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Exploring Potential Soil Bacteria for Sustainable Wheat (*Triticum aestivum* L.) Production

Rizwan Ali Sheirdil [1,2,*], Rifat Hayat [1,*], Xiao-Xia Zhang [2], Nadeem Akhtar Abbasi [3], Safdar Ali [1], Mukhtar Ahmed [4,5,6,*], Jabar Zaman Khan Khattak [7] and Shakeel Ahmad [8]

1 Department of Soil Science and Soil Water Conservation, PMAS Arid Agriculture University, Rawalpindi 46300, Pakistan; sherdil52@gmail.com
2 ACCC (Agricultural Culture Collection of China), Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China; xxzhang@caas.ac.cn
3 Department of Horticulture, PMAS Arid Agriculture University, Rawalpindi 46300, Pakistan; nadeemabbasi65@yahoo.com
4 Department of Agronomy, PMAS Arid Agriculture University, Rawalpindi 46300, Pakistan
5 Department of Biological Systems Engineering, Washington State University, Pullman, WA 99164, USA
6 Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences, 90183 Umeå, Sweden
7 Department of Biological Sciences, Islamic International University Islamabad, Islamabad 44000, Pakistan; jabar.khattak@iiu.edu.pk
8 Department of Agronomy, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan 60800, Pakistan; shakeelahmad@bzu.edu.pk
* Correspondence: rizwan_sheirdil52@yahoo.com (R.A.S.); hayat@uaar.edu.pk (R.H.); mukhtar.ahmed@slu.se (M.A.)

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Abstract: The application of plant growth-promoting rhizobacteria (PGPR) could allow growers to reduce the use of synthetic fertilizers and increase the sustainability of crop production. Wheat is the main staple food crop of Pakistan, and few studies have reported on the impact of PGPR on wheat crops. To determine if PGPR can maintain wheat productivity with reduced fertilizer applications, we isolated bacteria from the rhizosphere of wheat grown in sandy loam. We selected 10 strains based on in vitro assays for traits associated with PGPR: ACC deaminase activity, siderophore productivity, P-solubilization, and productivity of indole acetic acid (IAA). Furthermore, the strains were tested in three experiments (using a growth-chamber, pots with an experimental area of 0.05 m^2, and a field). Strains that possessed the four traits associated with PGPR increased the shoot length, root length, and fresh and dry weight of plants in the growth chamber study. Similarly, under the pot trial, maximum crop traits were observed under the consortium + half dose, while under field conditions maximum crop parameters were detected in the case of consortium 1 and consortium 2 along with half the recommended dose of fertilizer. This confirms that this consortium could provide growers with a sustainable approach to reduce synthetic fertilizer usage in wheat production.

Keywords: inoculation; PGPR; soil bacteria; wheat

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) are a group of free-living bacteria that can enhance plant growth and crop yield through several mechanisms. PGPR can produce hormones that stimulate plant growth, make nutrients available, fix atmospheric nitrogen, act as bio-control agents, and improve soil structure [1]. Soil bacteria produce a special type of organic acid, i.e., carboxylic acid [2], thus decreasing rhizosphere soil pH and dissociating bound forms of calcium phosphate in calcareous

soil. Soil bacteria help to increase uptake as well as the availability of nutrients for plants [3]. The advantageous effects of PGPR on growth and productivity are well documented and have been correlated with the production of phytohormones and higher nutrient supply [4]. Some potential bacterial candidates for biofertilizer include genera such as *Azospirillium, Pseudomonas, Bacillus, Azotobacter, Enterobacter, Burkholderia, Acinetobacter, Rhizobium, Erwinia, Flavobacterium*, and *Jeotgalicoccus*, etc. [5].

Wheat is a staple crop in Pakistan, but poor soils, lack of irrigation, and inefficient fertilizer use in the region prevent growers from reaching the potential yield of this crop. Soils in this region are low in organic matter (OM) content, which corresponds to low soil fertility and poor soil structure [6]. According to Wu et al. [7] microbial inoculum of two *Bacillus* species *(Bacillus megaterium* and *Bacillus mucilaginous*) improved the growth of the plant as well as the nutritional assimilation of the plant (total N and P, in addition to K). Egamberdiyeva [8] inoculated maize with the bacterial strains *Bacillus polymyxa*, *Pseudomonas alcaligenes*, and *Mycobacterium phlei* and reported a significant increase in root dry weight (19–52%) and increased maize total biomass by up to 38 percent. To develop a sustainable approach to wheat cultivation in Pakistan, here we explore and assess inoculation results of native rhizospheric bacteria on wheat (*Triticum aestivum* L.) crop growth and productivity under in vitro and in vivo conditions. Thus, the objective of current study was to isolate native PGPR and further assess the impacts on wheat crop growth and productivity with different combinations of inorganic fertilizers.

2. Results

2.1. Plant Growth-Promoting (PGP) Activity of Soil Bacteria

All 10 strains possess four PGP traits, i.e., production of indole-3-acetic acid (IAA), solubilization of insoluble tricalcium phosphate, ACC deaminase activity, and siderophore production (Table 1). All the strains produce IAA with tryptophan (1.84 to 12.02 μg mL^{-1}) and without the addition of tryptophan (1.24 to 2.42 μg mL^{-1}). All strains used in this study showed solubilized insoluble mineral phosphate ranges from 84 to 212 μg mL^{-1} along with a medium drop in pH. The maximum drop in pH was observed in case of strain RA-7 up to 4.38 from an initial pH of 7 during seven days of incubation. For plant growth-promoting bacteria, ACC deaminase activity was considered as an efficient marker because these strains have the potential to lower the level of ethylene inhibition in plants. All 10 strains utilize ACC as a sole source of nitrogen, and results depicted that different strains differed in their ACC activity, as shown in Table 1. Maximum ACC activity was observed in case of strain RA-8 (782 nmol h^{-1}) (*Pseudomonas brassicacearum* subsp. *neoaurantiaca*), and the minimum was observed in RA-4 (475 nmol h^{-1}) (*Pseudomonas corrugata*). Siderophore production of all the strains was confirmed by quantitative CAS assay and maximum activity was observed in case of RA-10 (*Pseudomonas azotoformans*). On the basis of absorbance value, siderophore activities were categorized into three levels: high (+++), moderate (++), and low (+).

Table 1. Plant growth-promoting traits of strains isolated from wheat rhizosphere.

	P-Solubilization		ACC-Deaminase Activity (nmol h^{-1})	Siderophore Activity Level	*A/Ar*	IAA mg L^{-1} with Tryptophan	IAA mg L^{-1} without Tryptophan
	(μgmL^{-1}) ± S.E	pH (7.0)				(μg mL^{-1}) ± S.E	(μg mL^{-1}) ± S.E
RA-1	**84.41 ± 1.66**	**5.74**	654 ± 54	+++	0.478 ± 0.025	2.08 ± 0.085	1.36 ± 0.13
RA-2	117.73 ± 2.41	5.12	754 ± 121	+++	0.514 ± 0.021	1.84 ± 0.060	1.24 ± 0.091
RA-3	93.34 ± 1.80	4.82	541 ± 47	+++	0.361 ± 0.017	2.04 ± 0.11	2.42 ± 0.40
RA-4	88.18 ± 1.77	5.02	475 ± 69	++	0.723 ± 0.029	3.50 ± 0.27	1.30 ± 0.17
RA-5	162.16 ± 1.46	4.58	589 ± 79	++	0.651 ± 0.031	2.3 ± 0.098	1.06 ± 0.067
RA-6	127.84 ± 1.59	4.75	671 ± 56	++	0.715 ± 0.042	2.61 ± 0.28	1.097 ± 0.035
RA-7	212.47 ± 2.72	4.38	621 ± 98	+++	0.586 ± 0.015	12.02 ± 0.61	2.408 ± 0.31
RA-8	104.34 ± 0.98	4.55	782 ± 79	+++	0.681 ± 0.034	9.51 ± 0.73	2.17 ± 0.28
RA-9	105.81 ± 1.80	5.12	480 ± 56	++	0.814 ± 0.044	1.91 ± 0.13	1.32 ± 0.32
RA-10	110.73 ± 2.82	4.98	590 ± 74	+	0.976 ± 0.036	2.12 ± 0.086	1.36 ± 0.16

All values are the average of three replicates. IAA: indole acetic acid.

2.2. 16S rRNA Gene Sequence Identification of Bacterial Strains

Diversity in rhizosphere bacteria with varying physiological and biochemical traits were identified to the species level of all isolates. Out of 10 bacterial strains, five strains were identified from the genus *Pseudomonas* as RA-1 (*Pseudomonas fragi*), RA-4 (*Pseudomonas corrugata*), RA-6 (*Pseudomonas arsenicoxydans*), RA-8 (*Pseudomonas brassicacearum subsp. neoaurantiaca*), and RA-10 (*Pseudomonas azotoformans*); four strains belonged to the genus *Bacillus*, these were RA-3 (*Bacillus sefensis*), RA-5 (*Bacillus cereus*), RA-7 (*Bacillus aryabhattai*), and RA-9 (*Bacillus thuringiensis*); and one strain belonged to the genus *Alcaligenes*, which was RA-2 (*Alcaligenes faecalis* subsp. *faecalis).* For phylogenetic tree construction (Figure 1) we obtained closely related taxa of our strains from BLAST search using the eztaxon server. All strains were also identified by analysis through the Sherlock microbial identification system (MIDI) (Library RTSA6 6.0, MIDI Sherlock software package, version 6.0) for cellular fatty acid profile composition (Table 2).

Table 2. Identification of soil bacteria on the basis of 16S rRNA gene sequencing.

	16S rRNA Gene (bp)	DDBJ Accession Number for the 16S rRNA Gene Sequence	Closely Related Taxa (Species)	Type Strain (Gene Bank ID)	DDBJ ACCESSION of the 16S rRNA Gene Sequence	Similarity (%)
RA-1	1330	KF848983	*Pseudomonas fragi*	ATCC 4973$^{(T)}$	AF094733	99.47
RA-2	1332	KF848984	*Alcaligenes faecalis* subsp. *faecalis*	IAM12369$^{(T)}$	D88008	99.1
RA-3	1321	KF848985	*Bacillus safensis*	FO-036b$^{(T)}$	AF234854	100
RA-4	1467	KF848986	*Pseudomonas corrugate*	ATCC 29736$^{(T)}$	D84012	99.23
RA-5	1335	KF848987	*Bacillus cereus*	ATCC 14579$^{(T)}$	AE016877	100
RA-6	1302	KF848988	*Pseudomonas arsenicoxydans*	VC-1$^{(T)}$	FN645213	99.31
RA-7	1323	KF848989	*Bacillus aryabhattai*	B8W22$^{(T)}$	EF114313	100
RA-8	1280	KF848990	*Pseudomonas brassicacearum* subsp. *neoaurantiaca*	ATCC 49054$^{(T)}$	EU391388	99.92
RA-9	1327	KF848991	*Bacillus thuringiensis*	ATCC 10792$^{(T)}$	ACNF01000156	100
RA-10	1311	KF848992	*Pseudomonas azotoformans*	IAM1603$^{(T)}$	D84009	99.62

All values are the average of three replicates.

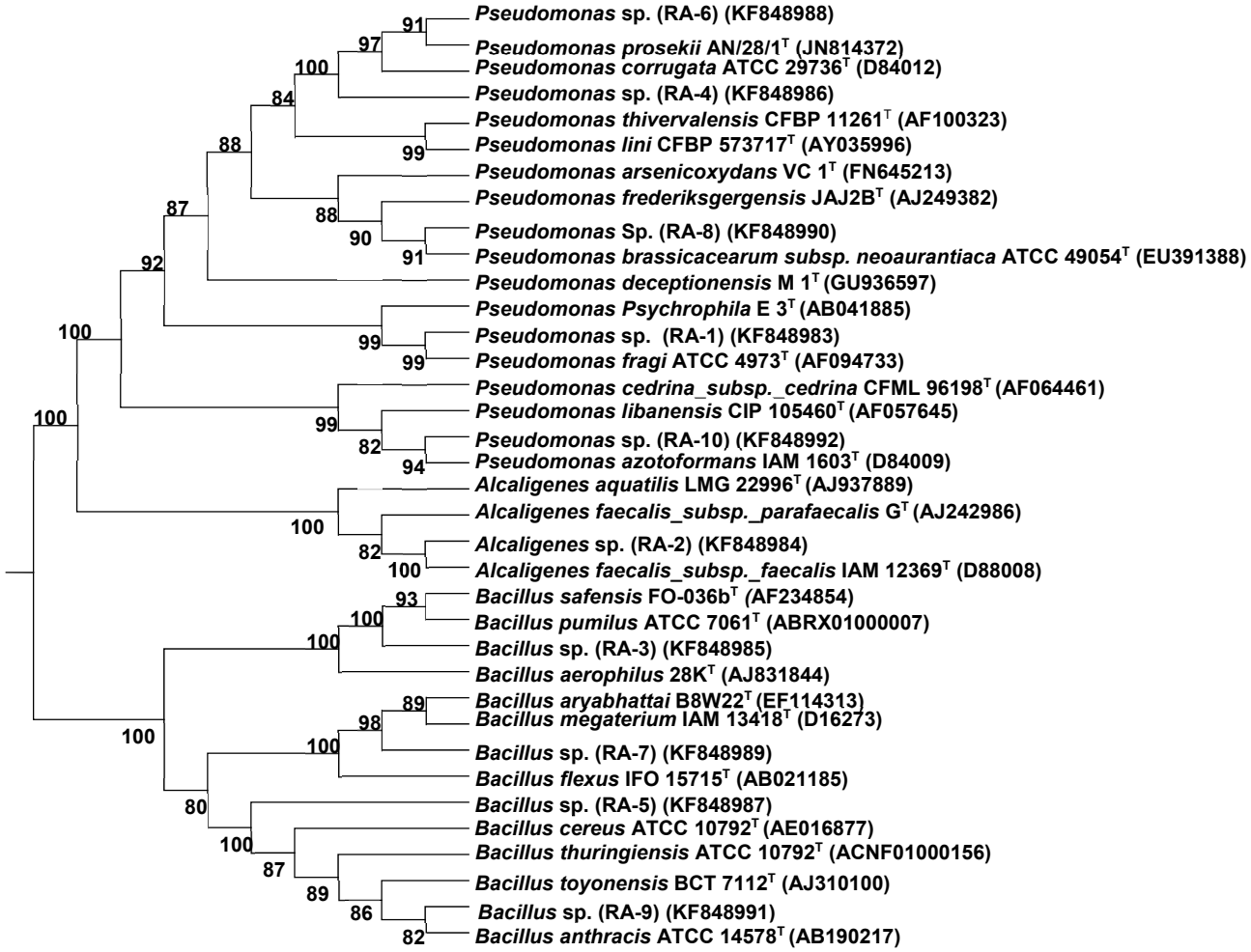

Figure 1. Neighbor-joining phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequences of the wheat rhizospheric representative isolates and some of their closest phylogenetic taxa.

Fatty acid profiles and metabolite utilization patterns of the isolates were consistent with the genera identified in Figure 1. The major fatty acids observed in bacterial strains were: $C_{16:0}$ 30.19 ± 0.01, summed feature 3 25.87 ± 0.01 in RA-1; $C_{16:0}$ 30.19 ± 0.01, summed feature 3 25.87 ± 0.02, summed feature 8 25.1 ± 0.03 in RA-2; anteiso $C_{15:0}$ 23.46 ± 0.01, iso-$C_{15:0}$ 27.42 ± 0.01 in RA-3; $C_{16:0}$ 28.11 ± 0.01, summed feature 3 21.68 ± 0.01, summed feature 8 22.97 ± 0.01 in RA-4; iso-$C_{15:0}$ 28.5 ± 0.01, iso-$C_{17:0}$ 9.62 ± 0.01 in RA-5; $C_{16:0}$ 27.16 ± 0.05, summed feature 3 24.31 ± 0.01, summed feature 8 24.26 ± 0.01 in RA-6; iso-$C_{15:0}$ 25.88 ± 0.05, anteiso-$C_{15:0}$ 29.72 ± 0.04 in RA-7; $C_{16:0}$ 29.95 ± 0.01, summed feature 3 28.05 ± 0.01 in RA-8; $C_{16:0}$ 17.73 ± 0.02, $C_{18:0}$ 11.56 ± 0.01 in RA-9; and anteiso-$C_{15:0}$ 33.49 ± 0.01, summed feature 3 10.31 ± 0.01, $C_{16:0}$ 15.96 ± 0.01 in RA-10. Details on other minor components are given in Table S1. Biolog results depicted the importance of these 10 strains (Table S2).

2.3. Response of Wheat to Soil Bacteria under Controlled and Field Conditions

Increase in shoot length, root length, and fresh and dry weight of plants in response to all treatments were observed (Table 3). A significant increase in shoot length was observed in all the treatments over the control. The maximum increase was observed in T 8 (*Pseudomonas brassicacearum* subsp. *neoaurantiaca*), which gave an 82% increase in shoot length, followed by 77%, 75%, 74%, 62% in T 7, T 3, T 11, and T 5, respectively. An increase of 161% in root length was observed in T 7 followed by 141%, 119%, 108%, and 93% in T 8, T 9, T 3, and T 11, respectively, when compared to the control. An increase in the fresh and dry weights of the plants was observed in all the treatments over control. The maximum increase in fresh weight was 335% by T 8 followed by 309%, 287%, and 258% in T 7, T 3, and

T 11, respectively. Six potential bacterial strains were screened on the basis of their performance in a growth chamber for further investigation in pot and field trials. Inorganic fertilizers were applied in these experiments for comparison with individual strains and consortium of strain with full and half recommended dose of fertilizer for wheat crop. The data taken at harvest stage in pot and field trial showed positive results for every parameter by all bacterial strain applications over the control. All the strains significantly improved shoot length over uninoculated control. The results showed that T 5 (*Bacillus cereus*) showed an increase of 25% in shoot length over the control, followed by T 2, T 4, and T 7 which showed increases of 20%, 19%, and 17%, respectively. A significant negative correlation (R^2 = 0.91) was observed with respect to percentage increase in crop parameters over various doses of inorganic fertilizers (Table 4). A similar trend was observed under field trial with respect to efficacy of inoculants at different doses of inorganic fertilizer. All the treatments applied in the pot experiment were repeated again at the field scale by dividing consortium into two different groups. In total, 15 treatments were applied in a field experiment including control, with full and half recommended doses of fertilizers individually and along with two different consortium groups as shown in Table 5. The maximum yield was observed in the case of consortium 1 and consortium 2 along with half the recommended dose of fertilizer.

Table 3. Effect of inoculation on shoot, root, and plant biomass under the growth chamber condition.

	Shoot Length		**Root Length**		**Fresh Weight (gm)**	**Dry Weight (gm)**
	(cm)	**(%)**	**(cm)**	**(%)**		
Control	15.9 ± 2.23 D	100	5.13 ± 0.93 F	100	1.47 ± 0.46 D	0.76 ± 0.15 E
RA-1	22.8 ± 3.18 BC	143	7.44 ± 1.01 DEF	145	2.96 ± 1.70 CD	1.24 ± 0.44 CDE
RA-2	27.9 ± 3.16 A	175	10.69 ± 2.63 ABCD	208	5.69 ± 1.94 AB	2.79 ± 1.39 AB
RA-3	22.1 ± 3.09 C	139	6.85 ± 1.21 EF	134	2.75 ± 1.34 CD	1.14 ± 0.81 DE
RA-4	26.7 ± 3.41 AB	168	8.81 ± 2.06 CDE	172	2.99 ± 1.14 CD	1.68 ± 1.34 BCDE
RA-5	22.3 ± 3.06 C	140	7.96 ± 1.04 CDEF	155	2.64 ± 1.19 D	1.37 ± 1.22 CDE
RA-6	28.2 ± 3.45 A	177	13.37 ± 3.17 A	261	6.01 ± 1.54 AB	3.98 ± 1.06 A
7-RA	28.9 ± 3.55 A	182	12.34 ± 2.03 AB	241	6.40 ± 1.09 A	3.96 ± 1.26 A
RA-8	25.7 ± 3.97 ABC	162	11.25 ± 2.45 ABC	219	4.53 ± 1.57 BC	2.35 ± 1.17 BC
RA-9	22.9 ± 3.19 BC	144	7.68 ± 1.24 DEF	150	3.13 ± 1.36 CD	1.71 ± 1.31 BCDE
RA-10	27.7 ± 4.09 A	174	9.91 ± 2.19 BCDE	193	5.26 ± 2.19 AB	2.39 ± 1.09 BCD
CV	10.44		21.42		26.40	37.16
p-value	0.0001		0.001		0.0000	0.0004

All values are the average of three replicates.

Table 4. Effect of inoculation with plant growth-promoting (PGP) traits on wheat crop under the pot trial.

	Shoot Length		Root Length		Number of Tillers	Fresh Weight (gm/plant)	Dry Weight (gm/plant)	Spike Length (cm)	Grain Yield	
	(cm)	(%)	(cm)	(%)					(kg ha^{-1})	(%)
Control	66.61 ± 5.16 F	100	19.56 ± 3.35 F	100	4 D	17.72 ± 2.54 GE	6.64 ± 1.23 E	6.70 ± 0.99 B	2900.43 ± 203 E	100
RA-2	88.39 ± 8.1 C	133	27.82 ± 3.45 BC	142	8 C	26.98 ± 3.19 DE	8.63 ± 2.65 DE	7.55 ± 1.27 B	4119.82 ± 249 C	142
RA-4	84.01 ± 8.6 E	126	24.97 ± 4.15 CDE	128	6 CD	21.87 ± 2.15 FG	7.92 ± 1.19 DE	6.99 ± 1.09 B	4158.98 ± 293 C	143
RA-6	6.96 ± 8.4 DE	131	25.87 ± 4.58 CD	132	6 CD	22.52 ± 2.19 EF	8.32 ± 2.19 DE	7.19 ± 2.14 B	3612.36 ± 353 D	125
RA-7	90.02 ± 8.9 C	135	21.56 ± 4.19 EF	110	9 BC	28.55 ± 2.48 CD	8.36 ± 2.22 DE	8.51 ± 2.06 B	4206.76 ± 393 C	145
RA-8	82.83 ± 9.5 E	124	22.21 ± 5.16 DEF	114	8 C	24.27 ± 3.76 DEF	8.66 ± 2.14 DE	7.59 ± 2.58 B	4103.22 ± 416 C	141
RA-10	86.44 ± 8.4 DE	130	21.57 ± 4.78 EF	110	7 CD	25.48 ± 2.93 DEF	9.87 ± 3.09 CD	7.85 ± 2.77 B	3805.54 ± 347 D	131
Full dose	94.42 ± 9.8 A	142	32.72 ± 5.9 A	167	12 AB	36.41 ± 2.41 B	12.92 ± 3.44 AB	10.9 ± 3.34 A	4812.96 ± 389 A	166
Half dose	86.42 ± 8.1 D	130	24.52 ± 4.9 CDE	125	8 C	28.55 ± 2.55 CD	9.96 ± 2.34 CD	7.41 ± 2.06 B	3706.56 ± 338 D	128
Consortium + half dose	97.83 ± 8.7 A	147	31.18 ± 5.5 AB	159	15 A	43.27 ± 5.97 A	14.66 ± 4.54 A	12.9 ± 2.67 A	4903.92 ± 416 A	169
Consortium + full dose	94.56 ± 8.9 B	142	28.12 ± 5.1 BC	144	14 A	32.48 ± 4.18 BC	11.87 ± 4.15 BC	10.8 ± 2.97 A	4605.84 ± 347 B	159
CV	2.84		9.14		2.99	9.84	14.24	14.86	21.64	
p-value	0.0000		0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	

All values are the average of three replicates and the % column indicates the change in percentage with reference to the control.

Table 5. Effect of inoculation with PGP traits on plant height, grain, and yield under the field condition.

Treatments	Plant Height		1000 Grain wt.		Grain Yield	
	(cm)	(%)	(g)	(%)	(kg ha^{-1})	(%)
Control	57.8 I	100	32.53 J	100	3902 I	100
Half dose	61.7 I	108	37.09 I	114	4700 G	120
Full dose	100.8 BC	177	61.08 D	188	5709 C	146
RA-2	79.3 E	139	55.86 E	172	4977 F	128
RA-4	73.8 FG	129	48.78 H	150	4439 H	114
RA-6	76.9 EF	135	54.22 EF	167	5206 E	133
RA-7	92.9 D	163	53.54 F	165	5452 D	140
RA-8	84.5 EF	148	56.28 G	173	5657 C	145
RA-10	86.1 E	151	48.92 H	150	4728 G	121
RA-2 + RA-4 + RA-6	73.0 H	128	54.91 F	169	5157 E	132
RA-7, RA-8, RA-10	77.8 G	136	53.43 F	164	5127 E	131
Consortium 1 + half dose	103.8 AB	182	69.76 BC	214	6337 B	162
Consortium 1 + Full dose	97.1 CD	170	68.58 C	211	6256 B	160
Consortium 2 + half dose	107.1 A	188	75.94 A	233	6597 A	169
Consortium 2 + Full dose	99.7 BC	175	70.64 B	217	6283 B	161
CV	1.36		1.80		1.26	
p-value	0.0000		0.0000		0.0000	

Consortium 1 (RA-2,4,6); consortium 2 (RA-7,8,10).

3. Discussion

The effect of inoculants on crop yields was only detected in pot experiments and very few examples were found when these inoculants were tested in field trials [9,10]. Our investigation was based on three experiments including in vitro and in vivo conditions. We focused on quantitation of the effect of inoculants individually and in consortia on wheat plants grown under full and half the recommended fertilization rate. The increase in the crop shoot length can be due to release of metabolites by bacteria [11] and mineralization of nutrients which are easily available for plants. Increases in dry weight of wheat plants by application of PGPRs were also reported in [12]. The PGPRs had a positive effect on the number of tillers, with an increase of up to 25% in the number of tillers in wheat by the application of PGPRs, as reported in [13]. The production of IAA by the rhizobacteria can increase tillers of the plant, but this factor cannot be the sole reason. In our study, as inorganic fertilizer rates increased, PGP efficacy decreased, showing a negative correlation ($R^2 = 0.91$) similar to that shown in other works [14,15]. Usage of PGPR could significantly reduce P and N fertilizer application without any reduction in wheat yield-related parameters [16]. Our results also showed that when PGPR inoculants were applied with the full recommended dose of fertilizer, the crop growth parameter and yield were lower than with half the recommended dose of fertilizer with PGPR inoculants. Furthermore, it has been reported under greenhouse conditions that the dry weight of tomato with 75% fertilizers and two PGPR inoculants was significantly greater than when using the full recommended dose of fertilizers without PGPR inoculants. Similarly, a significant increase in root length due to the application of PGPRs showed that phyto-hormone production by PGPRs is a major cause of increased root length of plants [14,15]. In another study, an increase in the shoot length of wheat plants due to the application of PGPRs was observed [17].

Numerous studies were conducted in which PGPR were used as inoculants for the improvement of crop growth and yield. The selection of inoculants is very vital and critical step as the inoculants used in our study were native and specific to the wheat crop thus, they showed maximum impact. Effective biofertilizer/biocontrol agent against soil-borne plant phytopathogen strains isolated from one region may not perform better in other soil and climatic conditions due to the variability and inconsistency of soil and climate effects, which could modify the beneficial influence of PGPR [18,19]. Our study main aim was to reduce the chemical fertilizers by utilizing potential wheat rhizospheric bacteria as inoculants, and all results showed that PGPR plays an important role and is useful

to reduce the rate of inorganic fertilizers, mainly because it makes nutrients available. In recent decades, investigations on PGPR revealed that it can promote plant growth directly or indirectly by producing ACC deaminase, as it reduces the level of ethylene in the roots of developing plants [20] by producing plant growth hormones like IAA [21], exhibits antagonistic activity against phytopathogenic soil-borne pathogens by producing siderophore [22], and causes mineral phosphates solubilization along with other nutrients [1]. ACC deaminase activity was considered as an efficient marker for plant-associated bacteria to improve plant growth by lowering the level of ethylene reserved in plants under stress conditions [23]. ACC deaminase activity producing PGPR significantly improved root growth under control conditions [14,15]. Siderophore production by rhizospheric bacteria improves strain colonization and it is also important for iron nutrition of plant antagonistic action [24] against phylopathogens [25]. Siderophores produced by *Pseudomonas sp.* are efficiently used against soil-borne plant pathogens as a biocontrol agent [26]. Indole acidic acid produced by bacterial strains promotes plant growth and has a positive effect on crop yield [27]. While inoculation was effective with inorganic fertilizer doses, its positive impact decreased with increasing rates of fertilizer application. Our findings could improve the sustainability of the whole system, as it will minimize the use of inorganic fertilizers which are major causes of global warming and climate change. Since consortium 2 + half-dose treatment resulted in the maximum production of thousand grain weight and grain yield, it should be used further to obtain sustainable crop yields in the future. Furthermore, it is recommended that multilocation trials be conducted in order to have more detailed information about available PGPR and its linkage with local industry.

4. Conclusions

Extensive use of inorganic fertilizers leads to the dangerous ecological effects, and therefore the biological approaches such as PGPR could be recommended to prevent further deterioration of the environment. Results showed that the application of PGPR in a consortium and alone improves wheat crop growth and yield. The isolation and usage of indigenous PGPR is more beneficial as it can reduce the rate of inorganic fertilizers. Similarly, the side effects of inorganic fertilizers on soil health could be mitigated by the application of PGPR with lower dose of N, P and K. Moreover, in the present study PGPRs were used with a lower dose of fertilizer; thus, it is an environmentally-friendly technology which can minimize soil pollution and maximize crop returns.

5. Material and Methods

5.1. Isolation and Screening of Soil Bacteria

Bacterial strains were isolated from wheat sandy loam rhizospheric soil (33°14′26.38″ N and 72°23′10.29″ E). Isolation of the stains was carried out by dilution plate technique using phosphate-buffered saline as a solution, with growth in Tryptic Soya Agar (TSA; Difco) medium at 28 °C for 48 h. Then, single bacterial colonies were picked and streaked on TSA medium plates with the aim of achieving single colonies.

5.2. Plant Growth-Promoting Assay and Biochemical Characterization of Soil Bacteria

Plant growth promotion activities like IAA production, phosphorus solubilization, and the presence of ACC deaminase activity, siderophore, and biolog of strains were determined following standard procedures. For IAA production, bacterial cultures were grown in Tryptic Soya Broth (TSB). The supernatant was then mixed with two drops of orthophosphoric acid and 4 mL of Salkowski reagents, and the optical density was determined at 530 nm using a spectrophotometer. Development of a pink color was an indicator of IAA production. IAA production by strains was measured by a standard curve graph where the standard range was up to 10 μg mL^{-1} [28]. P-solublization was determined quantitatively as described by Pikovskaya [29]. The supernatant was measured for available phosphorus by the protocol given by Watanabe and Olsen [30]. The optical density of the

supernatant was determined at 700 nm using a spectrophotometer and the values were determined by a standard curve graph; the standard range was up to 1 μg mL^{-1}. The estimation of quantitative siderophore produced by all 10 strains was done through chrome azurol-S (CAS) assay. The color obtained was measured by using spectrophotometer at 630 nm. The siderophore unit was estimated by using a proportion of CAS color shifted using the equation A/Ar, where A is the absorbance of the sample (supernatant + CAS solution) and Ar is the absorbance of reference (uncultured medium + CAS solution) [31]. ACC deaminase activity of all strains was measured following the procedure of Penrose and Glick [32]. The calibration curve was determined according to Bradford [33] and for protein calibration curve, we used bovine serum albumin (BSA) [32]. The absorbance value was measured at 540 nm wavelength.

5.3. Identification of Bacterial Strains Using 16S rRNA Gene Sequencing

The standard method of 16S rRNA gene sequencing was used to identify the strains. Strains were identified with 16S rRNA gene sequencing with further characterization by fatty acid and metabolite utilization profiling. Universal primers 9F and 1510R were used for PCR amplification [34]. The PCR product samples were sequenced using DNA sequencing service of MACROGEN, Korea. The sequence results were blasted through NCBI/Eztaxon [35] and the sequence of all related species was retrieved to get the exact nomenclature of the isolates. Phylogenetic analyses were performed using the bioinformatics software MEGA-5 [36]. Other software used for sequence alignment and comparisons were CLUSTAL X and BioEdit. DNA accession numbers of each strain were obtained from National Center for biotechnology information (NCBI). The accession number allotted by NCBI for strains from RA-1 to RA-10 was KF848983 to KF848992, respectively.

5.4. The Effect of Potential Soil Bacteria on Wheat Crops under Controlled and Field Conditions

Growth chamber, pot (area of 0.05 m^2), and field experiments on wheat crops were conducted in Rawalpindi, Pakistan. A one-month growth chamber experiment (GC) was conducted in trays using sterilized soil. In the growth chamber experiment all isolated strains were tested along with a control with four replications under CRD as the experimental design. In a pot trial, plastic pots were used with 4 kg of sterilized soil. Six potential strains were shortlisted on the bases of growth chamber experiment results and inoculated to wheat seeds. Similarly, same six strains were tested in a field trial. For all experiments, the wheat cultivar Chakwal 50 was used with a seed rate of 100 kg ha^{-1}. The field soil texture was sandy loam (clay 14%, silt 16%, sand 70%). Soil was alkaline with a pH of 7.2, with available P (7.2 μg g^{-1}), exchangeable K (119 μg^{-1}), NO_3-N (3.04 μg^{-1}), and total organic carbon (TOC; 0.47 g 100 g^{-1}). Plant parameters like shoot length, root length, and fresh and dry weight were recorded after a month of germination in the GC experiment and at the harvesting stage in the pot and field trial. The full recommended doses of NPK use for wheat were 100 kg ha^{-1}, 80 kg ha^{-1}, and 60 kg ha^{-1}, respectively. The inorganic sources of N, P, and K used were urea, DAP, and MOP, respectively, applied at the time of sowing along with different combinations of potential bacterial strains.

5.5. Whole-Cell Fatty Acid Analysis

All 10 strains were grown on TSA plates and incubated at 30 °C for 2 days. The Sherlock microbial identification system (MIDI) (MIDI, Newark, USA, Library RTSA6 6.0, MIDI Sherlock software package, version 6.0) was used for determination of cellular fatty acid composition. Strains were harvested and fatty acid methyl esters were prepared as described by Sasser [37].

5.6. Statistics

The obtained crop data were analyzed statistically by using statistix 8.1 through ANOVA, and the means were compared using the LSD test with a significance level of ≤0.05.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/11/12/3361/s1, **Table S1:** Cellular Fatty Acid Profiles (%) of bacterial strain.; **Table S2:** Biolog of bacterial strains.

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