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Analyzing Single and Combined Cultures of Plant Growth-Promoting Rhizobacteria Isolates from Afghanistan as a Potential Biofertilizer for Rice Growth and Development

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Abstract: Developing multi-functional, environmentally friendly biofertilizers can help reduce chemical fertilizer usage. This study examined single and combined application effects of four plant growth-promoting rhizobacteria (PGPR) strains: AF124 (*Rhizobium daejeonense*), AF74 (*Enterobacter cloacae*), AF96 (*Pseudomonas putida*), and AF42 (*Enterobacter cloacae*), isolated from alkaline salt-stressed soils from Afghanistan. Physiological features such as nitrogen (N_2) fixation, indole-3-acetic acid (IAA), organic acid production, and P and K solubilization of bacteria in single or combined cultures in vitro were examined. A single application of AF124 and AF74 showed significantly higher IAA production than the combined application of dual, triple, and quadruple PGPR treatments. Regarding acetylene reduction activity, an N_2-fixation activity indicator, a single application of AF124 showed the highest value, followed by a triple application of AF124 + AF96 + AF42. The P-solubilization activity and organic acid production were lower in the single application of AF124 but higher with other strains in the combined applications, which resulted in the secretion of multiple organic acids into the medium. The highest solubilized K value was obtained from the quadruple application compared to other treatments. In the rice inoculation test, all single and combined PGPR treatments increased its growth parameters, such as root length and root and shoot dry biomass. The present results indicate that these four PGPR strains can potentially contribute to developing multi-functional bio-fertilizer formulations.

Keywords: PGPR; rice; N_2-fixation; IAA production; nutrient solubilization

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1. Introduction

Rice is one of the most important staple foods for over half the world's population [1], and about 3.5 billion people worldwide rely on rice for over 20% of their daily calories [2]. Fertilizers are one of the major inputs in rice farming for increasing yield and productivity. However, excessive fertilizer use has adverse effects on ecosystems [3,4]. In this regard, using alternative methods such as multi-functional bio-fertilizers containing plant growth-promoting rhizobacteria (PGPR) can help reduce chemical fertilizer usage in agriculture [5,6].

Beneficial soil bacteria in the rhizosphere are referred to as PGPR, and they promote plant growth through various direct or indirect mechanisms. PGPR promotes plant growth directly through nitrogen (N_2) fixation [7,8], nutrient solubilization (e.g., phosphorus (P), potassium (K), and iron (Fe)) [9,10], phytohormone production [6,11], and indirectly

through the production of diverse chemical compounds such as chitinase, siderophores, and hydrogen cyanide [12–14]. Moreover, PGPR produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which reduces ethylene concentrations in plants [15] and protects plants under abiotic stress conditions.

A wide range of PGPR significantly increases the vegetative growth and grain yields of several important crops, such as wheat [16,17], rice [18,19], maize [20], and sugarcane [9] individually or in combination with inorganic fertilizers. The beneficial effects of PGPR depend on the abilities of each microorganism. Plant growth-promoting abilities of PGPR are typically associated with physiological functions, such as indole-3-acetic acid (IAA) production or acetylene reduction activity (ARA), which is an indicator of N_2-fixation activity. However, in the present study, none of the selected microorganisms showed all the desirable growth-promoting traits, such as high IAA production, ARA activity, and P- and K-solubilization abilities.

Single inoculations of PGPR have often resulted in inconsistent performance in agricultural fields, which could be attributed to various biotic and abiotic stresses that influence microbial activity in the field [21–23]. However, combining different PGPR with different beneficial functions may increase plant growth by providing multi-functional critical nutrient suppliers and increasing inoculant viability and consistency against biotic and abiotic influences [24,25]. Furthermore, PGPR maintains greater nutrient availability in soils [26], enhances the efficacy and reliability of their health effects on crops [27], and synergistically promotes plant growth [28]. Many studies have evaluated various combinations, such as PGPR with arbuscular mycorrhizal fungi [29,30], rhizobia with PGPR [31–33], and PGPR with other PGPR [18,26]. In most of these studies, co-inoculation was more efficient than single-species application [23,26,32].

In our previous study, 98 rice PGPR were isolated from soils collected from Afghanistan to develop suitable biofertilizers for alkaline salt-stressed soil conditions [34]. In the present study, we selected four strains from the 98 PGPR: AF124 with high ARA activity and IAA production, AF96 with high K-solubilization and siderophore production, and AF42 and AF74 with high IAA production and P- and K-solubilization [34], to assess the potential of these strains for multi-functional bio-fertilizer development. Single, dual, triple, and quadruple combinations of these four strains were examined in vitro to assess their physiological characteristics, such as IAA production, ARA activity, organic acid production, and nutrient solubilization, and their effect on rice growth.

2. Materials and Methods

2.1. Bacterial Strains

A total of 4 of the 98 PGPR isolates were obtained from alkaline salt-stressed soils in Afghanistan, namely AF124 (identified based on the *16S rRNA* sequence as *Rhizobium daejeonense*), AF74 (*Enterobacter ludwigii*), AF96 (*Pseudomonas putida*), and AF42 (*Enterobacter ludwigii*), were selected based on their physiological characteristics and their effect on plant growth performance [34]. The four PGPR were used as single and combined treatments to evaluate their physiological features, P- and K-solubilizing abilities, and effects on rice growth parameters.

2.2. Genomic-DNA Extraction, Amplification, and Sequencing of the rpoB Gene

Bacterial isolates were grown in nitrogen-free broth medium (NFb) for 2 days [35] at 28 °C. Bacterial cells were recovered by centrifugation at 13,000× *g* for 5 min and washed twice with sterile water purified by reverse osmosis (RO). Genomic DNA from bacterial cells was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The *rpoB* gene was sequenced as a complementary marker to the 16S rRNA gene [34] to determine the microbial diversity and taxonomic groups of the four isolates. A universal primer of the

rpoB gene [36], *rpoB*-F (5′-ATCGAAACGCCTGAAGGTCCAAACAT-3′), and *rpoB*-R (5′-ACACCCTTGTTACCGTGACGACC-3′) was used for PCR amplification. The PCR products were purified using a FastGene TM Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The purified products were reacted using an ABI Prism BigDye Terminator v 3.1 cycle sequencing kit (Thermo Fisher Scientific) and *rpoB*-F and *rpoB*-R primers. The sequences of the *rpoB* gene were then analyzed using an ABI PRISM 3500 genetic analyzer (Thermo Fisher Scientific) according to the manufacturer's protocols. The sequenced data were compared to the *rpoB* gene deposited in the GenBank database using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast, accessed on 10 June 2023). To illustrate isolated evolutionary relationships, a phylogenetic tree was constructed using the neighbor-joining tree and bootstrap method with 1000 replications using the MEGA11: Molecular Evolutionary Genetics Analysis software version 11 (https://www.megasoftware.net/, accessed on 10 June 2023).

2.3. Compatibility Study

Primary screening for the antagonistic effects of the isolates was performed using the perpendicular streak method, as described by Singh et al. [37]. Briefly, the AF124 isolate was inoculated through a single streak line in the middle of the agar plate and then incubated at 28 °C for 24 h. Then, other isolates (AF42, AF74, and AF96) were individually streaked at a 90° angle to the streaked AF124 isolates, and those inoculated plates were incubated at 28 °C for 4 days. The antagonistic effects of the bacterial strains were determined by observing the inhibition zones close to the intersection points of the streaked bacterial strains.

2.4. Indole-3-Acetic Acid (IAA) Production

Bacterial isolates were cultured on an NFb broth medium at 28 °C for 48 h to determine IAA production. The strains were then inoculated into NFb broth with 100 mg L^{-1} L-tryptophan at a cell density of 10^6 CFU/mL in single or combined inoculations (10^6 CFU/mL from each strain). The cultures were incubated in the dark for two days at 28 °C. The cell suspensions were centrifuged at 7000× *g* for 10 min to remove the cells, and the IAA content in the supernatant was evaluated using the Salkovski colorimetric approach [38] by measuring the absorbance at 530 nm using a spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Cambridge, UK). The plate dilution method was used to determine cell density.

2.5. Acetylene Reduction Assay (ARA)

Briefly, bacterial cultures were grown in NFb broth medium and incubated at 28 °C for 2 days. Then, 15 mL NFb semi-solid medium was inoculated in a 25 mL vial volume at 10^6 CFU/mL in individual and combined inoculations (10^6 CFU/mL from each strain) and incubated at 28 °C for 2 days. After that, acetylene was injected at 10% (*v*/*v*) into the culture vial and was incubated for 1 h at 30 °C. Ethylene production in the vials was determined using a Shimadzu GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Porapak N column (Chrompack, Middelburg, The Netherlands). Uninoculated vials were used as negative controls.

2.6. Phosphate Solubilization Estimation and Organic Acid Production

The P solubilization rate was estimated as described by Hu et al. [39]. Bacterial isolates were grown in NFb broth medium at 28 °C for 48 h. Then, bacterial cells were recovered by centrifugation at 7000× *g* for 10 min. Erlenmeyer flasks containing 30 mL of Pikovskaya medium [40], prepared with insoluble phosphate [$Ca_3(PO_4)_2$] [39], were inoculated with 10^6 CFU/mL in triplicate for single and combined inoculations (10^6 CFU/mL from each strain). Uninoculated flasks were used as controls. The flasks were incubated for 7 days at 28 °C. The cells were then removed by centrifugation at 10,000× *g* for 10 min. The amount of solubilized P in the medium was measured using the molybdenum blue assay [41], and

the absorbance of the solution was read using a microplate reader at 700 nm (SpectraMax Paradigm Multi-Mode Microplate Reader, Moleculr Devices Inc., San Jose, CA, USA). The pH of the medium was measured simultaneously.

For organic acid analysis, the supernatant of the medium was passed through a 0.22 µm nylon filter. Organic acids were analyzed using a high-performance liquid chromatography (HPLC) system equipped with Shim-pack column SCR102H and RSpack KC-811 (Shimadzu) at a column temperature of 40 °C and the UV detector SPD-20A (Shimadzu) at 210 nm. The mobile phase comprising 0.1% H_3PO_4 was used in isocratic mode with a flow rate of 0.5 mL.min^{-1}. Organic acids were quantified based on the peak areas obtained for the standards of shikimic acid, fumaric acid, citric acid, succinic acid, gluconic acid, oxalic acid, α-ketogluconic acid, malic acid, and tartaric acid. Each replicate was analyzed in a single run using HPLC, and the values were displayed as the mean of the three replicates.

2.7. Potassium Solubilization Estimation

Aleksandrov medium was used to estimate solubilized K [39]. Bacterial isolates were grown in NFb broth medium at 28 °C for 48 h. Then, the cells were recovered by centrifugation at 7000× *g* for 10 min. Erlenmeyer flasks containing 20 mL of Aleksandrov medium were inoculated with 10^6 CFU/mL in triplicate for single and combined inoculations (10^6 CFU/mL from each strain), and the uninoculated flasks were used as controls. The flasks were incubated for 7 days at 28 °C. The cells were then removed by centrifugation at 7000× *g*. The K content in the medium was estimated using a flame emission spectrophotometer (BWB Technologies, Newbury, UK). The pH of the medium was measured simultaneously.

2.8. Effects of Single and Combined Treatments of PGPR on Rice Growth

For the plant test, soil samples were collected from a paddy field of the Field Science Center at the Tokyo University of Agriculture and Technology (Honmachi, Fuchu City, Tokyo) at a depth of 0 to 20 cm. The nine soil samples were combined into a composite sample, which was then used for soil property analysis (Table 1) and rice cultivation. Available N, P_2O_5, and K_2O were determined as described by Ogawa et al. [42], Truog [43], and Kamewada et al. [44], respectively. Before the rice growth test, the soils were air-dried and passed through a 3 mm sieve. To remove the effects of indigenous soil microorganisms, soils were sterilized using gamma radiation at the Takasaki Advanced Radiation Research Institute, Japan Atomic Energy Agency (Takasaki, Gunma, Japan). The four bacterial strains were cultured for two days in 100 mL NFb broth medium at 28 °C. Then, bacterial cells were washed and dissolved in RO water. Seeds of one Afghan rice variety (*Oryza sativa* cv. Bada Doshi) were surface sterilized in 70% ethanol for 30 s, soaked in 3% sodium hypochlorite for 3 min, and then rinsed five times with sterilized distilled water. The germinated seeds were transplanted into 300 mL plastic pots containing 185 g of soil, and each bacterial strain at a cell density of 10^9 CFU/mL was applied to the seeds in the pot. For the combined treatments, a cell density of 10^9 CFU/mL of bacterial cells from each strain was mixed well and used in each pot. Un-inoculated pots were used as negatives. All pots were moved to a phytotron that was kept at 28 ± 2 °C during the day (16 h) and 25 ± 2 °C at night (8 h) at a humidity of 60%. Each pot was irrigated with sterilized RO water for 21 days. The experiment was performed using a completely randomized design consisting of three replicates for each treatment. Plants were harvested after three weeks, and the roots were washed thoroughly in tap water to remove any adhering soil. The fresh weights of roots and shoots were recorded, and then they were dried at 60 °C for 2 days to determine the dry weight. The statistical significance of differences among the treatments was determined using Tukey's honestly significant difference (HSD) and Dunnett's tests, and results were considered significant at $p < 0.05$ and $p < 0.01$. All statistical analyses were performed using JMP Pro 16 (JMP, Cary, NC, USA).

Table 1. Soil properties of the study site.

Site	pH(1:2.5) (Soil:H_2O)	Available N (mg/Kg DS)	Available P_2O_5 (mg/Kg DS)	Available K_2O (mg/Kg DS)
Honmachi paddy field	6.47 ± 0.1	88.5 ± 4.2	170.9 ± 6.8	183 ± 9.1

DS = dry soil. The value followed by the means represents standard error.

2.9. Nucleotide Sequence Accession Numbers

The DNA sequences were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC775306 to LC775309 (*rpoB*).

3. Results

3.1. Genetic Characterization of the Selected Bacteria Based on the rpoB Gene

In our previous study, four bacteria species were identified based on *16S rRNA* sequences: AF124 (*Rhizobium daejeonense*), AF74 (*Enterobacter ludwigii*), AF96 (*Pseudomonas putida*), and AF42 (*Enterobacter ludwigii*) [34]. Sequences of the *rpoB* genes were also analyzed to confirm the categories of these isolates. Based on the *rpoB* gene sequence, the AF124 isolate showed high similarity to *Rhizobium* sp. ACO-34A (97.5%) and *Rhizobium daejeonenese* CCBAU10050 (96.0%) (Figure 1). The AF96 isolate displayed maximum similarity to the *Pseudomonas putida* YC-AE1 (99.9%) species. Isolates AF42 and AF74 were categorized as highly identical to the *Enterobacter cloacae* complex sp. FDAARGOS 77 (99.6%). Thus, the bacterial categories based on the *16S rRNA* sequences and *rpoB* genes were almost consistent.

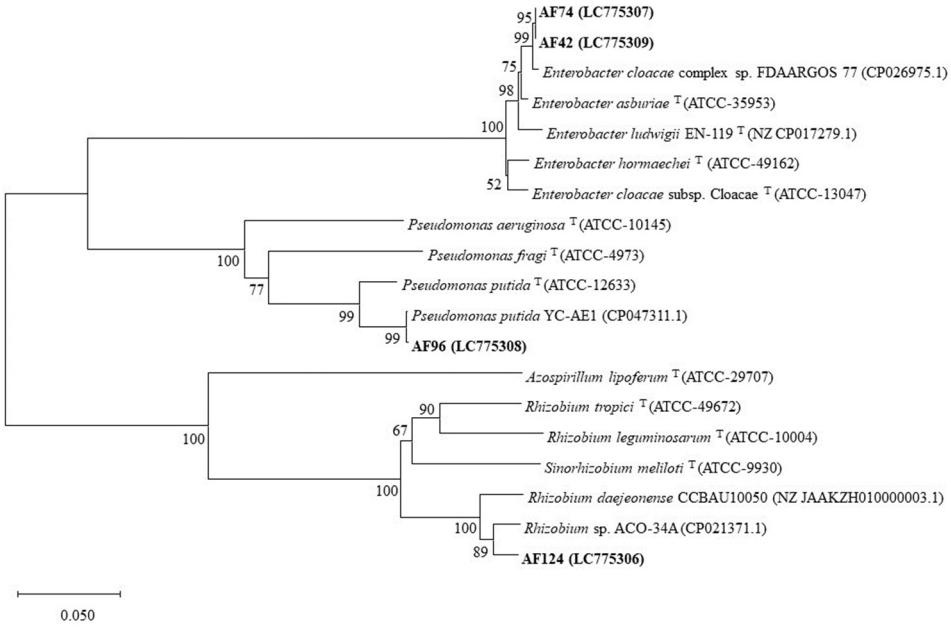

Figure 1. The phylogenetic tree of the *rpoB* gene was constructed using partial nucleotide sequences (1434 bp) on a neighbor-joining analysis using the bootstrap method for four bacterial isolates and type strains belonging to different genera and species.

3.2. Antagonistic Effects of Bacterial Strains

The perpendicular stroke method, which measured the antagonistic activity of the selected bacterial strains, showed that they had no inhibitory effects and most probably did not suppress the growth and reproduction of each other.

3.3. Indole-3-acetic Acid Production

The IAA production in media with single and combined applications of the four PGPR is shown in Table 2. In single applications, AF96 had the lowest IAA production among the four strains, which was consistent with the results of Habibi et al. [34]. IAA production with single applications of AF124 and AF74 was higher than with dual, triple, and quadruple applications. Furthermore, dual, triple, and quadruple applications containing AF96 produced higher IAA production than the single AF96 application.

Table 2. The effects of single and combined PGPR treatments on IAA production and nitrogen fixation. Different letters show statistically significant differences between the variables ($p < 0.05$).

PGPR Treatment	Bacterial Species	IAA (μg mL^{-1})	ARA (nmol tube^{-1})
	Control	N.D.	N.D.
Single	AF124 (*Rhizobium daejeonense*)	29.9 ± 1.7 ^{a}	944.0 ± 74.0 ^{a}
	AF74 (*Enterobacter cloacae*)	30.1 ± 2.6 ^{a}	7.2 ± 2.4 ^{hg}
	AF96 (*Pseudomonas putida*)	11.9 ± 1.0 ^{d}	N.D.
	AF42 (*Enterobacter cloacae*)	28.7 ± 1.1 ^{ba}	9.0 ± 1.0 ^{hg}
Dual	AF124 + AF74	21.0 ± 2.0 ^{cb}	417.5 ± 39.1 ^{d}
	AF124 + AF96	18.0 ± 0.9 ^{c}	134.1 ± 27.1 ^{e}
	AF124 + AF42	17.2 ± 1.2 ^{c}	10.0 ± 1.8 ^{hg}
	AF74 + AF96	25.2 ± 1.0 ^{b}	9.3 ± 1.5 ^{hg}
	AF74 + AF42	24.2 ± 1.1 ^{b}	11.0 ± 1.0 ^{g}
	AF96 + AF42	24.9 ± 1.0 ^{b}	4.5 ± 0.9 ^{h}
Triple	AF124 + AF74 + AF96	17.4 ± 0.4 ^{c}	233.1 ± 45.1 ^{ed}
	AF124 + AF74 + AF42	16.2 ± 0.3 ^{c}	552.5 ± 63.3 ^{c}
	AF124 + AF96 + AF42	16.2 ± 0.4 ^{c}	696.5 ± 71.2 ^{b}
	AF74 + AF96 + AF42	23.5 ± 0.6 ^{cb}	18.0 ± 2.2 ^{f}
Quadruple	AF124 + AF74 + AF96 + AF42	18.8 ± 0.2 ^{c}	291.5 ± 41.4 ^{ed}

N.D., not detected.

3.4. Acetylene Reduction Assay (ARA)

Among single applications, AF124 showed over 100-fold higher ARA activity than the rest, and AF96 showed no detectable activity, consistent with the results of Habibi et al. [34] (Table 2). Combined applications containing AF124 resulted in much higher activity than those without this strain. Among the combined applications, the triple application of AF124 + AF96 + AF42 resulted in the highest ARA activity, although this value was lower than that of a single application of AF124.

3.5. Phosphate Solubilization Assay and Organic Acids Production

In a previous study, the P- and K-solubilization activities of the four strains were analyzed based on the halo zones of the agar medium [34]. In the present study, P- and K-solubilization activities were analyzed using more accurate methods by measuring solubilized P and K in liquid medium inoculated with bacteria in vitro (Table 3). In a previous study, AF124 did not show P-solubilization activity based on the halo zone of the agar medium [34]. However, in the present study, this strain showed detectable P-solubilization activity in the liquid medium, although its activity was much lower than that of the other three strains (Table 3). Although the P-solubilization activity of AF124 was low in the single application, most of the combined applications containing this strain showed high activity, more than 60 μg mL^{-1}, suggesting that other strains in the combined applications complemented the defect of AF124 in P-solubilization. Organic acids produced by bacteria or plant roots play a role in P-solubilization in the soil [26,45–47]. Based on HPLC analysis, four types of organic acids were secreted into the medium by the inoculated isolates, of which malic acid and tartaric acid were the dominant organic acids; the other two organic acids were shikimic acid and oxalic acid. In single inoculations, AF124, with

the lowest phosphate solubilization activity, showed no detectable organic acid secretion (Table 3). AF96 showed the second-lowest P solubilization activity and contained only malic acid. In contrast, strain AF74, which had the highest P-solubilization activity, showed relatively high levels of the four organic acids (Table 3). In the combined application, the other strains compensated for defects in organic acid production by AF124. Four organic acids were detected in all combinations in the triple and quadruple applications, indicating high P solubilization. Compared to the uninoculated medium (negative control), the pH of the inoculated medium tended to decrease, but this drop was not correlated with the amount of released P in the medium.

3.6. Potassium Solubilization Activity

In a previous study, AF124 did not show K-solubilization activity based on the halo zone of the agar medium [34]. However, in the present study, this strain showed detectable K-solubilization activity in the liquid medium, although its activity was lower than that of the other three strains (Table 3). Interestingly, K-solubilization activity tended to increase in the order of single < dual < triple < quadruple application. The K-solubilization activity with quadruple PGPR treatment significantly increased by more than 4-fold compared with that of a single application. Inoculation of these PGPR reduced the pH of the medium to lower than 5, except for AF124 (Table 3); however, this reduction was not correlated with the K released in the medium.

3.7. Plant Growth Test

The plant height, root length, and shoot and root dry weights with single and combined inoculations are shown in Table 4. A single application of all four PGPR positively affected the growth parameters of rice plants, especially dry weight, as shown in a previous study [34]. Among single applications, AF74 was the most effective. In dual, triple, and quadruple applications, the combinations containing AF74 showed relatively high effects; notably, shoot dry weights with applications containing AF74 were greater than 400 mg per plant. Among all the treatments, the triple application of AF124 + AF74 + AF96 resulted in the highest shoot dry weight. As for root dry weight, the dual application of AF96 + AF42 resulted in the highest value, which was significantly higher than that of the single application of AF96 and AF42, suggesting synergistic effects of these two strains when they were combined.

Table 3. The effects of single and combined PGPR treatments on organic acid production, pH, phosphate solubilization, and potassium solubilization in vitro. Different letters show statistically significant differences between the variables ($p < 0.05$).

PGPR Treatment		Organic Acid Production (μmol)				Solubilized P	pH of P-Medium	Solubilized K	pH of K-Medium
	Bacterial Species	Shikimic Acid	Oxalic Acid	Malic Acid	Tartaric Acid	(μg mL^{-1})		(μg mL^{-1})	
	Negative control	N.D.	N.D.	N.D.	N.D.	N.D.	6.6 ± 0.1 ^{a}	13.5 ± 1.8 ^{e}	6.3 ±0.0 ^{a}
Single	AF124 (*Rhizobium daejeonense*)	N.D.	N.D.	N.D.	N.D.	5.8 ± 1.9 ^{b}	5.9 ± 0.1 ^{b}	29.0 ± 1.0 ^{e}	6.0 ±0.2 ^{a}
	AF74 (*Enterobacter cloacae*)	0.98 ± 0.18 ^{a}	4.31 ± 0.27 ^{ab}	121.8 ± 1.3 ^{b}	60.2 ± 0.8 ^{ab}	69.8 ± 1.5 ^{a}	5.6 ± 0.0 ^{c}	37.0 ± 2.0 ^{de}	4.4 ±0.1 ^{b}
	AF96 (*Pseudomonas putida*)	N.D.	N.D.	165.5 ± 5.7 ^{b}	N.D.	24.1 ± 4.2 ^{b}	5.8 ± 0.1 ^{b}	40.5 ± 1.5 ^{de}	4.4 ±0.0 ^{b}
	AF42 (*Enterobacter cloacae*)	0.99 ± 0.29 ^{a}	4.90 ± 0.46 ^{a}	N.D.	75.2 ± 17.2 ^{a}	62.6 ± 6.1 ^{a}	5.6 ± 0.0 ^{c}	46.0 ± 4.0 ^{de}	4.6 ±0.1 ^{b}
Dual	AF124 + AF74	0.71 ± 0.10 ^{a}	4.47 ± 0.79 ^{ab}	N.D.	37.2 ± 8.2 ^{bc}	62.9 ± 2.1 ^{a}	5.6 ± 0.1 ^{c}	70.0 ± 10.9 ^{dc}	4.9 ±0.0 ^{b}
	AF124 + AF96	N.D.	N.D.	245.7 ± 39.1 ^{a}	N.D.	21.3 ± 2.8 ^{b}	5.8 ± 0.1 ^{c}	52.5 ± 1.5 ^{dc}	4.4 ± 0.3 ^{b}
	AF124 + AF42	0.69 ± 0.00 ^{a}	3.24 ± 0.19 ^{bc}	132.4 ± 5.3 ^{b}	15.4 ± 1.7 ^{cd}	66.5 ± 6.0 ^{a}	5.6 ± 0.0 ^{c}	91.1 ± 6.0 ^{bc}	4.3 ± 0.1 ^{b}
	AF74 + AF96	0.83 ± 0.01 ^{a}	3.22 ± 0.10 ^{bc}	122.1 ± 1.8 ^{b}	36.3 ± 4.0 ^{bc}	61.8 ± 4.6 ^{a}	5.6 ± 0.1 ^{c}	86.0 ± 12.0 ^{bc}	4.2 ± 0.0 ^{b}
	AF74 + AF42	0.73 ± 0.01 ^{a}	4.36 ± 0.35 ^{ab}	122.9 ± 5.5 ^{b}	20.9 ± 2.0 ^{cd}	64.3 ± 5.2 ^{a}	5.6 ± 0.0 ^{c}	72.5 ± 15.4 ^{cd}	4.5 ± 0.1 ^{b}
	AF96 +AF42	0.71 ± 0.03 ^{a}	N.D.	113.3 ± 3.4 ^{b}	16.7 ± 1.2 ^{cd}	55.8 ± 2.8 ^{a}	5.6 ± 0.1 ^{c}	90.5 ± 2.6 ^{bc}	4.2 ± 0.1 ^{b}
Triple	AF124 + AF74 + AF96	0.75 ± 0.01 ^{a}	2.74 ± 0.03 ^{c}	122.9 ± 3.8 ^{b}	22.1 ± 1.1 ^{cd}	61.6 ± 4.6 ^{a}	5.6 ± 0.0 ^{c}	114.0 ± 8.1 ^{b}	4.3 ± 0.1 ^{b}
	AF124 + AF74 + AF42	0.65 ± 0.01 ^{a}	3.06 ± 0.14 ^{bc}	118.7 ± 3.9 ^{b}	25.3 ± 2.3 ^{cd}	63.9 ± 4.3 ^{a}	5.6 ± 0.1 ^{c}	104.3 ± 12.9 ^{bc}	4.4 ± 0.0 ^{b}
	AF124 + AF96 + AF42	0.80 ± 0.02 ^{a}	2.70 ± 0.05 ^{c}	132.9 ± 12.7 ^{b}	7.8 ± 0.3 ^{cd}	58.6 ± 1.5 ^{a}	5.6 ± 0.1 ^{c}	88.1 ± 3.0 ^{bc}	4.3 ± 0.1 ^{b}
	AF74 + AF96 + AF42	0.75 ± 0.03 ^{a}	3.34 ± 0.18 ^{bc}	120.8 ± 2.6 ^{b}	26.0 ± 1.4 ^{cd}	61.2 ± 4.2 ^{a}	5.6 ± 0.0 ^{c}	113.5 ± 7.5 ^{b}	4.2 ± 0.1 ^{b}
Quadruple	AF124 + AF74 + AF96 + AF42	0.76 ± 0.04 ^{a}	3.05 ± 0.05 ^{bc}	133.6 ± 3.5 ^{b}	23.7 ± 1.3 c^{d}	63.7 ± 3.0 ^{a}	5.6 ± 0.1 ^{c}	173.1 ± 39.0 ^{a}	4.3 ± 0.1 ^{b}

N.D., not detected.

Table 4. The effects of single and combined PGPR treatments on different rice plant growth parameters. Different letters show statistically significant differences between the variables (Tukey HSD, $p < 0.05$, and Dunnett's at * $p < 0.05$ and ** $p < 0.01$).

PGPR Treatment	Bacterial Species	Shoot Height (cm)	Root Length (cm)	Shoot Dry Weight (mg plant^{-1})	Root Dry Weight (mg plant^{-1})
	Control	49.1 ± 5.0 ^{a}	7.3 ± 1.0 ^{d}	277.5 ± 8.4 ^{c}	69.7 ± 5.5 ^{e}
Single	AF124 (*Rhizobium daejeonense*)	50.0 ± 2.0 ^{a}	12.7 ± 0.5 $^{abc\ *}$	406.1 ± 8.9 $^{ab\ *}$	130.2 ± 2.8 $^{abcd\ *}$
	AF74 (*Enterobacter cloacae*)	53.3 ± 1.7 ^{a}	10.7 ± 0.8 ^{abcd}	463.3 ± 9.6 $^{a\ **}$	139.0 ± 7.2 $^{abc\ *}$
	AF96 (*Pseudomonas putida*)	51.3 ± 1.3 ^{a}	11.1 ± 1.0 ^{abcd}	338.0 ± 28.5 ^{bc}	100.8 ± 6.7 ^{cde}
	AF42 (*Enterobacter cloacae*)	50.3 ± 1.8 ^{a}	11.0 ± 1.1 $^{abcd\ *}$	379.1 ± 27.6 $^{abc\ **}$	113.3 ± 8.8 $^{bcde\ *}$
Dual	AF124 + AF74	54.3 ± 3.7 ^{a}	8.7 ± 0.5 ^{cd}	427.1 ± 2.8 $^{ab\ **}$	150.3 ± 11.1 $^{ab\ **}$
	AF124 + AF96	56.6 ± 2.9 ^{a}	11.3 ± 1.7 ^{abcd}	404.3 ± 4.9 $^{ab\ **}$	133.4 ± 3.6 $^{abcd\ *}$
	AF124 + AF42	55.8 ± 1.7 ^{a}	11.0 ± 0.5 $^{abcd\ *}$	368.3 ± 35.7 ^{abc}	148.6 ± 17.1 $^{abc\ **}$
	AF74 + AF96	53.0 ± 3.2 ^{a}	10.3 ± 0.5 ^{abcd}	336.3 ± 31.5 ^{bc}	85.8 ± 12.2 ^{de}
	AF74 + AF42	55.3 ± 4.1 ^{a}	12.3 ± 1.5 $^{abc\ *}$	337.8 ± 23.4 ^{bc}	132.2 ± 9.8 $^{abcd\ *}$
	AF96 +AF42	56.1 ± 1.5 ^{a}	10.7 ± 0.6 ^{abcd}	428.0 ± 23.3 $^{ab\ *}$	164.4 ± 2.8 $^{a\ **}$
Triple	AF124 + AF74 + AF96	55.0 ± 1.5 ^{a}	11.3 ± 0.4 $^{abcd\ *}$	466.5 ± 32.0 $^{a\ **}$	110.1 ± 5.1 $^{bcde\ *}$
	AF124 + AF74 + AF42	59.1 ± 1.3 ^{a}	14.8 ± 0.1 $^{a\ **}$	429.8 ± 10.1 $^{ab\ *}$	146.6 ± 8.7 $^{abc\ **}$
	AF124 + AF96 + AF42	57.1 ± 3.0 ^{a}	9.6 ± 0.3 ^{bcd}	383.0 ± 15.3 $^{abc\ *}$	129.0 ± 12.5 $^{abcd\ *}$
	AF74 + AF96 + AF42	55.7 ± 2.3 ^{a}	10.5 ± 1.3 ^{abcd}	372.0 ± 23.1 $^{abc\ *}$	124.8 ± 11.2 $^{abcd\ *}$
Quadruple	AF124 + AF74 + AF96 + AF42	55.3 ± 2.0 ^{a}	13.7 ± 0.6 $^{ab\ *}$	405.2 ± 9.8 $^{ab\ *}$	130.0 ± 8.6 $^{abcd\ *}$

4. Discussion

The essential plant growth-promoting (PGP) properties of PGPR include IAA production, N_2-fixation, and mineral solubilization, including P and K. To further understand the effects of PGPR in combined multi-strain circumstances, we chose 4 PGPR from 98 isolates previously isolated from soils in Afghanistan [34] and studied their physiological interactions in single and combined inoculations.

Phytohormones are small signaling molecules that help plants regulate their growth and development, and auxins are one of the most essential and prominent groups [48–50]. More than 80% of microorganisms in the rhizosphere can generate IAA, which is a key regulator for stimulating plant development [51]. In the present study, we initially assessed IAA production by four PGPR strains in single and multiple combined inoculations. AF124 and AF74 showed the highest IAA production when applied individually, and IAA levels decreased when applied in combination with the other strains (Table 2). The variability in IAA production can be attributed to distinct bacterial synthetic processes, specific genes, and regulatory mechanisms [52]. Furthermore, the interactions between bacteria may have negatively affected these processes in the combined application in the current study.

Biological nitrogen fixation by bacterial nitrogenases, which provide nitrogen to crops, is an important mechanism of PGP. Various factors, such as temperature [53,54], salinity [55], and O_2 concentration [56] affect nitrogenase activity. We examined the nitrogen fixation potential of the four selected PGPR in an NFb semi-solid medium in single and combined applications. In a single inoculation, AF124 (*Rhizobium daejeonense*) showed more than 100-fold higher ARA activity than the other three strains (Table 2). Similarly, high nitrogenase activity in *Rhizobium daejeonense* was observed in a previous study [57]. Compared with AF124, the other three strains showed much lower ARA, but high activities were observed when they were combined with AF124. These results suggest that AF124 is a promising strain for maintaining high nitrogen-fixing activity during co-inoculation. Triple PGPR treatments using AF124 + AF74 + AF42 showed higher activity than dual and quadruple applications, suggesting that this triple PGPR combination is a promising candidate for multi-functional bio-fertilizers with good N_2-fixation potential.

Most P in soil is insoluble to varying degrees, making it unavailable to plants. A large portion of soluble inorganic phosphate, which is applied to the soil as chemical fertilizers,

is rapidly immobilized and becomes unavailable to plants [45]. Therefore, phosphate-solubilizing bacteria (PSB) play an important role in solubilizing P in the soil and making it available to plants. It is considered that the solubilization of insoluble phosphates in soils by PSB is related to the production of organic acids [46], as the carboxylic groups of organic acids chelate cations bound to the phosphate salts, resulting in the release of phosphate [26,45]. The levels and types of organic acids depend on the microbial strain and growth conditions. In the current study, the phosphate-solubilizing activities of the four PGPR strains were tested in a liquid medium. The two strains, AF74 and AF42, demonstrated a significantly better ability to solubilize P in a single application than the other two strains (Table 3). Both AF74 and AF42 isolates were categorized as *Enterobacter* species based on their 16S rRNA [34] and *rpoB* genes (Figure 1) and secreted multiple organic acids (Table 3). Enterobacter species have great potential for solubilizing inorganic P and promoting plant growth [58]. AF96 (*Pseudomonas*) produced only malic acid in a single inoculation, and malic acid may be the dominant organic acid type produced by *Pseudomonas* sp. [46]. AF124 (*Rhizobium*), which had the lowest P-solubilizing activity, did not secrete any organic acids into the medium (Table 3). When AF124 and AF96 were combined with AF74 and AF42 in dual, triple, and quadruple applications, the P-solubilizing activity and organic acid secretion were as high as those with the single application of AF74 and AF42. These results suggest that co-inoculation with a high organic acid-producing strain can effectively compensate for the P-solubilization ability of other bacteria with different PGP effects. Potassium is a macronutrient essential for plant growth and is involved in several plant physiological processes, including enzyme activation, protein synthesis, and photosynthesis. Through the formation and excretion of organic acids, including citric, oxalic, and tartaric acids, potassium solubilizing bacteria (KSB) can solubilize unavailable forms of K-bearing minerals such as micas, illite, and orthoclase [59,60]. Several studies have shown that different KSB can solubilize K in vitro and in the rhizosphere [55,61,62]. The four PGPR utilized in this study could solubilize K in vitro, and K-solubilizing activities increased in the combined treatments. Interestingly, the effects tended to increase in the order of single < dual < triple < quadruple combination, indicating positive interactions between strains to increase K solubilization.

From the in vitro analysis, it was found that the triple PGPR treatment, namely AF124 + AF74 + AF42, was a promising combination showing high activities in three PGP characteristics: ARA, P-solubilization, and K-solubilization. The combination of these three isolates provided fixed nitrogen, IAA, solubilized P, and K to the rice plant [63–65]. Promoted rice shoot and root growth might correlate with nutrient solubilization (P and K) and IAA production, while in single inoculation, the IAA effect was more visible in the rice shoot and growth promotion (Table 5). These results suggest that a combination of PGPR strains can be used for multi-functional bio-fertilizer formulations. This result was consistent with previous findings [19,66]. Moreover, these three combinations promoted the growth of rice plants when inoculated (Table 4); however, growth promotion by these three combinations was not significantly different from that using a single application. This may be because the soil used in the current experiment was not nutrient-deficient. The values of available N and P_2O_5 (Table 1) were above the improvement target value of basic soil fertility promotion guidelines for paddy fields in Japan, which are 80–200 $mg \cdot kg^{-1}$ DS for available N and above 100 $mg \cdot kg^{-1}$ DS for available P [67]. Combined applications with high ARA, P-solubilization, and K-solubilization activity can show more powerful PGP effects when soil nutrients are scarce.

Table 5. Correlation matrix of PGPR traits and shoot and root dry weights. The values in the table show correlation coefficients between the variables.

		IAA (μg mL^{-1})	ARA (nmol tube^{-1})	Solubilized P (μg mL^{-1})	Solubilized K (μg mL^{-1})	Shoot Dry Weight (mg plant^{-1})	Root Dry Weight (mg plant^{-1})
Single inoculation	IAA (μg mL^{-1})						
	ARA (nmol tube^{-1})	0.40					
	Solubilized P (μg mL^{-1})	0.62	−0.45				
	Solubilized K (μg mL^{-1})	0.64	−0.18	0.75 *			
	Shoot dry weight (mg plant^{-1})	0.91 **	0.27	0.67	0.54		
	Root dry weight (mg plant^{-1})	0.94 **	0.40	0.57	0.57	0.97 **	
Dual inoculation	IAA (μg mL^{-1})						
	ARA (nmol tube^{-1})	0.12					
	Solubilized P (μg mL^{-1})	0.83 *	0.14				
	Solubilized K (μg mL^{-1})	0.87 *	−0.02	0.91 **			
	Shoot dry weight (mg plant^{-1})	0.61	0.56	0.43	0.55		
	Root dry weight (mg plant^{-1})	0.57	0.33	0.56	0.63	0.87 *	
Triple inoculation	IAA (μg mL^{-1})						
	ARA (nmol tube^{-1})	0.24					
	Solubilized P (μg mL^{-1})	0.93 *	0.52				
	Solubilized K (μg mL^{-1})	0.95 **	0.33	0.97 **			
	Shoot dry weight (mg plant^{-1})	0.70 *	0.45	0.86 *	0.87 *		
	Root dry weight (mg plant^{-1})	0.79 *	0.67 *	0.90 *	0.82 *	0.69 *	

The symbols * and ** indicate significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively.

5. Conclusions

We investigated the IAA production, N_2-fixation activity, P- and K-solubilization capabilities, and influence on rice growth parameters of four PGPR (out of 98 isolates) in vitro, which were isolated from alkaline salt-stressed soils in Afghanistan. Based on the *rpoB* gene analysis, these four PGPR were categorized as AF124 (*Rhizobium daejeonense*), AF74 (*Enterobacter cloacae*), AF96 (*Pseudomonas putida*), and AF42 (*Enterobacter cloacae*), which is almost consistent with the previous categorization of the 16S rRNA gene [34]. IAA production was higher in the single application of AF124 and AF74 than in the combined applications, and the low IAA production by AF96 was compensated for in the combined applications by other strains. ARA in single AF124 was much higher than that in the other three strains, and the high ARA was maintained when AF124 was combined with other strains in a triple application, such as AF124 + AF74 + AF42 or AF124 + AF96 + AF42. The low P-solubilization of AF124 and AF96 was compensated when these strains were combined with AF74 and AF42, and P-solubilization correlated with the secretion of multiple organic acids. Quadruple application of the four PGPR resulted in a significantly greater solubilized K value than the other treatments, and dual and triple PGPR treatments had significantly greater solubilized K values than single treatments, suggesting synergistic interactions between bacteria in the process of K-solubilization. In the rice inoculation test, all single and combined treatments boosted plant growth. Therefore, these four PGPR strains could be used to develop multi-functional bio-fertilizer formulations for sustainable agriculture and a viable alternative to chemical fertilizers.

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