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Relative impact of PGPR inoculation on biofortification and yield of wheat under field conditions and their performance assessment through statistical tools

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Abstract
The production of quality food in an environment-friendly way from diminishing land and water resources is a formidable challenge of this century. Besides quality, agricultural production needs to be increased significantly on a sustainable basis to meet the continuously increasing food demand of mushrooming human population. Wheat is a cereal crop that provides plenty of nutrition. In this era, people are avoiding chemical fertilizers and replacing them with beneficial microorganisms that are now widely adopted for intensive agriculture in many parts of the world, which can stimulate the growth of their associated host through increased mobility, uptake and enrichment of nutrients in the plants. Thus, the present investigation was performed in search of potential plant growth promoting rhizobacteria to enhance the growth and productivity of wheat. Three bacterial isolates were screened during the study for plant growth promotary traits, including zinc and phosphate solubilization, IAA, ammonia and HCN production. Afterward, a field trial was conducted with wheat. The results demonstrated that among all bacterial isolates, PAWR 28 and HRM29 performed superior as they improved plant height, grain yield, biological yield, percent harvest index and grains zinc and iron content. The correlation coefficient, cluster and principal component analysis demonstrated that the performance of PAWR 28 and HRM29 bacterial isolates were remarkable and positively enhanced agronomical parameters, zinc and iron biofortification in grains. The results of the present investigation proved the potential of bacterial isolate PAWR 28 and HRM29 under field conditions and might be utilized as a biofertilizer to enhance wheat productivity and nutrition.

Keywords: Agriculture, biofortification, crop yield, food security, PGPR, wheat

Introduction
Cereals are referred as staple food in the world and are nonlegume in nature. Globally, around 734.32 million ha are used to cultivate cereals, with a production of around 2980.2 million tonnes (FAOSTAT, 2019) [9]. In India, cereal crops are grown in an area of 99.28 million ha, with an annual production of 245.49 million tonnes and a productivity of 2.47 t/ha (GOI, 2019) [12]. India's average productivity of cereals (2.47 t/ha) is almost 40% lower than the average cereals (4.11 t/ha). Wheat is the second most crucial staple food crop in the world, which meets most of the food requirements of the people. Further the quest for quality food has risen from the fact that the world's population is constantly increasing and is expected to be 9.8 billion by the end of 2050 (Kaneda *et al.* 2015) [13]. Additionally, in the year 2017/2018, wheat export exceeded 761.7 million tonnes (FAO, 2020) [8]. Hence, one must adopt precise and balanced nutrition improving practices for higher productivity of cereals because good nutrition is the key to good health (Upadhayay *et al*. 2022a) [34]. Regardless of a record increase in agricultural productivity during the twentieth century, the world faces insecurity in global food security. Chemical fertilizers are commonly used to supply essential nutrients to soil-plant systems in various cultivated crops. However, using high amounts of chemical fertilizers, especially nitrogen, has raised environmental concerns in the current agricultural systems. There is a need to find safe, alternative fertilization strategies to improve agro-ecosystems' sustainability, especially in cereal cultivation. One potential method is the application of plant growth-promoting rhizobacteria (PGPR). PGPR are free-living soil microorganisms that inhabit the rhizosphere of plant roots and improves plant growth and development. These microbes are indigenous to soil and plant rhizosphere and have proved to be potential tools for sustainable and productive agriculture.

Thorough studies suggested that they can increase nutrient concentration in the rhizospheric region by mobilizing various nutrients (zinc, phosphate, potassium, calcium), and also prevent their leaching (Khan *et al.* 2019) [17]. PGPR has been studied for several decades, and some are available in the market, which includes *Azosprillum, Azotobacter, Bacillus, Enterobacter Klebsiella, Pseudomonas, Serratia, Chryseobacterium and Variovorax* (Glick, 2012; Singh *et al.* 2013; Singh and Goel, 2015) [11]. The mode of action of PGPR that helps in plant growth promotion includes (i) tolerance towards abiotic stress in plants; (ii) nutrient acquisition for easy uptake by plant; (iii) plant growth regulators including IAA (indole acetic acid), GA(Gibberellic acid) etc.; (iv)siderophore production; (v) production of volatile; and (vi) production of intra/extracellular enzyme such as chitinase, glucanase, and ACC-deaminase for the prevention of plant diseases **(**Khan *et al.* 2020) [18]. Hence, the application of PGPR could emerge as efficient, eco-friendly, cost-effective and safer for augmenting wheat crop production. Thus, considering the importance of wheat, the present investigation was carried out to find a potential PGPR to raise wheat productivity and nutrients in an eco-friendly manner.

Material and Methods

Bacterial cultures and growth conditions

Twenty bacterial isolates were collected from the Microbiology Department, College of Basic Sciences and Humanities, GBPUA&T, Pantnagar, Uttarakhand, India. All the bacterial isolates were streaked on Nutrient Agar medium and incubated at an optimum temperature of 28 °C, and purity was verified through Gram's staining and microscopic examination at 100 x magnifications. All bacterial isolates were preserved in slants at 4 °C and at -20 °C in glycerol stocks for later usage.

Plant growth promoting traits- assessment

The ability of the bacterial isolates to promote plant growth was evaluated through the synthesis of zinc solubilization, HCN and ammonia production. The bacterial isolates were assessed quantitatively for IAA production and phosphate solubilization.

a. Zinc Solubilization Potential

The ability of bacterial isolates to solubilize zinc was examined on a minimal agar medium enriched with 0.1 percent $ZnCO_3$ (Ramesh *et al.* 2014). The spot inoculations of actively growing bacterial cultures were made on the appropriate medium, and plates were then incubated for 72–96 hours at 28 °C. The solubilization was demonstrated with a halo zone surrounding the bacterial colony. Zinc solubilization efficiency was determined using the formula below.

% solubilization efficiency = (Diameter of halo zone/diameter of colony) x 100

b. HCN Production

The picrate assay was used for determining HCN production. Bacterial cultures were streaked on nutrient agar supplemented with glycine (4.4 g/l). Parafilm sealed plates were incubated for 3 days at 28±2 ºC. Positive result was indicated by a color change of filter paper from yellowish to brownish orange (Donate Correa *et al.* 2005) [7].

c. Ammonia Production

Bacterial cultures were inoculated in 10ml peptone broth and incubated for 48 h at 120 rpm. After incubation, 0.5 ml of Nessler's reagent was added and incubated for 10 to 15 min. The appearance of yellow color indicates the positive results of ammonia production (Cappuccino and Sherman, 1992) [5].

d. Quantitative Estimation of IAA

In order to determine the production of IAA, bacterial isolates were inoculated in a nutrient broth supplemented with tryptophan (100µg/ml) and incubated for 5 days at 28±2ºC. The suspension was centrifuged at 10,000 rpm (4ºC) for 10 minutes. Afterward, 2ml collected supernatant was added with 4 ml salkowski's reagent and incubated for half an hour in the dark.: The pink color developed for IAA production was considered positive, and absorbance was taken at 530nm. Production of IAA was calculated with the help of a standard curve of pure IAA (Bric *et al.* 1991) [44].

e. Quantitative Estimation of Phosphorus

For estimation, 50 ml NBRIP broth was inoculated with bacterial isolates and incubated for 7 days at 28 $\pm$ 2°C (120 rpm). After incubation, broth was centrifuged at 5000 rpm, subsequently, 1 ml of supernatant was mixed with perchloric acid (0.4ml), ammonium molybdate solution (0.4ml) and coloring reagent (0.2ml), and then the volume was made up to 6 ml with distilled water. Absorbance was measured at 640 nm. The amount of solubilized P was inferred from the standard curve (Nautiyal, 1999) [21].

Field trial study

Plant growth promotion and biofortification potential of selected bacterial isolates were examined by conducting a field experiment at Norman E. Borlaug Crop Research Center, Pantnagar, on wheat (*Triticum aestivum* var. HD2967) crop. Each bacterial isolate was primed on seeds through charcoal-based bioformulation. Each bacterial bioformulation was independently used to bacterize the wheat seeds before sowing, allowing the bacteria to adhere to the seeds by drying in the shade for an hour. Afterward, seeds were planted in the corresponding field plot. Three replications of the randomized block design were used throughout the entire experiment.

Plant growth promotion assessment

At the Harvesting stage of the wheat crop, the height of the plant, spike length, number of grains per spike, 1000-grain weight, biological and grain yield, and harvest index were computed.

Estimation of iron and zinc in grains

The amount of accessible Zn and Fe was also measured in the seed samples as per the protocol described by Upadhayay *et al.* (2022a) [35]. In brief, 0.1g of wheat grains from each treatment were digested on a hotplate (at 95°C) in 10ml of a tri-acid combination [Nitric acid (10): Perchloric acid (4): Sulfuric acid (1) v/v/v], after which 6N HCL (5ml) was added to the digestion mixture. Subsequently, distilled water was added to make the volume upto 50ml, and the suspension was filtered through Whatman no. 42 filter paper. Finally, an atomic absorption spectrophotometer (AAS) was used to quantify Fe and Zn in the grains. The following formula was used to estimate the amounts of each element in the sample.

Element (mg/kg) = A x C where A = V / W
Where, V is the total volume of the sample solution (in ml); W stands for sample weight (in gm);C is Sample concentration (in ppm)

Statistical analysis
To ascertain the variance and significance between the treatments, One-way ANOVA followed by the Duncan test was carried out at *P*< 5%. Additionally, Pearson's correlation coefficient was examined to ascertain the relationship between the PGP features, grain, biological yield, and harvest index. The principal component analysis and cluster analysis were also used to confirm the impact of relative bacterial isolates on plant agronomical metrics.

Results and Discussion
PGP potential of bacterial isolates
All the bacterial isolates were examined for zinc and phosphate solubilization. Among all bacterial isolates, the maximum efficiency of zinc solubilization was exhibited by HRM29 and PAWR 28 i.e 233.33%, whereas the highest phosphate solubilization was demonstrated by CHC4 (286%) followed by HRM29 (Table 1). Similarly, in a study by Upadhayay *et al.* (2022b) [32], the zinc solubilization potential of bacterial isolates was investigated to identify the efficient PGPR. Moreover, Roshni *et al.* (2020) also screened rhizospheric bacterial isolates for zinc solubilization capability and further assessed them to improve wheat grain quality. In a recent study by Alam *et al.* (2022) [2] reported a positive effect of P solubilizing bacteria on wheat yield attributes. Furthermore, HRM29 also showed the highest IAA production i.e. 49.21±0.19 followed by PAWR 28 i.e. 46.20±0.19 (Table 1). Similarly, Abbasi (2015) [1] also estimated the IAA production and reported that the application of IAA-producing bacterial isolates enhances the growth and yield of wheat. In addition, none of the bacterial isolates showed a positive result for HCN production, but all isolates demonstrated a positive result for ammonia production (Table 1). The present study agrees with Yadav *et al.* (2022) [35] and Patel *et al.* (2021) [23], who has documented that PGPR producing ammonia help in enhancing plant growth.

Table 1: Plant growth promoting traits of bacterial isolates

	HCN production	Ammonia production	Phosphate solubilization	IAA production	Zn solubilization
PAWR 28	-	+	167±5.4	46.20±0.19	233.33±33.16
HRM29	-	+	256±6.9	49.21±0.19	233.33±35.11
CHC4	-	+	286±4.3	36.7±0.43	166.66±17.00
PWR16	-	+	-	26.4±0.73	180.00±12.76
PWR7	-	+	112±5.1	-	140.00±17.32
AWR 5	-	+	-	-	166.66±17.00

Mean± SD is shown in the table. Each value is the mean of three replicates.

Yield and growth performance
The current study showed that all bacterial isolates had a notable impact on the wheat crop's growth-promoting and yield-attributing characteristics compared to the control. The grain and biological yields are important attributes of wheat production. The highest grain yield (48.12±1.8 and 46.23±1.6 kg/ha) and biological yield (113.02 and 113.99 kg/ha) were observed for the treatment of PAWR 28 and HRM29 bacterial isolates, respectively, which is significantly higher compared to control i.e. 41.78±1.3 and 98.53±2.2 kg/ha, respectively (Table 2). Likewise, Ghadam *et al.* (2019) [10] also reported a significant enhancement in grain yield when wheat seeds were treated with potential PGPR. Concerning the plant height, seeds primed with PAWR 28 exhibited maximum plant height i.e. 91±2.5 cm followed by HRM29 (Table 2), which was far higher than control plants (85±2 cm). Similarly, Assainar *et al.* (2018) [3] also reported an increment in wheat shoot growth at maturity when inoculated with potential microbes over control. Moreover, among all treatments, the utmost spike length (12.43±0.66 and 12.36±0.64 cm) was recorded in CHC4 and PAWR 28 isolates, respectively. In addition, as compared to control (28.66±1.15), the number of grains per spike was utmostly observed in HRM29, and CHC4 treated plants (30±2 and 30±1), respectively (Table 2). These results are supported by the study of Naeem *et al.* (2018) [20], who observed 22% and 20% increase in spike length and number of grains per spike, respectively, when wheat seeds were treated with PGPR compared to the uninoculated control. The results of the present study confirmed the 1000 grain weight enhancement upon PAWR 28 bacterium primed plants was 61.56±1.42 g. Another principal concern of the study was No. of spike/m^2 and noted highest No. of spike/m^2 in PAWR 28 primed plants (Table 2). The present study also agrees with Sun *et al.* (2021) [31], who reported that wheat seeds treated with PGPR significantly increased in No. of spike/m^2. In addition to these findings, the present investigation demonstrated a significant harvest index 24.6±0.08 of wheat in HRM29 primed plants, which was higher than control plants (23.0±0.10). Similar results were also reported by Saber *et al.* (2012) [26] upon bioinoculant application. In addition, numerous recent PGPR studies conducted with various crops showed improvement in plant agronomical, yield and nutrient qualities, corroborating the current study (Khan *et al.* 2022; Sedri *et al.* 2022; Upadhayay *et al.* 2021; Parveen *et al.* 2018) [2, 34, 27, 22]. Plant inoculated with HRM29 and PAWR 28 showed enhanced zinc biofortification in grains i.e. 44.54±2.22 and 40.59±3.72 mg/kg, respectively, over control (33.37±1.22 mg/kg) (Table 3). Whereas wheat plants inoculated with PAWR 28 and CHC4 significantly improved grain iron content over control i.e. 26.72±1.52 and 24.04±1.66 mg/kg, respectively and showed a significant difference compared to control (20.22±1.79 mg/kg) (Table 3). Micronutrient content in grain indicated that PGPR inoculation significantly increased micronutrient uptake over non-inoculated control plants. These findings agreed with Khalid *et al.* (2022) [14], in which zinc solubilizing bacteria enhance the zinc content in wheat grains. A recent study by Shi *et al.* (2020) also reported enhancement of iron content in wheat grain by applying PGPR.

Table 2: Influence of PGPR on plant growth promotion and yield attributing traits of wheat

	Plant height (cm)	No. of spike/m2	No. of grain per spike	Spike length (cm)	Weight of 1000 grains (g)	Grain yield (kg/ha)	Biological yield (kg/ha)	% Harvest index
Control	85±2	72±1	28.66±1.15	11.83±0.76	59.83±0.78	41.78±1.3	98.53±2.2	23.0±0.10
PAWR28	91±2.51	80.66±5	29.33±2.08	12.36±0.64	61.56±1.42	48.12±1.8	113.02±2.9	23.6±0.15
HRM29	90.33±416	79±4.5	30±2	12.23±0.15	61.50±0.40	46.23±1.6	113.99±7.8	24.6±0.08
CHC4	88.33±1.15	74±7	30±1	12.43±0.66	61.43±0.40	46.19±2.8	111.67±4.6	24.2±0.24
S.Em	1.724	3.162	0.430	0.320	0.556	0.783	3.30	0.862

Data were analyzed at *P*< 0.05 level of significance. Mean±SD is shown in the table. Each value is the mean of three replicates.

Table 3: Estimation of Zn and Fe content in wheat grain

Treatment	Zn concentration (mg/kg)	Fe concentration (mg/kg)
Control	33.37±1.22	20.22±1.79
PAWR28	40.59±3.72	26.72±1.52
HRM29	44.54±2.22	23.44±1.05
CHC4	40.11±3.32	24.04±1.66
S.Em	1.6	0.796

Data were analyzed at *P*< 0.05 level of significance. Mean±SD is shown in the table. Each value is the mean of three replicates.

Correlation coefficient analysis

Statistical analysis showed a strong positive link to PGP characteristics and wheat yield parameters, which also showed that the magnitude of PGPR activity considerably improved wheat yield and other agronomical parameters (Table 4). The Pearson's correlation coefficient was checked between the PGP features and the yield attributes since the correlation coefficient shows the relationship between the parameters. The findings of Chandra and Sharma (2021) [6] also support the present study, which reported a highly positive correlation among plant growth enhancing activity and yield parameters like grain yield, height of the plant, spike length etc., in wheat and rice. Moreover, Khan and Singh (2021) [17], also recorded the positive correlation of PGPR on agronomical parameters of wheat.

Table 4: Correlation between PGP traits of bacterial isolates and oat agronomical parameters

	Phosphate solubilization	IAA Production	Zn solubilization	Zn concentration (mg/kg)	Fe concentration (mg/kg)	No. of grain per spike	Grain yield	Biological yield	% Harvest index
Phosphate solubilization		0.65889	0.51082	0.88547	0.26575	0.15585	0.1443	0.78413	0.41599
IAA Production	0.65889		0.14807	0.45565	0.92463	0.81473	0.80319	0.12524	0.92512
Zn solubilization	0.51082	0.14807		0.60371	0.77657	0.66667	0.65512	0.27331	0.92681
Zn concentration (mg/kg)	0.88547	0.45565	0.60371		0.61972	0.72962	0.74116	0.3304	0.46947
Fe concentration (mg/kg)	0.26575	0.92463	0.77657	0.61972		0.1099	0.12145	0.95012	0.15025
No. of grain per spike	0.15585	0.81473	0.66667	0.72962	0.1099		0.011545	0.93998	0.26015
Grain yield	0.1443	0.80319	0.65512	0.74116	0.12145	0.011545		0.92843	0.27169
Biological yield	0.78413	0.12524	0.27331	0.3304	0.95012	0.93998	0.92843		0.79988
HI	0.41599	0.92512	0.92681	0.46947	0.15025	0.26015	0.27169	0.79988	

Cluster and Principal component (CPC) analysis

The relative impact of PGPR on wheat plant yield attributes was determined through principal component and cluster analysis. The CPC analysis showed that HRM29 and PAWR 28 fall on the right side, confirming their maximal beneficial impact on plant growth and yield (Fig 1). Moreover, the cluster analysis also revealed that HRM29 and PAWR 28 were the most potential PGPR, as they clustered together on the left side (Fig 2). Similarly, Yagmur and Gunes (2021) [36] also determined the best PGPR that had maximum effect on agronomical parameters through principal component analysis (PCA). Moreover, Masmoudi *et al.* (2019) [19] confirmed the most effective isolates for growth promotion through principal component analysis.

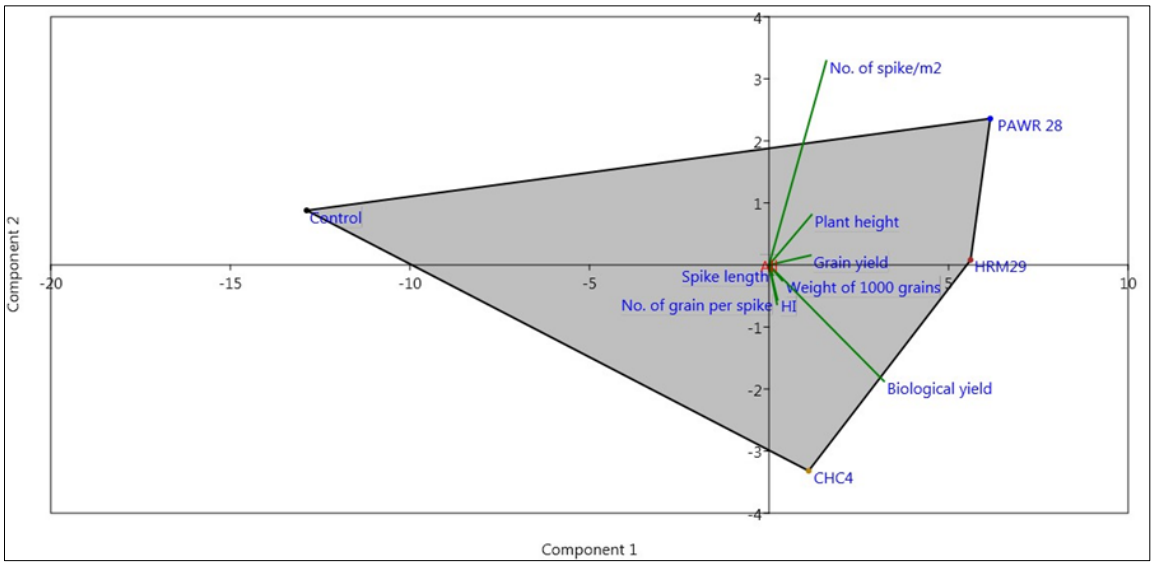

Fig 1: Principal component analysis depicting the influence of PGPR on growth and yield attributes of wheat crop

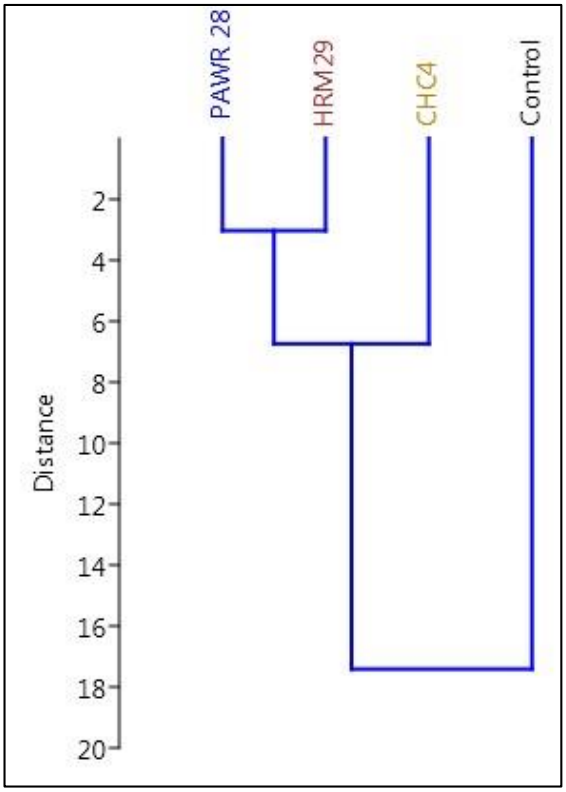

Fig 2: Cluster analysis of treatments depicting grouping based on their plant growth promotion potential

Conclusion

Wheat is an important staple food and meets most of the food requirements of the globe. In this scenario, supplementing biofertilizers can play an efficient role in agriculture to perk in agricultural production and soil fertility in a more sustainable and environmentally friendly manner. The present study concludes that inoculation with promising bacterial strains such as HRM29 and PAWR 28 can enhance wheat's micronutrient content and crop yield. Moreover, the correlation, principal component, and cluster analysis supported positive effects and their relative amplitude on yield. Therefore, such strains represent promising candidates for inclusion in integrated nutrient management practices for wheat crops.

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