	187.85 ± 12.88
Fse23	KJ733875	*Pseudomonas* sp.	99	-	+	59.63 ± 6.18	–	85.29 ± 8.02
Fse30	KJ733882	*Pseudomonas* sp.	100	-	+	45.08 ± 1.81	–	297.09 ± 32.93
Fse19	KJ733871	*Pseudomonas psychrotolerans*	100	-	+	18.87 ± 1.28	+	135.77 ± 3.86
Fse22	KJ733874	*Pseudomonas oryzihabitans*	100	7.61 ± 0.72	+	19.06 ± 4.46	–	30.47 ± 2.40
Fse24	KJ733876	*Pseudomonas oryzihabitans*	100	-	+	33.02 ± 1.38	–	120.46 ± 11.67
Fse15	KJ733867	*Xanthomonas* sp.	100	-	–	15.09 ± 1.22	–	78.73 ± 5.15
Fse20	KJ733872	*Xanthomonas* sp.	100	-	–	20.89 ± 1.53	–	-
Fse37	KJ733889	*Xanthomonas sacchari*	100	-	–	14.01 ± 0.35	–	207.06 ± 9.16
Fse44	KJ733896	*Xanthomonas sacchari*	100	-	–	54.29 ± 2.41	–	16.25 ± 4.15
Fse21	KJ733873	*Pantoea* sp.	100	-	+	72.97 ± 1.87	–	384.56 ± 29.09
Fse18	KJ733870	*Pantoea* sp.	99	-	+	89.70 ± 5.17	–	13.13 ± 2.95
Fse25	KJ733877	*Pantoea ananatis*	99	10.21 ± 0.94	+	47.25 ± 0.41	–	500.32 ± 56.05
Fse27	KJ733879	*Pantoea* sp.	99	15.41 ± 0.85	+	81.24 ± 2.01	–	280.90 ± 7.77
Fse28	KJ733880	*Pantoea* sp.	99	19.51 ± 1.64	+	90.11 ± 5.90	+	302.54 ± 8.80
Fse13	KJ733865	*Pantoea agglomerans*	99	13.23 ± 0.97	+	48.14 ± 2.73	+	193.74 ± 2.55
Fse29	KJ733881	*Acinetobacter* sp.	99	-	–	27.18 ± 0.98	+	209.67 ± 23.52
Fse1	KJ733853	*Bacillus subtilis*	99	12.24 ± 1.02	–	51.41 ± 2.04	–	-
Fse36	KJ733888	*Bacillus subtilis*	99	-	–	29.28 ± 4.17	+	43.83 ± 3.99
Fse7	KJ733859	*Bacillus velezensis*	100	-	–	6.02 ± 0.35	–	18.46 ± 3.85
Fse14	KJ733866	*Bacillus* sp.	100	8.12 ± 0.56	–	30.45 ± 1.83	+	102.29 ± 8.28
Fse47	KJ733899	*Bacillus* sp.	100	-	–	-	+	8.62 ± 1.45
Fse6	KJ733858	*Bacillus thuringiensis*	100	-	–	27.11 ± 1.35	+	92.75 ± 12.27
Fse8	KJ733860	*Bacillus thuringiensis*	100	-	–	3.37 ± 0.25	–	5.88 ± 0.69
Fse11	KJ733863	*Bacillus idriensis*	100	12.25 ± 1.25	–	-	–	-
Fse2	KJ733854	*Lysinibacillus sphaericus*	100	5.77 ± 0.44	–	8.66 ± 0.18	+	-
Fse17	KJ733869	*Facklamia tabacinasalis*	100	-	–	5.53 ± 0.21	+	61.53 ± 1.34
Fse3	KJ733855	*Microbacterium* sp.	100	-	+	7.88 ± 0.14	–	4.63 ± 0.79
Fse4	KJ733856	*Microbacterium* sp.	100	-	+	34.35 ± 2.35	–	29.09 ± 0.46
Fse12	KJ733864	*Microbacterium* sp.	100	-	+	6.16 ± 0.95	–	509.96 ± 39.41
Fse31	KJ733883	*Microbacterium* sp.	100	-	+	8.45 ± 0.46	–	151.88 ± 8.61
Fse34	KJ733886	*Microbacterium* sp.	100	-	+	54.52 ± 1.95	–	24.75 ± 4.31
Fse43	KJ733895	*Microbacterium* sp.	100	-	+	28.83 ± 3.65	–	-
Fse46	KJ733898	*Microbacterium* sp.	100	-	–	15.70 ± 2.78	–	60.49 ± 7.94

Table 2. *Cont.*

Strain Code	GenBank Number	Closest Relative from GenBank	Max. Identity	ACC Deaminase	N-Fixation	IAA (μg mL^{-1})	Siderophore	Phosphate Solubilisation (μg mL^{-1})
Fse9	KJ733861	*Micrococcus luteus*	100	2.12 ± 0.18	−	18.77 ± 0.69	+	-
Fse33	KJ733885	*Curtobacterium* sp.	100	-	+	8.58 ± 1.23	−	14.69 ± 1.91
Fse35	KJ733887	*Curtobacterium* sp.	99	20.72 ± 1.89	+	86.72 ± 5.40	+	112.35 ± 3.11
Fse38	KJ733890	*Curtobacterium* sp.	99	14.32 ± 0.23	+	72.94 ± 7.46	−	-
Fse39	KJ733891	*Curtobacterium* sp.	99	-	+	42.23 ± 5.49	−	-
Fse42	KJ733894	*Curtobacterium* sp.	100	12.12 ± 0.18	+	25.42 ± 2.26	−	6.90 ± 1.66
Fse45	KJ733897	*Curtobacterium* sp.	100	-	+	18.35 ± 0.33	−	84.77 ± 11.66
Fse16	KJ733868	*Curtobacterium oceanosedimentum*	100	15.74 ± 0.26	+	9.09 ± 0.34	+	191.90 ± 29.50

+ Presence of trait; − absence of trait; data represent the mean ± standard error (SE) based on three replicates. The ACC deaminase activity was measured spectrophotometrically at 590 nm and expressed as μmol mg^{-1} protein h^{-1}.

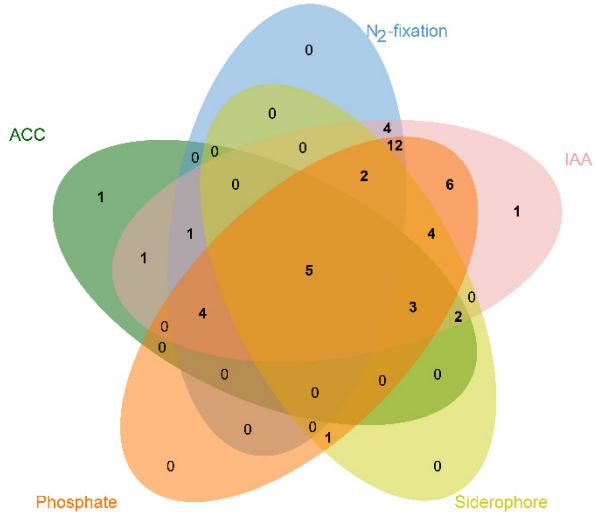

Figure 3. Venn diagram showing the distribution of seed-colonizing endophytic bacteria with different PGP traits isolated from DXWR seeds.

3.3. Effect of Bio-Inoculum Treatment on Rice Growth-Promoting under Growth Chamber

The effect of the two multi-functional PGP strains (Fse28 and Fse35) on seed germination and plant growth is shown in Table 3. The tested strains enhanced seed germination percentage, resulting in 75.67% germination when rice seeds were treated with the isolate Fse28 in comparison to the negative control 71.67%. Seeds inoculated with Fse28 and Fse35 presented higher results than the uninoculated seeds in terms of shoot and root length as well. Inoculation with *Pantoea* sp. Fse28 significantly ($p \leq 0.05$) increased shoot height and root length as compared to uninoculated plants, with a growth rate of 41.6% and 47.6%, respectively.

Table 3. Effects of inoculation with two putative PGPE strains on rice seed germination.

Treatments	3 Days	5 Days	7 Days		
	Germination Rate (%)	Germination Rate (%)	Germination Rate (%)	Sprout Length (cm)	Root Length (cm)
Control	47.57 ± 0.66 ^{c}	70.13 ± 1.98 ^{c}	94.62 ± 1.52 ^{b}	1.40 ± 0.18 ^{a}	1.28 ± 0.15 ^{a}
Fse28	56.44 ± 1.01 ^{a}	85.52 ± 2.11 ^{a}	96.67 ± 0.49 ^{a}	2.19 ± 0.28 ^{b}	2.53 ± 0.49 ^{b}
Fse35	50.86 ± 1.14 ^{b}	80.63 ± 2.09 ^{b}	95.33 ± 0.54 ^{b}	2.05 ± 0.29 ^{b}	2.07 ± 0.24 ^{b}

Germination rates represent averages based on 20 seeds (n = 20). Sprout and root lengths represent averages based on 20 seeds (n = 20). Mean values with the same letter(s) in the column do not differ significantly according to Duncan's multiple range test ($p < 0.05$).

After 10 and 20 days of the bacteria and host–plant association, the plant growth index was different between inoculated and uninoculated seedlings (Table 4, Figure 4). After 10 days, bacteria-inoculated rice seedlings showed significant increases in plant shoot length, dry weight, and chlorophyll content as compared to the uninoculated controls, although there was no significant difference in root length. Inoculation with Fse28 caused a strong increase in shoot length (18.84 cm), dry weight (28.34 mg), and chlorophyll content (1.82 mg/g), which was 31.3%, 50.7%, and 219.3% higher than the control, respectively. Data on root length, shoot length, dry weight, and chlorophyll content indicate that inoculation with Fse28 enhanced growth parameters significantly ($p = 0.05$) in comparison with the control after 20 days of development, and the seedling inoculated with Fse35 also had significant increases in shoot length and chlorophyll content. In summary, Fse28 was the most effective of the isolates tested in enhancing rice root, shoot, dry weight, and chlorophyll content.

Table 4. Effects of inoculation with two PGPE strains on different growth parameters in rice after transplantation for 10 and 20 days.

Days	Treatment	Shoot Length (cm)	Root Length (cm)	Dry Weight (mg)	Chlorophyll Content (mg g^{-1})
10 d	control	14.35 ± 0.22 ^{a}	6.60 ± 0.28 ^{a}	18.80 ± 2.29 ^{a}	0.57 ± 0.15 ^{a}
	Fse28	18.84 ± 0.69 ^{bc}	8.92 ± 1.46 ^{a}	28.34 ± 1.17 ^{b}	1.82 ± 0.15 ^{bc}
	Fse32	15.89 ± 0.45 ^{b}	7.35 ± 1.49 ^{a}	25.86 ± 2.47 ^{b}	1.11 ± 0.11 ^{b}
20 d	control	26.04 ± 0.40 ^{a}	9.06 ± 0.69 ^{a}	70.80 ± 9.53 ^{a}	1.68 ± 0.14 ^{a}
	Fse28	32.22 ± 1.00 ^{bc}	12.60 ± 1.12 ^{b}	168.87 ± 32.21 ^{b}	2.74 ± 0.37 ^{b}
	Fse32	29.55 ± 1.44 ^{b}	11.63 ± 1.42 ^{b}	118.79 ± 15.23 ^{b}	2.41 ± 0.12 ^{b}

Data represent averages based on 15 seedlings (n = 15). Chlorophyll contents represent averages based on three replicates (n = 3). Mean values with the same letter(s) in the column do not differ significantly according to Duncan's multiple range test ($p < 0.05$).

Figure 4. Effects of inoculating with PGPE bacteria on the growth of shoots and roots in rice seedlings after treatment for 10 and 20 days. Representative images are shown of rice seedlings with or without inoculation using endophytic bacteria for 10 and 20 days ((**A**) and (**B**), respectively).

4. Discussion

Plant seeds harbor complex and variable microbial communities that may be an important source of endophytic bacteria [11]. Most of the publications on seed endophytic bacteria have reported members belonging to Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes [21]. Strains assigned to *Pantoea*, *Bacillus*, *Methylobacterium*, *Rhizobiumr*, *Xanthomonas*, *Sphingomonas*, and *Microbacterium* have been found inside both the hybrid and cultivated rice seed [48,49]. In the present study, the molecular characterization of 47 strains revealed a significant taxonomic diversity, including members of α-, β-, and γ-proteobacteria, Firmicutes, and Actinobacteria, representing 13 different genera (Table 2 and Figure 1). The dominant genus observed in this study was *Bacillus* (at least eight out of 47 isolates), which is consistent with previous reports [50]. Apart from *Bacillus*, the Actinobacterial *Microbacterium* and *Curtobacterium* were the prevalent genera (Table 2), as also reported for mature seeds of rice varieties cultivated in the Philippines and Japan [51,52]. The core microbiome of *Bacillus* and *Microbacterium* from seeds is consistent with our previous results from Dongxiang wild rice [35], and is supported by the recent finding that

most seed transmitted bacteria are part of a core plant microbiome [17]. Some seed-borne pathogenic endophytes, such as *Burkholderia* spp. and *Xanthomonas* spp., were identified in our study (Figure 1b). However, Cottyn et al. [53] found that many *Burkholderia* spp. and *Xanthomonas* spp. isolated from rice seed fail to cause disease symptoms in common cultivated rice, suggesting they are in fact not pathogenic. Interestingly, we obtained strains of *Lysinibacillus sphaericus* and *Facklamia tabacinasalis*, which have not been obtained previously identified in other rice seed studies, suggesting that the study of different plant varieties, using different isolation protocols and distinct growth media can yield differences in the cultivatable population of bacterial endophytes species [54,55].

IAA is a plant hormone which stimulates the development of root systems and promotes plant-microbe interactions [56,57]. Almost all the isolates in the present study are capable of producing IAA, with the strain *Pantoea* sp. Fse28 showing the highest IAA production (90.11 $\pm$ 5.90 mg L^{-1}) (Table 2 and Figure 3), which representhigher levels of IAA production than typically found in other seed endophytic bacteria [24,58,59]. The solubilization of insoluble phosphates by PGP bacteria could increase the availability of the limiting nutrient phosphorus to the host [60]. Previous experiments have shown that endophytic bacteria possess the capacity to solubilize immobilized mineral phosphates [61], suggesting that during initial colonization, endophytic bacteria could enhance phosphate availability to the host plant. About 79% of bacteria have the trait of CaP-Solubilization, three of which strains were shown to result in high levels of soluble phosphorus release (>500 μg ml^{-1}) (Table 2 and Figure 3). Similarly high numbers of phosphate solubilizing bacteria were observed in rice cultivars grown in acidic P-limited soil, as previously documented by Hameed [62]. The abundance of phosphate solubilizing bacteria in Dongxiang wild rice seeds may be related to its long-term growth in low phosphate soil, where phosphate solubilizing bacteria could assist the plant to obtain more available phosphate from the red soil for the host plant to grow.

Plants form symbiotic relationships with microbes, including endophytes, in order to facilitate biological nitrogen fixation via the conversion of atmospheric nitrogen gas into a usable form of nitrogen [5,63]. In this study, 59.57% of the seed-associated microbes (*Sphingomonas*, *Pseudomonas*, *Pantoea*, and *Microbacterium*) could grow on N-free media (Table 2; Figure 3), which suggests that they were capable of either nitrogen fixation or N-scavenging [64–66]. The ability to produce siderophores is a trait that facilitates bacteria-plant associations and contributes to the colonization of the plant [67]. Some siderophore-producing bacteria have been reported to be found in rice, with the main genera being *Pantoea*, *Bacillus*, *Pseudomonas,* and *Burkholderia* [68,69]. Our findings showed 17 bacterial endophytes affiliated to 11 different genera (Table 2; Figure 3). Moreover, the diversity of the siderophore-producing bacteria was significantly higher in DXWR seeds than other studies [70,71]. It was noteworthy that we identified high siderophore production capacity in *F. tabacinasalis* for the first time.

Bacteria can help plants to withstand stress by reducing the level of the stress hormone ethylene through the activity of the enzyme ACC deaminase which hydrolyses ACC into α-ketobutyrate and ammonia instead of ethylene [72]. Organisms with ACC deaminase activiy of 0.062–2.664 μmol α-ketobutyrate mg^{-1} protein h^{-1} or higher can promote host plant growth [73]. In our study, among the 47 isolates, 17 strains were identified with ACC deaminase activity of 2.12–20.72 μmol α-ketobutyrate mg^{-1} protein h^{-1}, where Fse28 and Fse35 had the highest ACC deaminase activities of 19.51 $\pm$ 1.64 and 20.72 $\pm$ 1.89 μmol α-ketobutyrate mg^{-1} protein h^{-1}, respectively (Table 2). In addition, Fse28 and Fse35 have five PGP traits, such as IAA production, siderophore-producing, nitrogen fixation, phosphorus solubilization, and ACC deaminase activities. PGP validation experiments found that Fse28 (*Pantoea* sp.) and Fse35 (*Curtobacterium* sp.) significantly improved the agronomic traits of cultivated rice seedlings (Figure 4). *Pantoea* spp. has been reported to enhance plant development and improve the photosynthetic efficiency of crops [74], and *Curtobacterium* spp. could improve the drought resistance and metal tolerance of plants [75,76]. These studies suggested that the endophytic bacteria Fse28 and Fse35

could potentially be used to stimulate tolerance to environmental stress and promote plant growth.

5. Conclusions

Endophytic bacteria community structure was analyzed in the seeds of DXWR using culture-dependent methods. The seeds of DXWR harbored an abundant and diverse culturable endophytic bacteria community, some of which were capable of promoting plant growth. Most of the isolated endophytic bacteria were capable of producing IAA and solubilizing phosphate, and some could also produce siderophores, exhibit ACC deaminase activities, and fix nitrogen. The inoculation of rice seedlings with strains Fse28 and Fse35 exhibited a significant increase in root length, shoot length, dry matter, and chlorophyll content compared with the controls also grown under growth chamber conditions. We think that the mechanisms these microbes use to stimulate rice germination and growth work by increasing the nutrient availability of nitrogen, phosphorous, and iron as well as the production of phytohormones. In future studies, we will investigate the dynamics of plant nitrogen, phosphorus, and potassium after inoculation to further understand the mechanisms used by these plant growth-promoting bacteria to benefit their host.

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