



Article

Response of Upland Rice (*Oryza sativa* L.) Inoculated with Non-Native Plant Growth-Promoting Bacteria

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Abstract: A deep-rooting upland rice variety (Kinandang Patong) was evaluated for its growth response to bio-fertilization at early stages. Five non-native plant growth-promoting bacteria previously isolated from yams (*Dioscorea* spp.) were inoculated to upland rice under growth chamber and greenhouse conditions. Effects of the inoculation varied depending on bacterial strains and growing conditions. Growth of 14-day rice seedlings was improved by all tested bacterial strains. Under growth chamber, the strain S-333 increased plant length, shoot dry weight and nitrogen content as compared to the control, but total dry weight, nitrogen uptake, leaf chlorophyll content and number of tillers were higher with N fertilizer application. Under greenhouse conditions, most rice growth parameters were improved by inoculation with the strain S-7. The correlations between the bacterial plant-growth-promoting traits and rice growth parameters under growth chamber conditions were all negative for phosphate solubilization indexes. Our results suggest that bacterial inoculation can replace half (S-343 and S-611) of or the full (S-7) rate of chemical N fertilizer required, depending on bacterial strains and growing environments, although $\delta^{15}\text{N}$ value in control plants was lower than in inoculated plants under growth chamber conditions, suggesting that the bacteria improve rice growth through mechanisms other than biological nitrogen fixation.

Keywords: rice; biofertilizer; indole 3-acetic acid; phosphate solubilization; growth-promoting bacteria; non-native

1. Introduction

Rice (*Oryza sativa* L.) is one of the world's most important cereal crops. Rice holds a strategic position in world food security programs as nearly half of the world population utilizes rice. Although wet rice systems occupy the majority of rice production and land use for rice cultivation in the world [1], upland rice systems are important in some regions such as in tropical areas of Africa and are important in terms of preserving biodiversity.

Plant growth-promoting bacteria (PGPB) include rhizospheric and endophytic bacteria that promote growth and yields of their host plants. PGPB have been isolated from various environments such as soil [2,3] and plants [4–6]. In rice, several diverse PGPB were reported and found to increase biomass and grain yield as well as nutrients uptake and phytohormones production [7]. Bacteria genera such as *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Micrococcus*, among which plant growth-promoting

traits and effects have been reported, were found in the roots of rice [8]. Mechanisms used by the symbiotic bacteria are diverse and depend on the interactions of plant genotype, microbes and environment. Biological nitrogen fixation is an important bacterial trait that benefits the crop by acquiring all or part of its nitrogen requirement. In rice, the acetylene reduction assay and amplification of the *nifH* genes were used to confirm the nitrogen-fixing ability of bacterial strains of *Azospirillum amazonense*, *Burkholderia vietnamiensis*, *Peanibacillus kribbensis*, *Bacillus* spp., and *Microbacterium binotii* [9,10]. Endophytic bacterial strains belonging to the genera *Bacillus* and *Pseudomonas* have been reported among the most efficient phosphates solubilizers through the release of some organic acids. Strains of *Pseudomonas* spp. have been reported as beneficial to rice through enhanced root and shoot growth, nutrient uptake, and several yield attributes [11]. Several other plant growth-promoting traits involve iron mobilization and potassium solubilization [10,12]. Increase in rice grain yield of 20%–38% in pot and 20%–52% under field conditions has been attributed to the beneficial effects of potassium solubilizing bacteria such as *Pantoea agglomerans*, *Rahnella aquatilis*, and *Pseudomonas orientalis* [13]. Moreover, the host plant can acquire resistance against several pathogens [14] and other stresses under drought conditions [15]. In several cases, when growth-promoting traits were phenotypically studied, most PGPB showed positive responses to multiple traits [16,17]. However, the interactions between plants and the PGPB have been rarely evaluated on the angle of how all the studied bacterial traits affect the plant growth, even in cases where mixtures of bacterial strains were tested [18].

In the context of climate change, and environmental pollutions caused by the inappropriate use of chemical compounds in agriculture, the contribution of PGPB is gaining more interest both for research institutions and chemical industries as an alternative to chemical fertilizers and pesticides [19]. Consequently, the development and application of biofertilizers increased rapidly during the last decades. They have shown promising ability to increase crop productivity [20]. However, the use of biofertilizers on a broad range of host crops needs preliminary investigations. In this field, several studies have been conducted using bacterial strains native to the soil or plant as inoculants [21–23] and little attention has been given to the inoculation of PGPB as non-native (exogenous) strains to other important crops [24], which could increase the rapid adoption of biofertilizers for other crops with low research outputs. In a cross-inoculation study of the legume *Schizolobium amazonicum* inoculated with strains of *Rhizobium* sp. and *Burkholderia* sp., the authors found growth promotion in seedlings and increased biomass [25].

In our previous studies conducted on the sub-tropical farm of Tokyo University of Agriculture located on the Island of Miyako, Okinawa Prefecture (Japan) in 2017–2018, several PGPB were isolated from surface-sterilized organs of yams (*Dioscorea* spp.), a monocot tuber crop. Experiments of re-inoculation and cross-inoculation with other monocot crops in order to identify potential strains as candidates for bio-fertilization are being conducted. Kinandang Patong is a deep-rooting rice variety (tropical *japonica*) from which the DEEPER ROOTING 1 (DRO1) genes were reported. Despite its interesting agronomic traits, little interest has been given to the endophytic bacteria associated with this rice variety as well as to its interaction with non-native bacteria. The current study evaluates the response of this upland rice variety to its cross-inoculation with PGPB isolated from yams.

2. Materials and Methods

Experiments reported here were all conducted using the facilities available in Tokyo University of Agriculture, Japan, Setagaya campus between October 2019 and January 2020.

2.1. Source and Growth-Promoting Traits of Bacterial Strains

Bacterial strains used in these studies were isolated from surface-sterilized roots, leaves, and tubers of the water yam (*Dioscorea alata* L.) and lesser yam (*Dioscorea esculenta* L.) grown under different fertilizer regimes [26] in Miyako Island, Japan. These were taxonomically classified using the 16S rRNA gene sequences (Appendix A) subjected to similarity search in the 16S database of EzBioCloud [27]. The plant growth promotion ability of these strains was evaluated targeting their nitrogenase activity, hormone and siderophores production and inorganic phosphate solubilization. The acetylene reduction assay (ARA) was used to qualitatively evaluate the nitrogen fixation ability [28] of pure bacterial colonies inoculated to glass tubes containing a semi-solid nitrogen-free Rennie medium (MR) and incubated at 30 °C for five days. Acetylene gas was injected into the tubes to create an atmosphere at a final concentration of 10% (v/v). The amount of acetylene converted to ethylene was measured by injecting 1 mL of the atmosphere into a gas chromatograph equipped with flame ionization detector (FID) and a Porapak N column (GC 2014, Shimadzu, Japan).

Pakovskaya's agar [29] plates were prepared for qualitative and quantitative solubilization analysis of calcium phosphate. Bacteria were spotted on the plate in triplicates and incubated at 30 °C for seven days. Strains forming visible clear halos around the colonies were considered calcium phosphate solubilizers. The quantitative analysis was achieved by determining the solubilization index which was calculated as the ratio of total halo diameter (colony diameter plus diameter of the clear zone) to the colony diameter.

Indole 3-acetic acid (IAA) production was determined [30] in triplicates. Bacterial strains were grown in 100-mL flasks containing 50 mL Luria broth (LB) supplemented with L-tryptophan ($0.5 \text{ mg} \cdot \text{mL}^{-1}$) for 48 h on rotary shaker. The broth was centrifuged at $10,000 \times g$ for 15 min. The cell-free supernatant was collected. Two milliliters of Salkovski reagent (1 mL 0.5 M ferric chloride solution added to 50 mL of 35% perchloric acid (HClO_4)) were added to 1 mL of the collected supernatant with a drop of ortho-phosphoric acid. The solution was incubated for 30 min. Samples with a pink color were considered positive for IAA production. To quantify the amount of IAA produced, absorbance of positive samples was measured at 530 nm optic density by using a spectrophotometer and calculated using a standard curve prepared with pure IAA (99.8%).

Siderophore production was evaluated on chrome azurol S (CAS) agar plates following the step-by-step protocol described in [31]. Briefly, 1 mL CAS solution (prepared by dissolving 60 mg of CAS agar in 50 mL of double-distilled water) was mixed with 9 mL iron solution (prepared from 2.7 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mL of 10 mM HCl). The mixture was poured in a solution of hexadecyltri-methylammonium bromide (HDTMA, previously prepared from 73 mg in 40 mL double-distilled water), thoroughly mixed and autoclave-sterilized at 121 °C for 15 min. Piperazine-*N,N'*-bis(2-ethanesulfonic) acid (PIPES, 32.24 g) was dissolved in 850 mL of a solution containing 100 mL of minimal media 9 (MM9) salt solution, and 15 g agar was added and autoclaved at 121 °C for 20 min. This solution was cooled down to about 50 °C, then mixed with sterile Casamino acid solution and 20% sterile glucose solution. Plates were prepared from this mixture. Bacterial colonies were spotted on the CAS plates in triplicates and incubated at 30 °C for seven days. The presence of visible clear halos around the colonies was indicative of siderophore production, of which the index was determined as the ratio of total halo diameter (colony diameter plus diameter of the clear zone) to the colony diameter. Table 1 shows the bacterial strains used as inoculants in this study.

Table 1. The plant growth-promoting traits of the bacterial strains used in this study. [†] Bacteria were identified based on their partial 16S rRNA gene sequences. The supplementary file S1 describes the protocol used to taxonomically classify the strains used in this study. * Values in parenthesis are the accession numbers of the 16S rRNA gene sequences deposited in the DDJB database. S-7, S-33 and S-343 were isolated from the water yam (*Dioscorea alata* L.) while S-499 and S-611 were isolated from the lesser yam (*Dioscorea esculenta* L.). ¹ Acetylene Reduction Assay, ² Indole 3-Acetic Acid, ³ Phosphate solubilization, ⁴ Siderophores production.

Strains	Isolation Sources *	Taxonomy [†]	ARA ¹	IAA ² ($\mu\text{g/mL}$)	PS ³ Index	SP ⁴ Index
S-7	Root of accession A-18 (LC483213)	<i>Mesorhizobium</i> sp.	+	176.4 \pm 7.1	2.7 \pm 0.3	1.6 \pm 0.1
S-333	Root of accession A-18 (LC483343)	<i>Agrobacterium</i> sp.	+	32.9 \pm 0.4	1.5 \pm 0.1	1.7 \pm 0.1
S-343	Root of accession A-19 (LC533366)	<i>Agrobacterium</i> sp.	+	120.0 \pm 1.0	1.5 \pm 0.1	1.4 \pm 0.1
S-499	Leaf of accession E-3 (LC533367)	<i>Pantoea</i> sp.	+	27.9 \pm 0.4	2.6 \pm 0.1	2.7 \pm 0.0
S-611	Tuber of accession E-3 (LC483273)	<i>Enterobacter</i> sp.	+	45.5 \pm 0.7	1.4 \pm 0.1	3.0 \pm 0.3

2.2. Experiment 1. Effects of Bacterial Inoculation on Growth of Upland Rice Seedlings

Seeds of the upland rice variety Kinandang Patong (KP), which is a landrace variety (tropical *japonica*) kept by the International Rice Research Institute (Philippine origin) under the accession number IRGC23364 and identified as a long-rooted variety [32,33], were used in this experiment. Seeds were soaked in distilled water for two days to break dormancy. They were then surface-sterilized in ethanol 70% for 2 min, sodium hypochlorite 2.5% for 1 min, and rinsed in about 500 mL sterile double distilled water four times. The seeds were then placed on sterilized filter paper contained in glass Petri dishes (90 mm). Each Petri dish contained 10 seeds and each treatment consisted of 3 replications. The filter paper was treated either with 5 mL sterile double-distilled water as the control or 5 mL bacteria suspension. Bacteria inoculants were prepared by suspending the cells in sterile double-distilled water and the optic density at 660 nm adjusted so as to reach a final concentration of 1×10^6 colony forming units per milliliter (CFU mL⁻¹). This experiment was conducted for two weeks under growth chamber conditions at a day/night temperature of 28/25 °C, 65% air relative humidity and 14 h photoperiod with 25 Klux light intensity. This experiment was conducted in October 2019. Root and shoot length were recorded to evaluate the effect of bacterial inoculation on the initial growth of seedlings of upland rice as well as whole seedling dry weight. Dry weight was determined after drying the samples in an oven at 70 °C to constant weights (after 3 days).

2.3. Experiment 2. Effects of Bacterial Inoculation on Upland Rice Growth under Growth Chamber Conditions

Non-dehulled rice seeds of the variety KP were treated as in the previous experiment for dormancy break and surface-sterilization. In this experiment, small pots of 500 g capacity were filled with 300 g soil that was previously autoclaved at 121 °C for 1 h. The pots were placed in a growth chamber with environmental conditions set as in experiment 1. To avoid cross-contaminations, two compartments were created in the growth chamber; the compartment above served for the non-inoculated pots, while below was used for the inoculated plants. All the pots received an amount of P (as P₂O₅) and K (as K₂SO₄) equivalent to 25 kg ha⁻¹ before sowing. Three rice seeds were then directly sown in each pot and allowed to germinate and grow for two weeks. The plants were thinned to one seedling per pot and treated. Treatments consisted of the same bacterial strains as mentioned in Table 1 along with a non-inoculated control and one treatment with nitrogen fertilizer application at a rate equivalent to 25 kg N ha⁻¹ (as (NH₄)₂SO₄). No additional nitrogen was applied to the control and inoculated pots. Bacteria were grown in LB medium for 5 days and the optic density of the suspension was adjusted to reach a final concentration of 1×10^7 CFU mL⁻¹. A volume of 20 mL of bacteria suspension

was applied to the appropriate pots. The strain S-7 was prepared and final concentration adjusted to 1×10^7 CFU mL⁻¹ LB medium and autoclaved at 121 °C for 20 min. 20 mL of this autoclaved LB medium was applied to the control and N treated pots. The pots in each compartment were arranged in a completely randomized design with three replicates and pots were moved around every week. Pots were watered on a daily basis to avoid water stress with about 50 mL tap water, but not under submerged condition. The soil used in this experiment was analyzed for its water holding capacity, pH, and nitrogen content as well as its nitrogen isotope composition. Plant were grown for 30 days after inoculation (DAI) between December 2019 and January 2020.

Soil was sieved to pass 2 mm pores and air dried. Three subsamples were used for all analysis. Soil pH was determined after suspending 20 g in 50 mL distilled water and an agitation for an hour. pH was recorded using a digital pH meter. The air-dried soil was ground and sieved with a 250 µm sieve for total N and nitrogen isotopes analysis. 20 mg were used in a NC analyzer (NCH-22F, Sumigraph, Japan). The percentage of nitrogen was directly recorded. Hippuric acid (N 10.36% and C 71.09%) was used as an internal standard and for calibration. Nitrogen isotopic ratios ($\delta^{15}\text{N}$) of 6 mg soil were determined using a Delta VTM Isotope Ratio Mass Spectrometer (IRMS). Three in-house references previously calibrated against IAEA reference materials were used: L-alanine ($\delta^{15}\text{N}$ of +13.7), Glycine (+1.12), and L-Histidine (-7.58) as internal references. The $\delta^{15}\text{N}$ was calculated as:

$$\delta^{15}\text{N} (\text{‰}) = \frac{R(\text{sample}) - R(\text{standard})}{R(\text{standard})} \times 1000, \quad (1)$$

where R is the ratio $^{15}\text{N}/^{14}\text{N}$.

Number of leaves, plant length, and number of tillers were recorded 7, 14, 21, and 30 DAI. At 30 DAI, the plants were destructively sampled. Plants were carefully removed from the pots, soaked in water to remove root-attached soil particles, and brought to the laboratory in individual plastic bags. Roots were separated from the shoot and fresh weights were immediately recorded. Leaf chlorophyll content was determined using the method of [34]. Four replicates of 25 mg (*w*) each were immersed in 1 mL N, *N*-Dimethylformamide and stored at 4 °C overnight. 300 µL of the leaf extract were added to 600 µL N, *N*-Dimethylformamide. Absorbance (A) was read in a spectrophotometer at 647 nm and 664.5 nm. Contents of chlorophyll a and b were calculated as follows:

$$\text{Chlorophyll a } (\mu\text{g}/\text{mg fresh weight}) = \frac{(12 \times A_{664.5}) - (2.79 \times A_{647})}{w}, \quad (2)$$

$$\text{Chlorophyll b } (\mu\text{g}/\text{mg fresh weight}) = \frac{(20.78 \times A_{647}) - (4.88 \times A_{664.5})}{w}, \quad (3)$$

The total chlorophyll content was calculated as the sum of chlorophyll a and chlorophyll b contents.

Plant shoot and root samples were chopped and oven-dried at 70 °C for 72 h. Dry weights were recorded and samples were mixed and pulverized. The powder was used for both total nitrogen content and nitrogen isotopic ratio analysis on the whole plant basis. For total N and $\delta^{15}\text{N}$, 20 mg and 3 mg samples were used, respectively, in the same conditions as for the soil as mentioned above. All analyses were performed in three replications.

2.4. Experiment 3. Effects of Bacterial Inoculation on Rice Growth under Greenhouse Conditions

This experiment was conducted in pots from November 2019 to January 2020 under greenhouse conditions. Temperature in the greenhouse was recorded on the daily basis during plant growth. Daylength was 9.5 h.

Rice variety KP was used and seeds were surface-sterilized as in above. Seedlings were generated on solidified 300 mL agar medium contained in 500 mL volumetric flasks for 14 days at the 2-leaf stage in a growth chamber. Pots were filled with 2 kg of the same soil used in the previous experiment without autoclave. The pots were arranged in a randomized bloc design with three replications.

Two seedlings were transplanted to each pot. One week later, one seedling was removed and pots were treated. All pots received an equivalent of 25 kg ha⁻¹ of P and K fertilizers respectively as P₂O₅ and K₂SO₄. The control treatment received no fertilizer N application, as did the five bacterial inoculation treatments. Two levels of N fertilizer application (as (NH₄)₂SO₄) were evaluated: full rate (1 N) and half rate (0.5 N), making a total of eight treatments. In 1N, an equivalent of 25 kg ha⁻¹ N was applied as basal application 7 DAI (early growth stage) and of 25 kg ha⁻¹ N applied 40 DAI (active tillering stage) as top-dressing application, while in 0.5 N, half of these amounts were applied at the same stages. The bacterial strains listed in Table 1 constituted the inoculation tests. The inoculants were prepared similarly as in the experiment under growth chamber, except that 200 mL of inoculant was applied in equally-split volume following the dates of N application in other treatments. All the pots were watered with tap water as needed after daily check while avoiding submersion. Data on number of leaves, number of tillers, plant height, leaf chlorophyll contents, root and shoot dry weights, and whole plant nitrogen concentration and uptake were determined 60 DAI following the methods and procedures described above. Plant nitrogen isotopic composition was not determined due to the low growth rate and dry matter.

2.5. Statistical Analysis and Other Calculations

All data were subjected to Shapiro–Wilk test in order to check the normal distribution. Data that were not normally distributed were log-transformed (untransformed data are reported here). The one-way analysis of variance (ANOVA) with the Duncan test for multiple comparisons of the means was conducted ($p = 0.05$). In addition, Pearson's correlation analysis was performed to define possible relationships among variables. All data were analyzed using SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY, USA) and graphs were made using Microsoft Excel 2019. Moreover, in order to find out the possible contribution of the bacterial plant growth-promoting traits on plant nitrogen uptake, root, shoot and total dry weights, a regression analysis between these parameters was conducted using the data analysis tools available on Excel 2019 at 95% confidence interval.

3. Results

3.1. Effects of Bacterial Inoculation on Growth of Rice Seedlings

Inoculation of the upland rice (variety KP) with the selected non-native bacteria showed significant growth promotion effects in comparison with the control, depending on bacterial strain. Except for the bacterial strain S-7, root length was promoted by all tested strains in comparison with the control treatment. However, only strain S-343 showed increased shoot length. Among bacterial strains, significant differences were observed. Strain S-343 significantly increased shoot length as compared to S-611, but was not statistically higher than S-7, S-333, and S-499. Root length was lower in plants treated with strains S-7 and S-611 compared to S-333, while no significant differences were observed with the other strains (Table 2). When the whole seedling dry weight was determined, all seedlings inoculated with the bacterial strains accumulated more dry matter than the non-inoculated. Inoculation increased seedlings dry matter by 27%–42%, while roots were 30%–89% longer in inoculated seedlings.

Table 2. Growth parameters of upland rice seedlings as affected by bacterial inoculation. ¹ Data were collected 14 days after inoculation.

Treatments ¹	Dry Weight (mg seedling ⁻¹)	Root Length (mm)	Shoot Length (mm)
control	7.3 ^b	118.0 ^c	45.0 ^b
S-7	9.5 ^a (30.1)	153.8 ^{bc} (30.4)	49.8 ^{ab} (10.7)
S-333	10.4 ^a (42.5)	223.3 ^a (89.4)	49.2 ^{ab} (9.4)
S-343	10.4 ^a (42.5)	186.6 ^{ab} (58.2)	52.6 ^a (17.0)
S-499	9.8 ^a (34.2)	185.3 ^{ab} (57.1)	49.0 ^{ab} (8.9)
S-611	9.3 ^a (27.4)	169.3 ^b (43.5)	47.0 ^b (4.4)

Means follow by different letters in columns are significantly different according to Duncan test ($p = 0.05$). Values (in percentage) in parenthesis represent average percentage increases compared to the control.

3.2. Effects of Bacterial Inoculation of Rice under Growth Chamber Conditions

The soil used in these experiments had the following initial characteristics: pH (water) 6.2 (± 0.05), water holding capacity of 454 (± 7.6) g kg⁻¹ soil, 0.15% N content. The nitrogen isotopes composition of the soil showed a $\delta^{15}\text{N}$ value of +8.2‰ (± 0.8).

Non-destructive data collected 7, 14, and 21 DAI are presented in the Supplementary Table S1. At 7 DAI, plant length was affected by the treatments, however none of the bacterial strains could promote plant growth compared to the control and the N fertilized pots. The strain S-343 significantly increased plant length compared to S-7 and S-499. Plant treated with S-333 had the highest number of leaves as compared to control and S-611. The number of tillers was higher for treatments S-333 and N fertilizer as compared to others treatments.

At 14 DAI, plants under S-333 treatment were taller than all other treated and control plants, but number of leaves was similar. The number of tillers was significantly higher under N treatment in comparison to the other treatments.

21 DAI, S-333 increased plant length as compared to the N fertilized and S-499 treated plants. S-343 and S-611 promoted plant length than S-499, but S-7 and control treatments showed similar results than all other treatments. The number of leaves and tillers was significantly higher under N treatment as compared to the other treatments where no difference was observed.

Data collected 30 DAI are represented in Table 3. At the end of this experiment, N-fertilized plants were significantly shorter than those in all treatments, but had the highest number of leaves and tillers. Strain S-333 significantly increased plant length than the control and S-7 treatments. The other bacterial strains showed no significant differences as compared to the control. Root dry weight was higher with S-333 treatment and lower under S-7, N and S-499 treatments. However, N fertilization significantly increased shoot and plant total dry weights as compared to other treatments, except S-611 for shoot dry weight. Leaf chlorophyll content was highly increased by N fertilizer application as compared to all treatments, except S-499 and S-7. Chlorophyll content in control plants was not significantly different from in inoculated plants, but S-333 and S-343 had lower total chlorophyll content as compared to S-7 and S-499. Nitrogen concentration in plant tissues was significantly affected by treatments. The highest N content was found in N-fertilized plants and the lowest in the control. Among the bacterial strains, S-333 and S-611 had similar low N contents, while S-499 significantly increased N content. The total amount of N accumulated in the plant was higher in N-fertilized plants and lower in control ones. No difference in plant N uptake among bacteria strains was observed. The isotopic composition of plant nitrogen revealed that control plants had the lowest value as compared to the inoculated plants.

Table 3. Response of upland rice on bacterial inoculation under growth chamber conditions. Plant length (PL), number of leaves (NL), number of tillers (NT), root dry weight (RDW), shoot dry weight (SDW), total plant dry weight (TDW) and total chlorophyll content (Chl, mg g⁻¹ fresh weight). Nitrogen (N) uptake is expressed in mg pot⁻¹. ND denotes not determined values. Values (in percentage) in parenthesis and bold represent the bacterial contribution to N uptake in comparison to the control and chemical N fertilizer treatments, respectively.

Treatments	PL (cm)	NL	NT	RDW (g)	SDW (g)
Control	50.7 ^{bc}	13.6 ^b	2.6 ^b	0.35 ^{ab}	0.76 ^d
S-7	47.3 ^c	16.6 ^b	3.0 ^b	0.24 ^b	0.74 ^d
S-333	57.6 ^a	15.6 ^b	3.3 ^b	0.40 ^a	0.98 ^{bc}
S-343	53.3 ^{abc}	17.0 ^b	3.0 ^b	0.34 ^{ab}	0.85 ^{cd}
S-499	52.0 ^{abc}	15.6 ^b	3.3 ^b	0.24 ^b	0.77 ^d
S-611	55.6 ^{ab}	15.0 ^b	2.6 ^b	0.36 ^{ab}	1.04 ^{ab}
N	41.0 ^d	26.6 ^a	7.0 ^a	0.27 ^b	1.15 ^a
	Chl	TDW (g)	N (%)	N Uptake	$\delta^{15}\text{N}$ (‰)
Control	1.16 ^{bcd}	1.11 ^{bc}	0.88 ^f	9.97 ^c	1.61 ^c
S-7	1.33 ^{abc}	0.98 ^c	1.40 ^c	13.8 ^b (38.4)/(28.2)	3.55 ^{ab}
S-333	0.97 ^d	1.38 ^{ab}	1.10 ^e	15.5 ^b (55.5)/(31.7)	3.13 ^b
S-343	0.92 ^d	1.20 ^{bc}	1.27 ^d	15.3 ^b (53.5)/(31.3)	3.46 ^{ab}
S-499	1.41 ^{ab}	1.02 ^c	1.54 ^b	14.6 ^b (46.4)/(29.9)	3.63 ^a
S-611	1.12 ^{cd}	1.41 ^{ab}	1.13 ^e	16.3 ^b (63.5)/(33.3)	3.19 ^{ab}
N	1.44 ^a	1.60 ^a	3.41 ^a	48.9 ^a	ND

Means follow by same letters for each measured parameter denote non-significant differences according to Duncan test ($p = 0.05$).

The Pearson's correlation matrix is presented in Table 4. There was no significant correlation between all the tested parameters and root dry weight and plant total dry weight. However, shoot dry weight was positively correlated with plant total dry weight and nitrogen uptake, while plant length showed negative correlations with nitrogen concentration and number of leaves. Nitrogen concentration and uptake highly increased with number of leaves per plant.

Table 4. Pearson's correlation between upland rice growth parameters at 30 DAI under growth chamber conditions. Plant length (PL), number of leaves (NL), root dry weight (RDW), shoot dry weight (SDW), total plant dry weight (TDW). Nitrogen (N) uptake was expressed in mg pot⁻¹.

	RDW	SDW	PL	TDW	N (%)	N Uptake
SDW	0.426					
PL	0.455	-0.214				
TDW	0.723	0.827 *	0.159			
N (%)	-0.296	0.664	-0.819 *	0.221		
N uptake	-0.088	0.800 *	-0.735	0.420	0.976 **	
NL	-0.223	0.708	-0.790 *	0.313	0.981 **	0.979 **

* and ** respectively indicate significance at the level 0.05 and highly significance at the level 0.01 confidence intervals.

3.3. Effects of Bacterial Inoculation on Rice Growth under Greenhouse Conditions

The average temperature during the experiment was 22 ± 3 °C. Data were collected 40, 47, 54, and 60 DAI. No significant differences were observed at sampling dates before 60 DAI. Therefore, only data of 60 DAI are presented in this report (Table 5). Treatment did not affect the number of leaves per plant. However, only the strain S-7 could significantly increase plant length as compared to the control. Nitrogen fertilizer applied did not significantly increased plant length in comparison to the

tested bacteria strains. Root, shoot and plant total dry weights were improved by the application of the strain S-7 compared to the control treatment.

Table 5. Growth response of upland rice to bacterial inoculation 60 DAI under greenhouse conditions. Plant length (PL), number of leaves (NL), root dry weight (RDW, mg), shoot dry weight (SDW, mg), total plant dry weight (TDW, mg plant⁻¹), and total chlorophyll content (Chl, mg g⁻¹ fresh weight). Nitrogen (N) uptake was expressed in mg pot⁻¹.

Treatments	PL (cm)	NL	RDW	SDW	TDW	Chl	N (%)	N Uptake
control	19.6 ^b	5.6 ^a	50.1 ^b	109.3 ^d	167.2 ^c	1.08 ^{bc}	2.87 ^c	4.56 ^c
0.5N	26.0 ^{ab}	6.3 ^a	51.8 ^b	172.7 ^{bcd}	234.0 ^{bcd}	1.30 ^{ab}	3.17 ^b	7.11 ^{bc}
1N	25.0 ^{ab}	6.6 ^a	66.7 ^b	193.0 ^{abc}	267.8 ^{bc}	1.15 ^{bc}	3.64 ^a	9.42 ^{ab}
S-7	32.6 ^a	5.6 ^a	113.9 ^a	245.8 ^a	365.3 ^a	1.45 ^a	2.93 ^c	10.58 ^a
S-333	21.0 ^b	6.0 ^a	65.4 ^b	149.9 ^{bcd}	220.3 ^{bcd}	1.03 ^c	2.40 ^d	5.17 ^c
S-343	27.3 ^{ab}	6.3 ^a	74.2 ^b	177.3 ^{abcd}	259.4 ^{bc}	1.47 ^a	2.79 ^c	7.06 ^{bc}
S-499	22.3 ^b	5.6 ^a	58.4 ^b	130.0 ^{cd}	195.5 ^{cd}	1.54 ^a	2.80 ^c	5.29 ^c
S-611	26.6 ^{ab}	6.0 ^a	66.6 ^b	207.7 ^{ab}	279.2 ^b	1.46 ^a	2.90 ^c	7.97 ^b

Means follow by same letters for each measured parameter denote non-significant differences according to Duncan test ($p = 0.05$).

The strain S-611 showed growth improvement as compared to the control in shoot and total dry matter accumulation, but not for the root dry weight. Plant dry weight was not significantly affected by the N treatments compared with the control. Except for the strain S-333, all strains increased leaf chlorophyll content compared to the control and the 1N treatments. Nitrogen concentration was higher under N fertilizer applications. No significant difference in N concentration was observed between the control treatment and the bacterial inoculation treatments, except in S-333 which resulted in the lowest N content. N uptake was highly increased by inoculation with strains S-7 and S-611 compared to the control. Strain S-7 improved N uptake as compared to the 0.5N treatment and showed similar N uptake as the 1N treatment. Increase rate of N uptake by bacteria inoculation in comparison to the control and fertilizer N treatments is presented in Table 6.

Table 6. Relative increase in N uptake (%) in upland rice inoculated with non-native PGPB. Increase in N uptake of inoculated plants relatively to the control (^a), the half (^b) and full (^c) doses of chemical N fertilizer applied.

Bacterial Strains	Control ^a	Half N Rate ^b	Full N Rate ^c
S-7	131.9	48.7	12.3
S-333	13.3	-27.3	-45.1
S-343	54.8	-0.7	-25.0
S-499	15.9	-25.6	-43.9
S-611	74.6	12.0	-15.4

Compared to the control, N uptake was increased by from 13% to 131% by inoculation. As applied rate of N increased, the contribution of inoculation to N uptake decreased. Strains S-7, S-343, and S-611 could provide the plant with similar N uptake as the half N rate. When full fertilizer N rate was applied, only strain S-7 could provide similar N to the plant, while plants treated with the other bacteria strains lacked 15 to 45% of N.

The result of the correlation analysis between plant parameters is presented in Table 7. Root dry weight was significantly correlated with shoot dry weights, plant length and plant nitrogen uptake, and highly correlated with plant total dry weight. Plant length highly affected shoot and total dry weights as well as N uptake. Plant length and dry weights highly correlated with N uptake.

Table 7. Pearson’s correlation matrix between growth parameters of upland rice grown under greenhouse conditions 60 DAI. Plant length (PL), number of leaves (NL), root dry weight (RDW), shoot dry weight (SDW), total plant dry weight (TDW). Nitrogen (N) uptake was expressed in mg pot⁻¹.

	RDW	SDW	PL	TDW	N (%)	N Uptake
SDW	0.799 *					
PL	0.823 *	0.928 **				
TDW	0.899 **	0.981 **	0.943 **			
N (%)	-0.039	0.314	0.26	0.23		
N Uptake	0.744 *	0.943 **	0.880 **	0.928 **	0.574	
NL	-0.199	0.226	0.115	0.11	0.603	0.322

* and ** respectively indicate significance at the level 0.05 and highly significance at the level 0.01 confidence intervals.

3.4. Regression Analysis

Results of the regression analysis between the growth-promoting traits as determined in the bacterial strains and plant growth parameters in both growing conditions are reported. Slopes between siderophore production indexes of the bacteria and the plant parameters were not significant in both growth conditions, and therefore are not presented here. For IAA, only root dry weight under greenhouse conditions was positively and significantly affected ($R^2 = 0.8544$) and could be expressed as: root dry weight (g) = 0.3131 × (IAA) (μg/mL) + 50.526. Figure 1 shows the regression equations between P solubilization index of the bacteria and plant growth parameters. All the data under greenhouse conditions were not significant. On the other hand, nitrogen uptake, root, shoot, and total plant dry weight under growth chamber conditions were all negatively significantly correlated with bacterial P solubilization index.

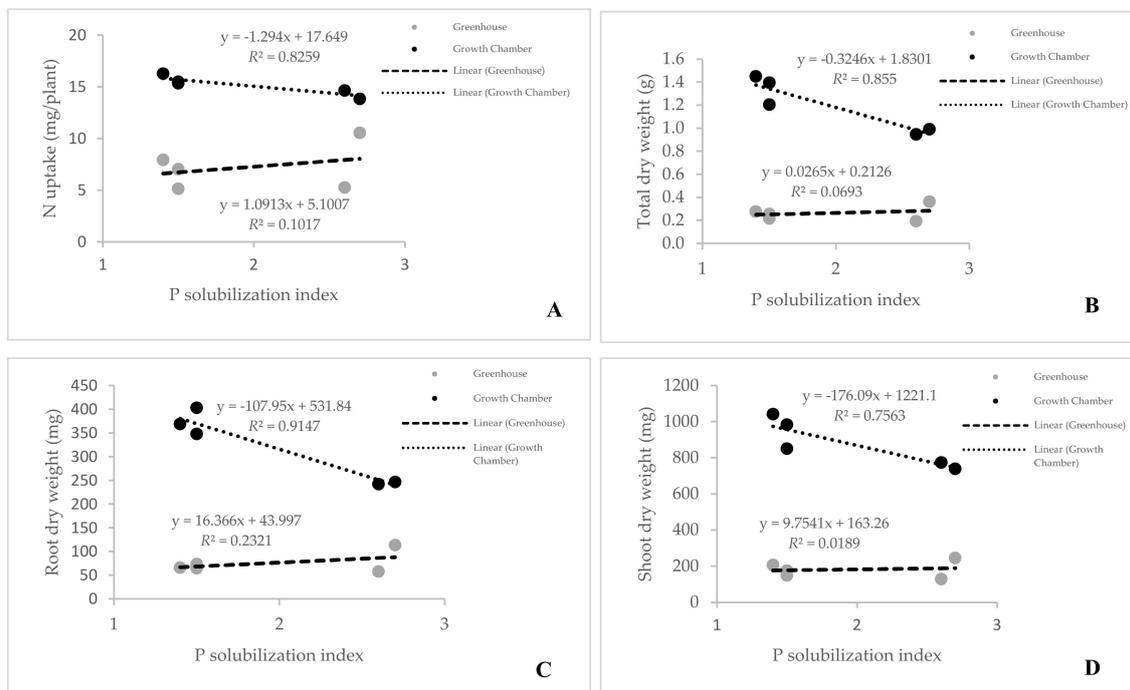


Figure 1. Regressions between bacterial inorganic phosphate solubilization index and N uptake (A), total plant dry weight (B), root dry weight (C), and shoot dry weight (D) of upland rice under greenhouse and growth chamber conditions. Data under growth chamber were collected 30 DAI, and 60 DAI under greenhouse conditions.

For leaf chlorophyll content, positive and significant correlations ($R^2 = 0.8887$ under growth chamber and $R^2 = 0.9296$ under greenhouse) were found only with bacterial inorganic phosphate solubilization trait. The relations between P solubilization index and leaf chlorophyll are expressed in Equations (4) and (5) under growth chamber and greenhouse conditions, respectively.

$$\text{Chlorophyll content } (\mu\text{g}/\text{mg fresh weight}) = 0.321 \times \text{PSI} + 0.4742 \quad (4)$$

$$\text{Chlorophyll content } (\mu\text{g}/\text{mg fresh weight}) = 0.3038 \times \text{PSI} + 0.4907 \quad (5)$$

4. Discussion

The identification of plant growth-promoting bacteria with a large host range is important and agronomically relevant to increase the use of bio-fertilizers in replacement of part of chemical fertilizers in the context of sustainable agricultural production. The current study tested the effects of cross inoculation with non-native bacteria on upland rice growth. The upland rice Kinandang Patong, a drought tolerant and deep-rooting variety [32,33] was used in order to evaluate its interactions with non-native bacteria selected based on their plant growth-promoting traits. Rice seedling and plant early growth stages were observed under growth chamber and greenhouse conditions.

The response of the upland rice variety studied in this experiment varied considerably depending on bacterial strain and growing environment in all three trials. Although root and shoot length of rice seedlings was not promoted by all bacterial strains, seedling total dry weight was increased with bacterial inoculation in the Petri dish experiment. The beneficial effects of bacterial inoculation on rice seedlings has been reported in previous studies. In a study where a Malaysian rice variety was inoculated with strains of *Bradyrhizobium* sp. or *Burkholderia* sp., shoot length and root growth were increased with vigor seedlings [35]. In our study, seedling growth parameters were affected by bacteria strains. In our study, seedling growth parameters were affected by bacteria strains. Such strain-dependent effects were reported in rice seedlings. For example, it was reported that while a strain of *Bacillus sphaericus* increased rice seedling growth, strains of *Rhizobium* sp. showed varying results from higher than to similar to the non-inoculation treatment [36]. Our results also showed positive effects of inoculation on seedling dry weight. Similarly, in a study where rice seedlings were inoculated with 35 nitrogen-fixing bacterial strains, all strains promoted rice seedling growth parameters, including the dry weight after 12-day growth period [36]. Moreover, these authors found that growth parameters of inoculated rice (cv. Swarna) seedlings were correlated with in vitro plant growth promotion traits of the bacteria [37].

Grown under growth chamber conditions with sterilized soil, rice initial growth was affected by bacteria inoculation, however the response varied among the tested strains. The varying effect of bacterial strains on plant growth and yield, even of the same genus, has been reported [38]. In maize crops grown under controlled conditions, these authors documented the growth promoting effects of several strains of the genus *Herbaspirillum* and other genera, and found significant differences among the *Herbaspirillum* strains, indicating a specific plant-bacteria interaction. The impact of environmental conditions, both biotic and abiotic, on these interactions has been less studied [39]. The importance of N fertilizers in improving rice growth, tiller numbers, and grain yield is well established [40,41], as confirmed in our study due to the availability of this nutrient in the root environment. However, plants can increase their root length and biomass under nutrient-deficient conditions [42], reducing the effects of chemical N fertilizers on root parameters. Our results showed that 28%–33% of chemical N fertilizer could be replaced by bacterial inoculation in rice under growth chamber conditions for nitrogen uptake. The fact that root dry weight was not significantly affected by inoculation as compared to the control could be attributed to the deep-rooting trait of Kinandang Patong as it was revealed in breeding populations of varieties IR64 and Kinandang Patong, with the identification of the DEEPER ROOTING 1 (DRO1) [32]. When the plant overall nitrogen isotopic composition was determined, surprisingly, the uninoculated control plants had the lowest value compared to the inoculated plants, although their

$\delta^{15}\text{N}$ values were all lower than that of the soil. $\delta^{15}\text{N}$ in N-treated plants was not determined, assuming that its value will be between that of the soil and the chemical N applied, thus not informative for the purpose of this study. The results suggest that the tested strains could help rice derive nitrogen from the air, but with lower efficiency, and that other mechanisms might be used to increase nutrients uptake. Rice organs have been reported to harbor several endophytic bacteria [43,44], some of which have been identified as nitrogen-fixing bacteria including *serratia marcescens* [45], *Azospirillum* sp., and *Peanibacillus* [46]. Hence, although few attempts have been made to isolate and identify beneficial bacteria from this variety, Kinandang Patong might be inhabited by efficient diazotrophic bacteria for biological nitrogen fixation. Moreover, when rice variety IR72 was reinoculated with an endophytic diazotroph bacterial strain identified as *serratia marcescens*, although root length and dry weight were increased, N uptake was not affected [45] and inoculated rice plant did not show acetylene reduction in the absence of an additional source of carbon. This suggests that biological nitrogen fixation can be limited under low-carbon soil conditions.

The response of Kinandang Patong grown in soil to the inoculated strains varied depending on bacterial strains under greenhouse conditions. The short-day length and low air temperature (18–23 °C) in the greenhouse during the growth period can explain the low dry matter accumulation and overall growth of the rice, especially at 60 DAI. Although long day lengths would be better for evaluating the ability of growth-promoting bacteria, our experiment was carried out under above conditions. As compared to the control, the response of rice plant to bacterial inoculation changed greatly. The impacts of environmental factors on the performance of bacterial inoculants applied to soils have not been extensively evaluated. However, it is likely that rhizo-competitiveness and varying functioning of the growth-promoting traits strongly affect the beneficial effects of inoculation [47], resulting in inconsistent promotion of plant growth from controlled conditions to the open field conditions. In our study, the strain S-499 had no significant effect on rice growth in both growth chamber and greenhouse conditions. The correlations analysis showed a strong positive effects of root dry weight on other plant physiological parameters including nitrogen uptake under greenhouse conditions unlike in growth chamber conditions. The volume of soil (pot size) might affect root growth. Three out of the five strains used in this study were able to improve nitrogen uptake at a level similar to the half dose chemical N applied while one (S-7) could provide a level similar to the full N rate application. This suggests that bacteria S-343, S-611 can replace half of rice N requirement and strain S-7 can totally replace chemical N fertilizer under our growing conditions in greenhouse.

Several studies have tested the plant growth promotion effects of bacterial strains either as sole strains or as a consortium based on their beneficial traits [48–52]. Even when these traits were quantified, few attempts have been made to correlate these traits to plant growth parameters. In our study, the correlations between bacteria IAA and siderophore production and phosphate solubilization ability and rice growth parameters were determined through a regression analysis. Siderophore production of individual bacterial strains have been found to promote early growth in rice and mung bean (*Vigna radiata* L.) [53]. In our study, the siderophore production index had no significant correlation with the plant growth parameters. However, in both growing conditions, bacteria with the high siderophore production index (S-499 and S-611) show similar results as the control, suggesting that the expression of this functional trait could be limited during the experiment period. IAA production showed a positive correlation with plant root dry weight only under greenhouse conditions. Previous studies have shown that the response of crops to IAA-producing bacteria depends on plant genotype as well as bacteria strains [54]. In their study, the authors found improvement in root biomass, except in the millet crop. The effects of P solubilization indexes on plant growth were strongly affected by growing environment. Under greenhouse, no significant correlations were found as it was reported previously [55], however under greenhouse conditions all plant growth parameters were negatively affected by high P solubilization indexes, particularly the root dry weight. Taken individually, several P-solubilizing bacteria have shown beneficial effects on plant growth [17,56], however little attention has been given to the relative contribution of high index of P solubilization on plant growth parameters.

Although most of these plant growth parameters in inoculated plants were higher than the uninoculated, our findings revealed differential effects among bacterial strains. More investigations are necessary to understand the physiological response of important crops to increasing values of the bacterial growth-promoting traits. To the best of our knowledge, this study is the first to evaluate the relative contribution of such bacterial traits isolated from yams on upland rice growth.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/6/903/s1>, Table S1: Growth parameters of rice, variety Kinandang Patong at three weeks interval.

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Appendix A

The 16S rRNA genes of the pure bacterial colonies were sequenced for identification purpose. Bacterial cell suspension (20 µl) was treated with Proteinase K (5 µl) at 60 °C for 20 min and 95 °C for 5 min to disrupt cell membranes. The 16S rRNA genes were amplified from the extracted DNA template with the universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') using the Thermal Cycler C1000 Touch (BIO-RAD). The PCR reaction mixture consisted of 10× ExTaq Buffer 5 µl, dNTP Mix 4 µl, 1 µl of each primer, distilled water (nuclease-free) 37.75 µl and Enzyme ExTaq 0.25 µl. Proteinase K, 10× ExTaq Buffer, dNTP Mix and ExTaq were provided from Takara (Takara-Bio Inc., Japan). PCR was performed in 35 cycles as follows: denaturation at 95 °C for 30 s, annealing at 55 °C for 15 s, extension at 72 °C for 60 s and final extension at 72 °C for 1 min. PCR products were sent to Macrogen-japan company for sequencing. Obtained sequences were about 1000 bp long and were used for similarity search in the bacterial 16S database of EzBioCloud [27].

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