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Research article

Bacteria from native soil in combination with arbuscular mycorrhizal fungi augment wheat yield and biofortification

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ABSTRACT

Plant growth promoting bacteria (PGPB) have been used to enhance crop productivity. The effect of native PGPB and arbuscular mycorrhizal (AM) fungi in combination on wheat yield, biofortification and soil enzymatic activity is a relatively unexplored area. Twenty seven bacterial isolates from three different soils were characterized for their plant growth promoting traits. A total of three native and five non-native bacteria were used with and without arbuscular mycorrhizal (AM) fungi in an open greenhouse pot experiment with two wheat varieties to evaluate their effect on wheat yield, nutrient uptake, and soil health parameters. Wheat plants subjected to native PGPB (CP4) (*Bacillus subtilis*) and AM fungi treatment gave the best results with reference to macronutrient (nitrogen and phosphorus), micronutrient (iron and zinc) content in wheat grains and yield-related parameters, including thousand grain weight, number of grains per spike and total tillers per plant in both wheat cultivars. Treatment with CP4 and CP4 plus AM fungi enhanced total chlorophyll in wheat leaves indicating higher photosynthetic activity. Significant improvement in soil health-related parameters, including soil organic matter and dehydrogenase activity, was observed. Significant correlation among grain yield-related parameters, nutrient enhancement, and soil health parameters was observed in PGPB and AM fungi treated plants, especially HD-3086. These results provide a roadmap for utilizing native PGPB and AM fungi for enhancing wheat production in Punjab state of India and exploring their utility in other parts of the country with different soil and environmental conditions.

1. Introduction

Wheat is a dominant staple food which constitutes about 50% of the diet (Shewry and Hey, 2015). The world population has increased around four-fold in the last 100 years and is predicted to reach 9.5 billion by 2050. Due to various aspects of man-made global warming, worldwide food production will be significantly reduced (IPCC et al., 2018). Soils and fertilizers are non-renewable resources that contribute to food production. Environment-friendly approaches that mitigate climate change effects and reduce or eliminate the use of fertilizers are needed.

One such environment-friendly approach is the use of soil microbial communities, which are involved in nutrient cycle, regulating soil fertility, mobilizing or immobilizing heavy metals and maintaining plant diversity and improving plant fitness (Jacoby et al., 2017). Plant growth promoting bacteria (PGPB) are known to either colonize plant roots or exist as free-living to promote plant growth and development (Glick, 2012). Plant genotype significantly influences the native microflora in the rhizosphere (Horner et al., 2019). Bacterial diversity is imperative since it comprises the majority of earth's species diversity and it is one of the most useful resources with significance in bioremediation, bioprospecting, and intercropping systems (Li and Wu, 2018).

Enhancing yield and essential micronutrients in wheat grain is the main goal of modern agricultural practices. Biofortification is one of the potential solutions for malnutrition in developing countries like India. Biofortification is a process to enhance nutrient bioavailability and concentration in the edible parts of plants, which is generally accomplished through conventional plant breeding and genetic engineering (Garg et al., 2018). Biofortification of crops can also be achieved employing PGPB, which increase the micronutrient content of staple crops besides improving yield and soil fertility (Rana et al., 2012; Arif et al., 2017). There are a few studies reported that PGPB and PGPB consortium inoculation enhance the micronutrients, especially iron and zinc in grains of rice and wheat, as well as legumes (Rana et al., 2015; Gopalakrishnan et al., 2016; Dogra et al., 2019). Rising demand for food and environmental concerns have focused on the importance of PGPB in agriculture. Growth responses in inoculated wheat depend on

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various factors like plant genotype, nature of PGPB, as well as environmental conditions (Sheirdil et al., 2019). *Bacillus subtilis*, *Bacillus licheniformis*, *Achromobacter xylosoxidans*, *B. pumilus*, *Brevibacterium halotolerans* and *Pseudomonas putida* are potential PGPB with a role in cell elongation, activation of ACC deaminase and plant growth promotion (Backer et al., 2018). PGPB synthesize various biomolecules such as IAA, siderophores, organic acids, and exopolysaccharides, which enhance mineral availability at the plant root interface, increase plant growth and tolerance to abiotic stresses including drought and salinity (Barnawal et al., 2019). PGPB have been used to enhance the productivity of soybean, wheat and corn up to 30% in Latin American countries (Diaz-Zorita and Fernandez-Canigia, 2009). Also, *Pseudomonas fluorescens* and related pseudomonads are being used as biocontrol agents to reduce soil phytopathogens (Glick, 2012; Chaudhary and Shukla, 2019a). Current agricultural practices lead to poor soil in terms of macro and micronutrients, especially in wheat rice cropping systems (Shahane et al., 2019). PGPB have attracted considerable attention in agriculture, and are being used in fields as soil inoculum to improve plant growth and yield (Boostani et al., 2014; Enebe and Babalola, 2018). They can partly replace chemical fertilizers to make nutrient management economically viable and ecologically sustainable (Shahane et al., 2019). Although, the current use of biofertilizers globally is about 5% of the chemical fertilizers, the use of microbial inoculation is gaining importance due to a higher price and the adverse impact of chemical fertilizers (Timmusk et al., 2017).

The employment of PGPB as a biofertilizer requires understanding of their interactions with plants. Plants can be positively or negatively affected depending on the nature of microbial species. A well-known example is a mutualistic association between arbuscular mycorrhizal fungi (AMF) and plants. Mutualistic rhizospheric microbes like PGPB and AMF can increase the plant nutrient uptake, especially when applied in combination (Varinderpal-Singh et al., 2019). PGPB and AMF consortium enhanced growth, micro and macronutrient uptake in red pepper (Kim et al., 2010). Similar results were reported from our research group using PGPB (*Pseudomonas* sp.) and AMF on maize and sorghum (Dhawi et al., 2015, 2016). The interaction between AMF and microbes depends on the background phosphorus availability and the exchange of exudates between plants and AMF. Inoculation of wheat plants with PGPB and AMF increased the biomass yield, as well as N and P uptake in above-ground plant parts (Saia et al., 2015a, 2015b). Triple inoculation of AMF and two PGPB species enhanced the tobacco yield (Subhashini and Murthy, 2015; Subhashini, 2016). Inoculation of wheat, rice, black gram, and maize with PGPB and AMF increased grain yield, protein, and mineral nutrients (Mäder et al., 2011; Rocha et al., 2019). Further, rhizospheric and endophytic PGPB and their consortia have been reported to be a promising option to enhance the quality of cereals (Emami et al., 2018).

Crop productivity can be enhanced not only by increasing yield but also by reducing losses due to biotic and abiotic stresses. Although, most of the research on plant stress responses have concentrated on a single abiotic or biotic stress, efforts are being made to understand the role of phytohormone, transcription factors, and ROS as common regulators of concurrent stresses (Shaik and Ramakrishna, 2014; Ramakrishna and Kumari., 2017; Dangi et al., 2018). PGPB that enhance yield and nutrient content would be good candidates for evaluation of their role in conferring resistance to combined stress. Identification of biochemical and molecular mechanisms involved in the process would open up novel avenues for improving crop productivity.

Identification and application of native PGPB at field level can be an alternative, which is an environment-friendly and sustainable approach (Gopalakrishnan et al., 2016). Although some studies have used PGPB isolated from one location to enhance plant growth in a different location, it is preferred to utilize PGPB isolated from a specific area to support plant growth in that region to avoid the introduction of non-native microorganisms. The aim of the present study is to identify native soil bacteria with plant growth promoting attributes and compare their ability with non-native bacteria, to enhance yield-related parameters, macro and micronutrients of wheat grain and promotion of soil health when used alone and in combination with arbuscular mycorrhizal fungi. This study will lay the foundation to evaluate them in field studies including the fields of local farmers.

2. Material and methods

2.1. Plant growth and treatments

Two wheat cultivars HD-3086 and HD 2967 procured from Punjab Agricultural University (PAU) were used in this study. Plastic pots (7-inch diameter) were filled with 5 kg native soil collected from the Central University of Punjab city campus (NL 30°10′20″ EL 74° 57′58″) after sieving to remove root debris. Soil samples were amended with compost in a ratio of 1:3. Wheat seeds were subjected to three microbial treatments, bacteria, vesicular arbuscular mycorrhiza and combination of both (B, My, and My + B) in addition to a control (C). Each treatment had 9 replicates with three wheat seedlings per pot. B represents eight different bacterial isolates, i.e., ARP8, AHP3, AHP4, CP4, CP6, PR30, PR29, and RA6, whereas My represents Symbion-VAM PlusTM (www.tstanes.com/products-symbion-vam.html), which is a commercial bio-fertilizer. My + B represents each of the eight bacterial isolates in combination with vesicular arbuscular mycorrhiza (VAM). A total of 108 pots were used for growing the plants with 54 pots for each wheat variety, including the control. Plants were grown in an open greenhouse.

2.2. Soil physicochemical properties and antioxidant enzyme activity

Soil samples were collected from four sites: site A (Central University of Punjab (CUP) campus, Bathinda), site B (Aizawl roadside, Mizoram), site C (Aizawl hillside, Mizoram) and site D (cotton field, Bathinda). All soil samples were collected from the same depth and in triplicates in aseptic falcon tubes (50 mL) and immediately transported in a cooling box to the laboratory and stored at 4 °C. The samples were analyzed for physicochemical properties like soil texture, pH, soil organic carbon (SOC), soil organic matter (SOM), nitrogen (TN), phosphate (TP), C:N ratio and soil dehydrogenase activity using standard methods. Soil pH measurements were performed with soil to water ratio of 1:1 as per McLean (1982). Soil organic carbon and organic matter were determined by the rapid titration method (Walkley, 1947; Kumar et al., 2016). Briefly, 1 g of dried soil was added to 10 mL $K_2Cr_2O_7$ (1N) and 20 ml H_2SO_4 with shaking. The solution was diluted by the addition of 200 ml of distilled water followed by the addition of a few drops of o-phenanthroline–ferrous complex indicator. The solution was titrated against a 0.5N ferrous ammonium sulphate solution. At the endpoint, the green-colored solution changed from blue to red or maroon tinge. Available phosphorus of soil samples was determined as per Olsen (1954). Total nitrogen content was determined using the Kjeldahl digestion method (Pelican Kelplus Kelvac VA equipment) (IS 5194, 1969). Soil C:N ratio was determined by calculating the ratio of soil organic carbon and total nitrogen.

The soil used in this study was collected from CUP campus, Bathinda. Prior to sowing the seeds and after harvesting the plant samples of each treatment group, the soil physicochemical properties were analyzed as described above. In addition, soil dehydrogenase activity was assayed before and after harvest using 2, 3, 5 triphenyltetrazolium chloride (TTC) as the substrate by the method proposed by Casida et al. (1964). Triphenylformazon (TPF) production per gram of soil (dry weight) per 24h ($\mu g \cdot g^{-1} \cdot 24\ h^{-1}$) represents the soil microbial dehydrogenase activity.

2.3. Isolation of phosphate solubilizing bacteria from soil samples

Soil sample (1 g) was suspended in 10 mL of sterile saline solution

(0.85%) and agitated on a shaker (200 rpm) for 15 min at room temperature. One mL of this soil suspension was used to prepare serial 10-fold dilutions with saline. A 100-μL aliquot of the 10^{-3} and 10^{-4} dilutions was used for plating in triplicate on modified Luria-Bertani agar (100 μg/mL ampicillin) and incubated at 37 °C for 48 h. All isolates were further streaked onto PVK (Pikovskaya, 1948) and NBRIP media (Nautiyal, 1999) plates. The isolates grown successfully on both media were selected and evaluated for plant growth promoting attributes. Two bacterial isolates, PR29 (*Pseudomonas chlororaphis*) and PR30 (*Bacillus subtillis*), were procured from Prof. Ramteke (Sam Higginbottom University, Allahabad, India). The other isolate previously collected from Bathinda region was characterized as *Pseudomonas* sp. RA6 based on biochemical and DNA sequence analysis (Adhikary et al., 2019; Accession number KM594398).

2.4. Identification of bacterial isolates by ribosomal DNA sequencing

Total genomic DNA from ARP8, AHP3, AHP4, CP4, and CP6 was extracted using Wizard genomic DNA isolation kit (Promega). In these bacterial isolates, partial 16S rRNA gene (about 1.5 kb) was amplified using primers (5′-AGAGTTTGATCATGGCTCAG-3′ and 5′-TACGGCTAC CTTGTTACGAC-3′). The PCR product was purified using HiPurA PCR product purification kit (Himedia). BigDyeTM Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used to perform DNA sequencing reactions. The sequencing reaction was precipitated using ethanol and the pellet was resuspended in 10-μl Hi-DiTM formamide before running the samples on a 3730xl DNA analyzer (Applied Biosystems). The high-quality sequences obtained from DNA sequencer were used for BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify similarity to sequences in GenBank.

2.5. Evaluation of plant growth promoting attributes

Indole acetic acid (IAA) production was estimated (quantitative assay) with the calorimetric technique proposed by Gordon and Weber (1951). Phosphate solubilization was assayed on Pikovskaya's medium containing tricalcium phosphate. Individual isolates were spot inoculated on the center of each plate and incubated at 37 °C for 48 h. A clear halo zone around the spot was considered positive for phosphate solubilization. This experiment was carried out in triplicate with an uninoculated plate serving as the control. The positive isolates for P-solubilization were used to estimate soluble phosphate spectrophotometrically by the ascorbic acid method (Bray and Kurtz, 1945). These bacterial isolates were tested for their ability to produce siderophore by the chrome azurol S (CAS) method proposed by Schwyn and Neilands (1987). Briefly, an overnight culture of the bacterial isolate was spot inoculated onto a CAS agar plate and incubated at 28 °C for 48 h and quantitative estimation was performed by the method described by Arora and Verma (2017).

2.6. Seed bacterization

Wheat seeds were surface sterilized with 70% ethanol for 2 min, followed by sterile water washes multiples times. Bacteria growing at the exponential phase were harvested and centrifuged. The pellet was resuspended in saline buffer (0.85% NaCl) to adjust to 10^8 colony-forming units (CFU) mL^{-1}. The culture was amended with 1% carboxymethyl cellulose (CMC) (final) as an adhesive prior to the sowing of seeds (Dogra et al., 2019). Twenty seeds were dipped in 30 mL of a bacterial culture for 1 h and then each seed was dibbled into the soil at a depth of 1.5–2 cm in the pots with the help of forceps. Five seeds from each treatment and control were grown in one pot and were thinned to three after germination. 30 mL of saline suspension of PGPB (10^8 CFU per mL) was inoculated again after 15 days, 30 days, and 45 days in each pot with application to the soil surface at five points at 1.5 cm from the plant shoot. Pots were grown in an open greenhouse and allowed to grow till maturity. Plants were watered three to four times per week until the final harvest to maintain appropriate soil moisture. Potential water loss was minimized by placing a saucer at the bottom of each pot.

2.7. Plant growth and yield analysis

Plants were harvested when they reached the full maturity stage in mid-April 2019 at Zadoks growth stage Z99 (Zadoks et al., 1974). Plant height, total number of tillers per plant, spike length, 1000 grains weight (TGW), and the total number of grains per spike were recorded. Plants were hand thrashed to retain all the grains. Dry biomass of the whole plant was estimated by oven drying at 60 °C until it was completely dry.

2.8. Inductively coupled plasma mass spectrometry (ICP-MS) based micronutrient analysis of grains

Nutrient content of wheat grains was analyzed with inductively coupled plasma mass spectrometry (ICP-MS). Exactly 0.5 g of dried grain samples were taken and mixed in tubes designed for microwave digestion containing 10 mL of digestion solution (8 mL 70% HNO_3 and 2 mL H_2O_2) (Krachler et al., 2002). The digestion vessels were kept for 4–5 h in Milestone Ethos UP™. The digested samples were filtered through the Whatman filter paper followed by syringe filtration through a 0.45 μm filter. The filtrate was diluted 1000 times with sterile water (Becker et al., 2008). The samples were submitted to CIL (Central Instrumentation Laboratory) for ICP-MS analysis.

2.9. Nitrogen and phosphorus quantification

Nitrogen estimation in wheat grain was performed using the method described by Dogra et al. (2019). Briefly, 5 g of wheat grains were weighed per plant and digested for 3 h in Kjeldahl flask with a mixture of cupric sulphate and potassium sulphate (1:10 ratio) and 10 mL sulfuric acid. The ammonia released during the distillation process was absorbed by boric acid. In the end, the total nitrogen was calculated as per the Bureau of Indian Standards (IS 5194, 1969). Total phosphorus in wheat grain samples was estimated as described earlier (Dogra et al., 2019).

2.10. Indirect protein estimation

Indirect estimation of proteins is based on two assumptions: first, all the proteins have a nitrogen content of 16%, and second, total nitrogen content was derived from proteins. However, amino acid analysis revealed that the conversion factor of 6.25 is generally an overestimate of the protein content in most of the food due to variation in amino acid profiles and non-proteins nitrogen (Krul, 2019). However, in order to adjust these variations, several species-specific conversion factors have been suggested to make this nitrogen to protein estimation more precise (Lourenço et al., 2002; Mariotti et al., 2008). In this study, we used the average conversion factor of 5.8 for cereals, as described by Krul (2019). Wheat grain protein content was determined by an indirect method where nitrogen content (percentage) calculated by the Kjeldahl method was multiplied by a conversion factor (5.8) to estimate the total protein.

2.11. Chlorophyll content

The total chlorophyll content of wheat leaves was determined as per the method described by Arnon (1949). Briefly, 100 mg of fresh wheat leaves were homogenized with 2 ml of chilled acetone and incubated overnight at 4 °C. The total chlorophyll was calculated using the following formula: mg total chlorophyll/g = 20.2(A645) + 8.02 (A663)*V/1000*W. where A = absorbance at specific wavelength,

V = final volume of chlorophyll extract in 80% acetone W = fresh weight of plant tissue extracted.

2.12. Correlation matrix and statistical analysis

Plant growth, nutrient analysis and biochemical parameters reported in this study were subjected to two-way ANOVA (analysis of variance) (GraphPad Prism™ software) and a pair-wise *t*-test of each treatment with the control was performed. The correlation among grain yield and soil health parameters and macro and micronutrients in PGPB and AM fungi treatments was determined in wheat cultivar HD-3086 using Pearson correlation with XLStat software (Addinsoft).

3. Results

3.1. Identification and characterization of bacterial isolates with plant growth promoting attributes from diverse soils

Physicochemical properties of four soil samples from Punjab and Mizoram states of India indicate that Aizawl (Mizoram) soil was highly acidic compared to Bathinda soil (Punjab) which was alkaline with low levels of soil organic carbon and organic matter (Table A1). The two soil samples from Aizawl and one soil sample from Bathinda were rich in soil organic carbon and soil organic matter, whereas the soil sample from the Central University of Punjab (CUP) showed relatively lower organic carbon and organic matter. Phosphorus levels in the soil samples showed a similar trend. However, the soil sample from the cotton field (Bathinda) showed the highest nitrogen level, whereas the other three soil samples showed similar levels of nitrogen. Further, the dehydrogenase activity of the soil samples was the highest for Aizawl soil samples and lowest for CUP soil sample. The C:N ratio of different soil samples showed a similar trend as soil dehydrogenase activity.

Analysis of the plant growth promoting attributes of twenty-seven bacterial isolates with phosphate solubilizing activity from different soil samples identified AHP3 and AHP4 as the top two isolates in terms of phosphate solubilization (quantitative) (Table 1). A similar analysis for

Table 1
Screening of bacterial isolates for plant growth promoting traits.

Source of soil sample	Isolates	Indole acetic acid (μg/mL ± SE)	Phosphorus conc (μg/ml ± SE)	Siderophore activity
Aizawl Hill Side	AHP 1	13.71 ± 0.65	96.96 ± 0.83	–
	AHP 2	13.75 ± 0.51	53.24 ± 0.51	–
	AHP 3	12.40 ± 1.68	**101.88 ± 1.04**	+
	AHP 4	13.50 ± 0.24	**116.72 ± 1.36**	+
	AHP 5	15.04 ± 1.04	41.86 ± 0.46	–
	AHP 6	12.72 ± 0.84	46.62 ± 1.55	–
	AHP 7	9.67 ± 0.59	45.43 ± 0.26	–
	AHP 8	10.83 ± 0.25	54.84 ± 0.48	+
	AHP 9	18.15 ± 8.18	49.20 ± 0.89	–
Aizawl Road Side	ARP 1	62.21 ± 1.37	52.66 ± 1.74	–
	ARP 2	63.39 ± 1.24	39.64 ± 0.76	–
	ARP 3	10.57 ± 6.93	32.01 ± 4.43	–
	ARP 4	46.75 ± 5.87	15.30 ± 1.91	+
	ARP 5	38.11 ± 8.34	18.27 ± 9.00	–
	ARP 6	41.31 ± 13.16	36.77 ± 2.45	–
	ARP7	**71.60 ± 0.61**	36.24 ± 0.65	–
	ARP 8	**67.62 ± 0.84**	67.05 ± 0.91	+
	ARP 9	66.23 ± 2.49	21.15 ± 2.26	–
Cotton field, Bathinda	CP1	2.63 ± 0.30	24.41 ± 2.71	-
	CP2	3.14 ± 0.41	44.06 ± 0.64	–
	CP3	3.30 ± 0.36	13.89 ± 2.00	-
	CP4	47.52 ± 1.85	51.02 ± 2.51	+
	CP5	3.33 ± 0.40	28.85 ± 0.18	–
	CP6	5.35 ± 0.25	48.93 ± 1.83	+
	CP7	28.84 ± 1.18	20.41 ± 2.13	–
	CP8	35.47 ± 1.64	13.29 ± 1.36	–
	CP9	5.22 ± 0.38	29.97 ± 0.60	+

All values are the average of three replicates.

Table 2
Effect of different PGPB treatments on wheat growth and yield related parameters.

Treatments	Plant height (cm)		Dry weight/Plant #(g)		Total tillers/plant		Spike length (cm)		No. of grains/spike	
	HD-3086	HD-2967	HD-3086	HD-2967	HD-3086	HD-2967	HD-3086	HD-2967	HD-3086	HD-2967
Control	68 ± 1.03	68.5 ± 2.78	5.88 ± 0.2	5.15 ± 0.2	2.33 ± 0.2	1.83 ± 0.31	12.97 ± 0.15	12.67 ± 0.66	52.33 ± 0.92	50.83 ± 0.4
ARP8	74.3 ± 3.3*	72 ± 1.88	6.83 ± 0.23	7 ± 0.28*#	3.33 ± 0.33*	2.33 ± 0.21	13.36 ± 0.29	14.2 ± 0.77*	55 ± 0.63	55.17 ± 0.6*
CP6	77.3 ± 0.67*	75.3 ± 0.49*	7.84 ± 0.19*#	7.36 ± 0.2*#	3.67 ± 0.21*	2.83 ± 0.3*	14.55 ± 0.1*#	15.58 ± 0.5*#	61.83 ± 0.4*#	59 ± 0.37*#
CP4	**78.7 ± 1.4*#**	77.2 ± 1.1*#	**8.25 ± 0.18*#**	**7.76 ± 0.2*#**	**4 ± 0.26*#**	**3.17 ± 0.48*#**	14.62 ± 0.3*#	15.08 ± 0.5*#	63.5 ± 0.5*#	60.33 ± 1.1*#
VAM	68.3 ± 2.14	69.17 ± 2.57	6.06 ± 0.14	6.49 ± 0.3*	2.5 ± 0.43	2.5 ± 0.22	13.15 ± 0.23	13.8 ± 0.47	58.67 ± 0.9*#	55.33 ± 0.6*#
AHP4	71.17 ± 1.54	69 ± 1.26	6.65 ± 0.24	7.06 ± 0.2*#	2.33 ± 0.49	1.83 ± 0.3	13.73 ± 0.3	14.46 ± 0.6*	60.5 ± 0.67*#	58.33 ± 0.2*#
AHP3	69.15 ± 1.95	71.8 ± 1.76	7.43 ± 0.17*#	7.16 ± 0.2*#	3.5 ± 0.43*	2.83 ± 0.17*	14.4 ± 0.23*#	14.6 ± 0.23*	60.83 ± 0.8*#	59.5 ± 0.92*#
PR30	69.3 ± 3.4	69.8 ± 0.7	6.19 ± 0.37	6.1 ± 0.39	2.5 ± 0.34	2 ± 0.26	13.07 ± 0.34	13.9 ± 0.86	58.33 ± 0.3*#	56.33 ± 0.56
PR29	74.5 ± 2.45	75.8 ± 2.2	5.97 ± 0.22	5.6 ± 0.3	2.67 ± 0.33	2.5 ± 0.22	13.04 ± 0.41	13.83 ± 0.83	56.83 ± 0.9*	55.5 ± 1.02*
RA6	69 ± 1.65	69.24 ± 0.95	6.68 ± 0.34	6.27 ± 0.23*	2.5 ± 0.22	2.17 ± 0.3	13.35 ± 0.24	14.38 ± 0.5*	58.17 ± 0.54*	54.5 ± 0.34*
ARP8 + VAM	72.33 ± 0.88*	73.7 ± 1.84	7.13 ± 0.23*#	7.26 ± 0.2*#	2.83 ± 0.17*	3 ± 0.37 *	13.2 ± 0.32	13.2 ± 0.44	58.5 ± 0.85*#	56.17 ± 0.8*#
CP6 + VAM	75.8 ± 1.6*	71.8 ± 1.8	7.6 ± 0.35*#	7.37 ± 0.3*#	3.5 ± 0.3*#	2.67 ± 0.2*	14.28 ± 0.3*#	15.57 ± 0.5*#	60.83 ± 0.4*#	58.5 ± 0.85*#
CP4 + VAM	78.5 ± 0.9*#	**78 ± 2 *#**	8.16 ± 0.14*#	7.65 ± 0.2*#	**4 ± 0.37*#**	**3.17 ± 0.3*#**	**14.96 ± 0.6*#**	**16.35 ± 0.5*#**	**63.83 ± 0.4*#**	**60.17 ± 0.3*#**
AHP4 + VAM	70.5 ± 3.66*	68.8 ± 1.05	7.26 ± 0.29*#	7.3 ± 0.1*#	3.33 ± 0.4*	2.83 ± 0.3*	14.3 ± 0.44*	15.58 ± 0.6*#	61.33 ± 0.8*#	56 ± 0.63*
AHP3 + VAM	76.17 ± 1.6*	70.5 ± 1.1	7.55 ± 0.27*#	7.13 ± 0.26*#	3.83 ± 0.3*#	2.33 ± 0.33	14.03 ± 0.18*	14.34 ± 0.38*	60.17 ± 0.6*#	59.33 ± 0.5*#
PR30 + VAM	74.3 ± 1.74*	75 ± 1.8	6.66 ± 0.34	6.08 ± 0.3	2.67 ± 0.22	1.83 ± 0.17	12.34 ± 0.28	12.98 ± 0.75	58.33 ± 0.42	50 ± 0.45*
PR29 + VAM	74.5 ± 1.54*	72.3 ± 1.1	6.8 ± 0.3	6.23 ± 0.15	2.5 ± 0.22	2 ± 0.26	14.1 ± 0.68	14.3 ± 0.64	58.67 ± 0.56	54.83 ± 0.3
RA6 + VAM	68.8 ± 4.6	72.17 ± 2.3	6.96 ± 0.27	6.21 ± 0.2	2.5 ± 0.17	2.33 ± 0.2	13.99 ± 0.27	14.63 ± 0.2*	56.67 ± 0.76	57.5 ± 0.72*

The value shown is Mean ± Standard error, n = 3; *and # represent p value < 0.05 using *t*-test and ANOVA, respectively for comparisons of treatments with control.

the production of IAA identified ARP 7 and ARP8 as the top two isolates. Siderophore activity was detected in eight isolates, including AHP3, AHP4, ARP7, and ARP8. The majority of siderophore producing bacteria also showed the ability for phosphate solubilization and IAA production. Two isolates, CP4 and CP6 showed the best phosphate solubilization activity among the nine isolates analyzed from Bathinda cotton field soil. Based on the above analysis, five potential PGPB isolates, ARP8, AHP3, AHP4, CP4, and CP6 with phosphate solubilization activity, IAA and siderophore production were selected for testing their effects on wheat plants grown in pots in open greenhouse. Sequencing of 16S ribosomal DNA and BLAST analysis revealed that three of the isolates, AHP3, ARP8, and CP4 belong to *Bacillus* sp. and the other two, AHP4 and CP6 belong to *Paenibacillus* sp. and *Staphylococcus* sp., respectively, based on the best match in NCBI GenBank (Table A2). The sequences were deposited in NCBI GenBank with accession numbers MN607034 (ARP8), MN607032 (AHP3), MN607033 (AHP4), MN607035 (CP4), and MN607036 (CP6).

3.2. Plant growth and yield-related parameters enhance by specific combinations of microbial inoculation

Five bacterial isolates described above and three other isolates, PR29, PR30, and RA6, which showed plant growth promoting activity in other studies/locations, were used alone and in combination with VAM for comparison to test their effects on the wheat cultivars, HD-3086 and HD-2967. Plants treated with the isolates, CP4, CP6, and ARP8 alone and in combination with VAM, showed a significant plant height increase in wheat cultivar HD-3086, whereas in the case of HD-2967 treated with CP4 alone and in combination with VAM, showed significant increase compared to the control plants (Table 2). The highest increase (12%) in plant height was observed in HD-3086 plants (78.7 cm) treated with CP4. In comparison, only 8% increase was seen in HD-2967 (78 cm). CP4 + VAM treated plants showed 12% increase in plant height compared to the control in both HD-3086 and HD-2967. A few other treatments showed significant increase in plant height when used alone or in combination with VAM. The next step was to determine the dry biomass of the plant at the harvesting stage. The highest increase in dry biomass was recorded in plants treated with CP4 alone in both HD-3086 and HD-2967. CP4 showed 50% increase in HD-2967 (7.76 g), and 40% increase in HD-3086 (8.25 g) compared to the control. Other single and combined treatments with VAM, which showed a significant increase in plant height, include CP6 and AHP3 in both HD-3086 and HD-2967 and ARP8 and AHP4 in only HD-2967.

The number of tillers per plant is an important yield trait for wheat. The total number of tillers per plant (TTP) showed a significant increase in plants treated with CP4 and CP6 alone and in combination with VAM in HD-3086 (72%) and HD-2967 (73%) compared to the control (Table 2). In the case of HD-3086, plants treated with ARP8 and AHP3 alone and in combination with VAM also showed a significant increase compared to the control. Treatments with CP4 alone and in combination with VAM resulted in 72% and 73% increase in the number of tillers per plant in HD-3086 and HD-2967, respectively. HD-2967 and HD-3086 plants treated with CP6 AHP3 and CP4 alone and in combination with VAM, showed a significant increase in spike length as compared with control plants with the highest increase of 29% recorded in CP4 + VAM treated HD-2967 and 15% in HD-3086.

Thousand grain weight (TGW) is an important agronomic trait for crop productivity and reflects the final yield of a crop. ARP8, CP6, CP4 and AHP4 alone and in combination with VAM treated plants showed a significant increase in TGW in both HD-2967 and HD-3086 (Fig. 1). The highest increase of 40.5% in TGW was recorded in plants treated with CP4 + VAM in HD-3086 (43.3 g) and 34.8% increase in TGW in HD-2967 (40 g). The average number of grains/spike showed a significant increase in CP6, CP4, AHP3, and AHP4 treated (alone and in combination with VAM) HD-2967 and HD-3086 where CP4 + VAM showed a maximal increase of 22% in HD-3086 and 18.4% in HD-2967 (Table 2). In general, plants treated with PR30, PR29, and RA6 alone and in combination with VAM did not show a significant increase in most parameters related to yield.

3.3. Macro and micronutrient enhancement of wheat by microbial inoculation

Macronutrients (Ca, K, Mg, N, P, S) are needed in higher quantities and micronutrients (B, Cl, Cu, Fe, Mn, Zn) are needed in lower quantities for optimal plant growth. Nitrogen (N) content in grains showed significant increase in CP6, CP4, and AHP3 treatments alone and in combination with VAM in both varieties (Fig. 2). A similar observation was made in the case of AHP4 in HD-2967. The highest increase was recorded in CP4 + VAM inoculated HD-2967 (112%) reaching 2.7% and HD-3086 (137%) reaching 3.43% compared to the control. Nitrogen content of HD-3086 was 14% higher compared to HD-2967 control plants. This difference doubled to 27% in CP4 + VAM treated plants.

The total protein content was estimated using the nitrogen protein conversion factor of 5.8 for wheat. The increase in the protein content in various treatments showed the same pattern as the nitrogen content with the highest increase in CP4 + VAM treated plants compared to the control in both cultivars reaching a maximum of 19.89% in HD-3086 (Table A3). A significant increase in wheat grain phosphorus (P) content was observed in CP6, CP4, AHP4, and AHP3 treatments alone and in combination with VAM in both varieties (Table 3). The highest increase in phosphorus was recorded in CP4 + VAM inoculated HD-3086 (79%) and HD-2967 (70%). Phosphorus levels in grains were 20% higher in control plants of HD-2967 compared to HD-3086. The phosphorus levels increased in CP4 + VAM treated plants to similar levels in both HD-2967 (1.07%) and HD-3086 (0.99%). Calcium levels of wheat cultivar HD-3086 showed significant increase with CP6, CP4, and AHP4 treatments alone and in combination with VAM. The highest increase in calcium concentration (492.6 mg/kg) was recorded in HD-3086 inoculated with CP4 + VAM (60%). However, CP4 treatment alone increased calcium by only 29%. In the case of HD-2967, the only isolate which showed a significant increase in calcium when inoculated alone (452 mg/kg) and in combination with VAM (42%) was AHP3 (468 mg/kg). CP4 + VAM showed an increase of 40% in calcium level compared to the control.

Iron and zinc are key micronutrients for the biofortification of grains. HD-3086 treated with ARP8, CP6, CP4, AHP4, and AHP3 alone and in combination with VAM, showed a significant enhancement in iron level compared to the control plants (Table 3). The highest increase in iron level (25 mg/kg) was recorded in CP4 + VAM (62.5%) treated HD-3086 plants. In comparison, only CP4 treatment alone and in combination with VAM, showed a significant increase in iron with maximum increase recorded in CP4 + VAM (37.4%) treated HD-2967 (25.6 mg/kg). The pattern of accumulation of iron in grains in control plants of HD-2967 (20% higher) compared to HD-3086 and their similar levels in CP4 + VAM treated plants of both HD-2967 and HD-3086 was similar to that of phosphorus. Zinc levels increased significantly in both varieties treated with CP6 and CP4 alone and in combination with VAM. The best values with 38% increase in HD-3086 (50.7 mg/kg) and 53% increase in HD-2967 (55.5 mg/kg) were recorded for CP4 + VAM treatment. In addition to common observations for both varieties, AHP4 in the case of HD-3086 and ARP8 and AHP3 in the case of HD-2967 showed a significant increase in zinc levels when inoculated alone and in combination with VAM. Similar levels of zinc were recorded in control plants, whereas approximately 10% higher values were recorded in CP4 + VAM treated HD-2967 compared to HD-3086. Boron levels showed a significant increase in ARP8, CP4, CP6, AHP3, and AHP4 alone and in combination with VAM in both HD-2967 and HD-3086. PR30 and RA6 alone, as well as PR30 and PR29 in combination with VAM, showed a significant increase in HD-2967. The highest boron levels in grains of CP4 + VAM treated plants were observed to be 3.4 and 2-fold in HD-3086 and HD-2967, respectively. Boron levels in

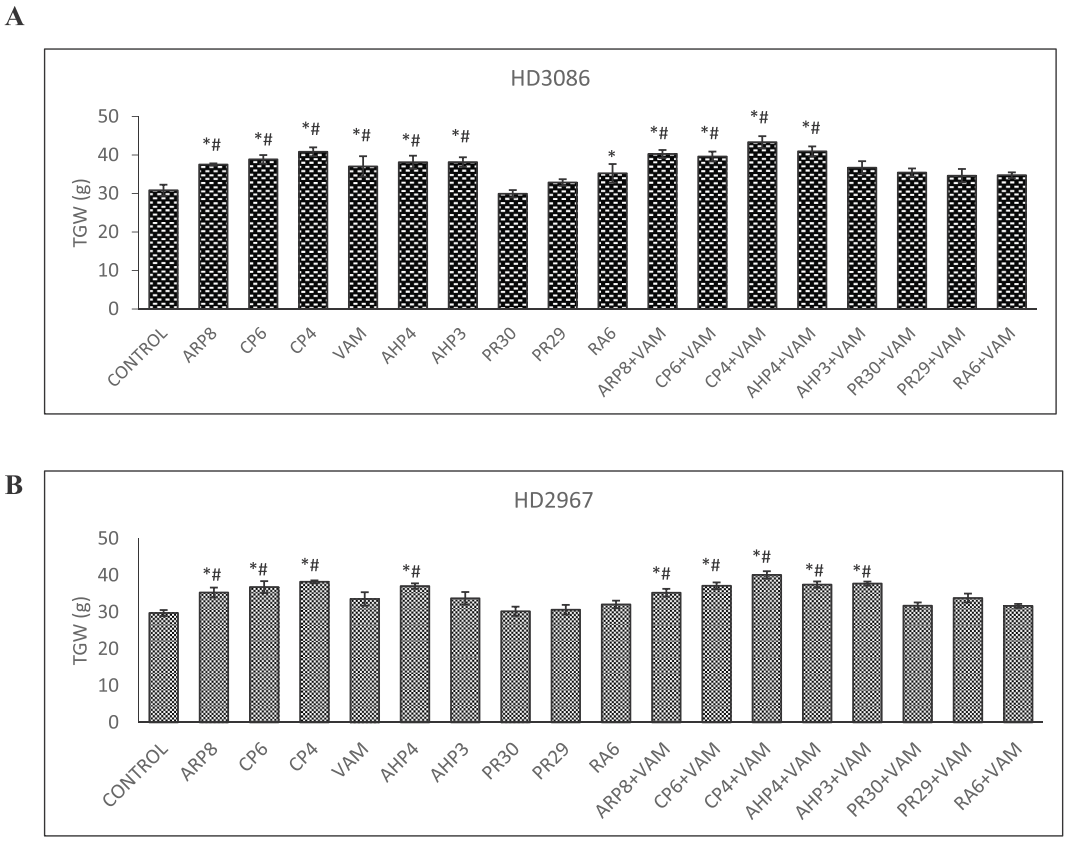

Fig. 1. Thousand grain weight of wheat varieties inoculated with different combinations of bacteria and VAM. A. HD-3086 B. HD-2967. Values are shown as Mean ± Standard error, n = 3; * and # indicate statistically significant differences between treatment and control based on *t*-test and ANOVA test, respectively.

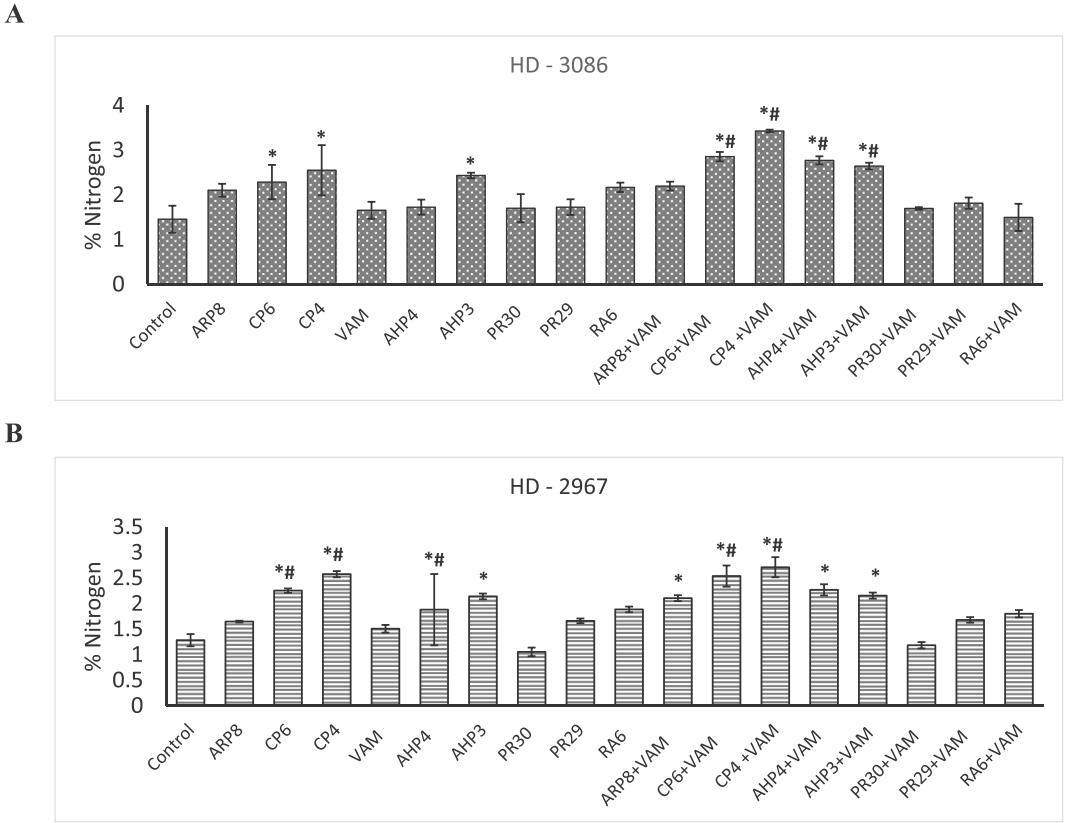

Fig. 2. Nitrogen content of grains of wheat varieties inoculated with different combinations of bacteria and VAM. A. HD-3086 B. HD-2967. Values are shown as Mean ± Standard error, n = 3; * and # indicate statistically significant differences between treatment and control based on *t*-test and ANOVA test, respectively.

Table 3
Macro and micronutrient levels of wheat grains.

	% P		Ca mg/kg		Zn mg/kg	
	HD-3086	HD-2967	HD-3086	HD-2967	HD-3086	HD-2967
Control	0.55 ± 0.01	0.636 ± 0.01	306.46 ± 19.47	330.56 ± 38.33	36.80 ± 2	36.3 ± 2
ARP8	0.716 ± 0.02	0.730 ± 0.001	391.95 ± 58.72	399.41 ± 39.65	38.26 ± 1.2	41.7 ± 1.6*
CP6	0.796 ± 0.02*#	0.96 ± 0.014*#	371.24 ± 14.66*	392.40 ± 14.8	44.70 ± 3.2*	43.0 ± 1.5*
CP4	0.868 ± 0.02*#	1.022 ± 0.02*#	396.81 ± 35.47*	361.57 ± 16.1	44.97 ± 0.2*	40.7 ± 1.1*
VAM	0.623 ± 0.03*	0.7 ± 0.02*	366.21 ± 15.77*	366.73 ± 46.5	37.28 ± 0.9	36.7 ± 3.2
AHP4	0.718 ± 0.03*#	0.776 ± 0.02*#	408.06 ± 27.82*	403.17 ± 41.75	40.67 ± 2.6*	41.1 ± 0.9
AHP3	0.720 ± 0.02*#	0.943 ± 0.02*#	339.05 ± 38.46	**467.8 ± 42.7***	39.44 ± 0.8	44.7 ± 1.8*
PR30	0.646 ± 0.023*	0.692 ± 0.02*	331.20 ± 13.83	345.48 ± 17.6	37.34 ± 0.4	38.0 ± 1.9
PR29	0.678 ± 0.02*#	0.853 ± 0.01*#	308.21 ± 35.64	333.00 ± 15.12	37.67 ± 2.9	36.5 ± 1.6
RA6	0.677 ± 0.01	0.706 ± 0.02	308.28 ± 13.53	342.71 ± 17.54	36.87 ± 2.8	41.4 ± 0.3
ARP8 + VAM	0.687 ± 0.02	0.928 ± 0.02*#	429.75 ± 34.24*	401.37 ± 52.57*	37.95 ± 0.86	49.2 ± 1.3*#
CP6 + VAM	0.906 ± 0.01*#	0.938 ± 0.02*#	462.53 ± 14.3*#	458.89 ± 21.2*	46.59 ± 1.2*#	49.0 ± 3.4*#
CP4 + VAM	**0.987 ± 0.03*#**	**1.074 ± 0.02*#**	**492.56 ± 43*#**	464.12 ± 22.6*	**50.7 ± 2.5*#**	**55.5 ± 6*#**
AHP4 + VAM	0.907 ± 0.02*#	0.922 ± 0.02*#	348.62 ± 26.15*	335.33 ± 24.4	42.88 ± 0.94*	49.5 ± 1.1*#
AHP3 + VAM	0.718 ± 0.02*#	0.919 ± 0.01*#	361.16 ± 21.96	452.40 ± 30.6*	39.35 ± 2.5	52.0 ± 2.1*#
PR30 + VAM	0.713 ± 0.03	0.742 ± 0.02	314.82 ± 27.05	412.31 ± 33.48	42.01 ± 0.45	39.4 ± 1.2*
PR29 + VAM	0.705 ± 0.03	0.547 ± 0.02	326.78 ± 20.62	366.12 ± 13.13	37.55 ± 1.6	38.2 ± 2.1
RA6 + VAM	0.588 ± 0.03	0.696 ± 0.01*	323.37 ± 25.72	374.70 ± 38.6	39.35 ± 3.8	35.2 ± 0.7

	Cu mg/kg		Fe mg/kg		B mg/kg	
	HD-3086	HD2967	HD-3086	HD-2967	HD-3086	HD-2967
Control	29.67 ± 1.6	23.88 ± 1.4	15.36 ± 0.57	18.66 ± 2.55	9.72 ± 0.27	9.48 ± 0.09
ARP8	30.03 ± 1.7	**31.03 ± 3.04**	21.43 ± 1.39*	22.61 ± 2.48	12.74 ± 0.27*#	10.81 ± 0.72#
CP6	36.19 ± 0.4*	28.46 ± 2.19	22.38 ± 1.01*#	22.13 ± 3.09	15.54 ± 0.13*#	11.99 ± 0.26*#
CP4	34.80 ± 1.9*	26.19 ± 1.03*	24.19 ± 1.92*#	24.43 ± 0.75*	21.50 ± 0.62*#	16.15 ± 0.08*#
VAM	29.90 ± 1.5	25.05 ± 1.88	19.79 ± 0.97*	22.49 ± 2.27*	13.74 ± 0.31*#	9.70 ± 0.44
AHP4	32.78 ± 2.4	25.87 ± 1.15	23.01 ± 0.99*#	20.87 ± 3.15	17.32 ± 0.33*#	13.09 ± 0.45*#
AHP3	32.21 ± 2.7	25.12 ± 1.88	21.94 ± 2.38*#	20.54 ± 1.76	16.39 ± 0.6*#	14.82 ± 0.48*#
PR30	31.49 ± 6.7	25.69 ± 1.78	16.22 ± 2.54	19.43 ± 0.43	12.23 ± 0.84	11.89 ± 0.11*#
PR29	30.15 ± 1.4	24.72 ± 2.51	15.86 ± 1.08	19.06 ± 1.52	12.47 ± 0.11	9.73 ± 0.46
RA6	30.39 ± 4.2	25.78 ± 0.6	17.54 ± 0.39	22.36 ± 3.01	11.50 ± 0.43	13.27 ± 0.2*#
ARP8 + VAM	31.10 ± 3.7	25.00 ± 1.5*	23.94 ± 0.82*#	21.98 ± 2.03	24.47 ± 0.44*#	10.25 ± 0.57
CP6 + VAM	37.25 ± 0.5*	28.34 ± 3.5	24.38 ± 2.40*#	23.28 ± 2.84*	20.33 ± 0.67*#	14.78 ± 0.11*#
CP4 + VAM	**39.94 ± 1.3***	**31.02 ± 3.1***	**24.97 ± 0.2*#**	**25.64 ± 0.3***	**32.55 ± 0.36*#**	**18.82 ± 0.34*#**
AHP4 + VAM	30.91 ± 0.88	26.79 ± 2.6	21.17 ± 1.91*	22.05 ± 1.4	25.22 ± 0.6*#	15.30 ± 0.22*#
AHP3 + VAM	37.50 ± 3.1*	28.50 ± 5.3	22.34 ± 1.30*#	23.18 ± 1.5*	29.28 ± 1.1*#	17.26 ± 0.41*#
PR30 + VAM	30.12 ± 2	25.20 ± 4.7	14.04 ± 1.51	21.92 ± 1.9	12.39 ± 0.22*	14.35 ± 0.41*#
PR29 + VAM	30.12 ± 1.3	25.53 ± 1.5	16.67 ± 0.99	19.23 ± 1	10.65 ± 0.61	13.39 ± 0.54*#
RA6 + VAM	32.51 ± 4.6	25.95 ± 0.9	19.34 ± 0.72*	18.89 ± 1.9	11.73 ± 0.42	11.01 ± 0.41

Data show concentration of B, Ca, Cu, Fe and Zn in mg/kg dry matter; % P - Phosphorus content of dry matter; Mean ± Standard error, n = 3.

Table 4
Chlorophyll content of wheat leaves.

	Total Chlorophyll (mg g^{-1})	
	HD-2967	HD-3086
Control	0.76 ± 0.08	0.80 ± 0.03
ARP8	1.00 ± 0.05*	1.03 ± 0.32
CP6	0.97 ± 0.08*	0.96 ± 0.14#
CP4	1.02 ± 0.18*#	1.11 ± 0.13*#
VAM	1.01 ± 0.07	0.92 ± 0.58
AHP4	0.99 ± 0.62*#	1.30 ± 0.44*#
AHP3	0.93 ± 0.57*#	**1.46 ± 0.37*#**
PR30	0.97 ± 0.02	1.08 ± 0.01*
PR29	0.84 ± 0.02	0.93 ± 0.03
RA6	0.77 ± 0.01	0.84 ± 0.01
ARP8 + VAM	0.99 ± 0.03*#	1.01 ± 0.04
CP6 + VAM	0.95 ± 0.14	1.12 ± 0.26*
CP4 + VAM	**1.11 ± 0.00*#**	**1.33 ± 0.04*#**
AHP4 + VAM	1.00 ± 0.05	0.93 ± 0.07
AHP3 + VAM	0.92 ± 0.02	1.06 ± 0.10*
PR30 + VAM	0.81 ± 0.03	0.92 ± 0.01
PR29 + VAM	0.86 ± 0.03	0.99 ± 0.18
RA6 + VAM	0.91 ± 0.33	0.87 ± 0.39

Cholorophyll content in leaves represented as Mean ± Standard error, n = 3.

grains of control plants showed similar levels, whereas their levels in HD-3086 were close to 2-fold compared to HD-2967. The only bacterial isolate that showed a significant increase in copper in both varieties of wheat when used alone and in combination with VAM was CP4 with a 35% increase in HD-3086 and a 30% increase in HD-2967. Copper levels in grains were 20% higher in control plants of HD-3086 compared to HD-2967, as well as CP4 + VAM treated plants.

3.4. Chlorophyll enhancement by microbial inoculation

Wheat cultivar HD-3086 treated with ARP8, CP4, CP6, AHP3, AHP4, ARP8 + VAM, and CP4 + VAM showed a significant increase in total chlorophyll content with the highest increase of 83% in the case of AHP3 (1.46 mg/g) and 66.5% increase in CP4 + VAM treated plants compared to the control (Table 4). A similar observation was made in the case of wheat cultivar HD-2967, with the highest increase of 47.4% in plants treated with CP4 + VAM (1.11 mg/g) compared to the control plants. The effect of PGPB inoculation was more promising in the case of HD-3086 as compared to HD-2967. Both cultivars treated with CP4, CP6, AHP3, AHP4, and CP4 + VAM showed a significant increase in total chlorophyll content compared to the control plants. These bacterial isolates are more effective in terms of total chlorophyll content leading to higher photosynthesis.

3.5. Improvement of soil health parameters by microbial inoculation

There was a significant improvement in soil health parameters after harvesting the plants compared to the soil samples before sowing the seeds. It was observed that all the soil samples inoculated with PGPB isolates increased soil organic content and soil organic matter. A significant increase in SOC and SOM was recorded in soil inoculated with CP4 and AHP4 alone in the case of HD-3086 and CP4, CP6, and ARP8 alone in the case of HD-2967 compared to the control soil where plants were grown without inoculation (Table 5). When inoculated in combination with VAM, CP4, CP6, and AHP4 showed a significant increase in both HD-3086 and HD-2967, whereas AHP3 and ARP8 showed a significant increase in only HD-3086. The maximal increase in SOC was recorded in soil treated with CP4 + VAM in the case of HD-3086 (54%) and AHP4 + VAM (45%) treated soil in the case of HD-2967.

Soil enzyme activity is a parameter used to assess soil health. Soil dehydrogenase activity is used as an index of endogenous respiration in soil. Soil dehydrogenase activity at the harvest stage increased in soil inoculated with ARP8, CP4, CP6, AHP3, and AHP4 alone and in combination with VAM in both cultivars (Table 5). The best values for soil dehydrogenase activity were recorded for soil where HD-3086 (7.04 μg TPF/g) and HD-2967 (6.67 μg TPF/g) were grown with AHP3, which showed a 2.7-fold increase compared to soil with non-inoculated plants.

Pearson correlation analysis in HD-3086 identified a significant positive correlation among thousand grain weight and total number of tillers per plant (yield parameter), soil dehydrogenase activity and organic carbon (soil health parameters), nitrogen, phosphorus and calcium (macronutrients), iron, zinc, copper and boron (micronutrients) in different microbial treatments except for soil dehydrogenase activity with phosphorus and zinc and nitrogen with boron (Table 6). In comparison, 42 out of 55 comparisons showed a significant positive correlation in the case of HD-2967 with an increase in thousand grain weight, nitrogen, and iron positively correlated with all other variables. The other end of the spectrum was the total number of tillers per plant, which showed a significant positive correlation in five out of ten comparisons. In summary, the combination of CP4 and AM fungi gave the best results with reference to wheat grain yield, protein and nutrient content.

4. Discussion

Rice and wheat are the two staple cereal crops, which represent an important source of nutrition for people all over the world, especially developing countries (FAO, 2019). Significant efforts are required to fortify wheat and rice with selected macro and micronutrients to ameliorate the malnutrition from developing countries. Plant growth promoting bacteria can contribute towards this goal. Native bacteria have been hypothesized to perform better than non-native bacteria based on previous studies that have shown multiple benefits in terms of reduced use of harmful chemical fertilizers, enhanced plant growth, and bio-fortification of agriculturally important crops inoculated with native PGPB (Rana et al., 2012, 2015; Dogra et al., 2019). Wheat and other crops treated with various combinations of PGPB and VAM resulted in different effects ranging from biomass increase to nutrient uptake in shoots and grains (Dhawi et al., 2015, 2016; Prasanna et al., 2016; Dogra et al., 2019; Yadav and Ramakrishna, 2019). Enhancement in micronutrients in the plant due to PGPB inoculation can lead to efficient metabolic activities which in turn has positive effect on plant growth (Li et al., 2014; Dhawi et al., 2016). Endophytes and rhizospheric microorganisms possess several mechanisms to enhance the uptake of micronutrients in plant tissues and stimulation of plant growth by the secretion of phenolic-like substances, siderophores, organic acids in the root exudates, eliciting modification in root morphology and anatomy of crop plants, and the production of signaling molecules such as auxin and gibberellic acid (Khare et al., 2018; Pankievicz et al., 2019). The present study is a significant step towards understanding the role of native and non-native PGPB alone and in combination with AM Fungi on yield, plant growth, and biofortification of nutrients in wheat grain. Our results emphasize the integration of AM fungi and native PGPB to enhance crop yield, nutrient content in grains, and total chlorophyll.

A meta-analysis to study the effect of AM fungi on seven cereal crops, including wheat, identified a 16% increase in yield in AM fungi inoculated plants (Zhang et al., 2019). A recent study does not recommend managing AM fungi in farmland due to a lack of clear evidence of their benefits in field studies to improve crop yield (Ryan and Graham, 2018). Our study showed marginal improvement in yield and plant nutrition parameters when inoculated with AM fungi alone, which is largely in agreement with the above studies. However, a significant improvement was observed in wheat yield and nutrient content when wheat cultivars were inoculated with AM fungi in combination with PGPB which may be due to the allocation of more nutrients to reproductive tissues as a result of a three-way interaction among plant,

Table 5
Soil organic carbon and soil organic matter after harvesting.

	% Soil organic carbon		% Soil organic matter		Soil dehydrogenase activity (μgTPF/gram soil/24hr)	
	HD-3086	HD-2967	HD-3086	HD-2967	HD-3086	HD2967
Control	0.77 ± 0.08	0.751 ± 0.123	1.33 ± 0.14	1.295 ± 0.212	2.56 ± 0.37	2.50 ± 0.16
ARP8	0.98 ± 0.13	0.991 ± 0.091*	1.62 ± 0.20	1.708 ± 0.120*	6.40 ± 0.12*#	6.61 ± 0.13*#
CP6	0.90 ± 0.01	0.944 ± 0.027*	1.55 ± 0.02	1.628 ± 0.100*	5.61 ± 0.23*#	4.76 ± 0.05*#
CP4	1.09 ± 0.05#	1.011 ± 0.102*	1.93 ± 0.05*#	1.743 ± 0.221*	5.95 ± 0.40*#	5.48 ± 0.03*#
VAM	1.01 ± 0.09	0.958 ± 0.115	1.75 ± 0.16#	1.651 ± 0.199	5.44 ± 0.44*#	3.06 ± 0.13
AHP4	1.14 ± 0.13*#	0.938 ± 0.064	1.91 ± 0.21#	1.617 ± 0.111	5.17 ± 0.18*#	5.61 ± 0.20*#
AHP3	0.85 ± 0.12	0.785 ± 0.074	1.45 ± 0.20	1.353 ± 0.128	**7.04 ± 0.32*#**	**6.67 ± 0.17*#**
PR30	0.90 ± 0.09	0.845 ± 0.225	1.47 ± 0.10	1.456 ± 0.388	2.88 ± 0.32	3.95 ± 0.12
PR29	0.96 ± 0.07	0.838 ± 0.060	1.60 ± 0.08	1.445 ± 0.103	2.19 ± 0.08	4.47 ± 0.09
RA6	0.79 ± 0.07	0.984 ± 0.082	1.42 ± 0.08	1.697 ± 0.141	4.03 ± 0.68	3.59 ± 0.25
ARP8 + VAM	1.02 ± 0.04*	0.891 ± 0.131	1.71 ± 0.04*	1.536 ± 0.226	5.04 ± 0.56*#	4.29 ± 0.19*#
CP6 + VAM	1.10 ± 0.03*#	0.984 ± 0.088*	1.86 ± 0.02*#	1.697 ± 0.152*	4.68 ± 0.36*#	5.70 ± 0.17*#
CP4 + VAM	**1.19 ± 0.14*#**	0.991 ± 0.165*	**2.00 ± 0.23*#**	1.708 ± 0.284*	5.69 ± 0.26*#	6.05 ± 0.16*#
AHP4 + VAM	1.10 ± 0.12*#	**1.091 ± 0.073*#**	1.82 ± 0.16*#	**1.880 ± 0.011*#**	5.86 ± 0.45*#	4.39 ± 0.15*#
AHP3 + VAM	1.06 ± 0.06#	0.924 ± 0.153	1.85 ± 0.10*#	1.594 ± 0.264	7.00 ± 0.20*#	6.21 ± 0.04*#
PR30 + VAM	0.81 ± 0.10	0.851 ± 0.088	1.43 ± 0.16	1.467 ± 0.152	3.23 ± 0.46	3.59 ± 0.29
PR29 + VAM	0.81 ± 0.12	0.978 ± 0.094	1.35 ± 0.19	1.685 ± 0.163	3.00 ± 0.70	4.52 ± 0.08
RA6 + VAM	0.80 ± 0.07	0.958 ± 0.208	1.36 ± 0.12	1.651 ± 0.358	4.73 ± 0.17*#	3.53 ± 0.13

Values are Mean ± Standard error, n = 3; * and # represent p value < 0.05 using *t*-test and ANOVA, respectively for comparisons of treatments with the control; TPF - Triphenylformazan.

Table 6
Pearson correlation among macro and micronutrients, yield and soil health parameters in wheat varieties HD-3086 and HD-2967.

Variables	TPF	SOC	N	P	Ca	Fe	Zn	Cu	B	TGW	TTP
TPF		*0.22(0.36)*	***0.60(0.008)***	***0.58(0.01)***	***0.71(0.001)***	***0.48(0.043)***	***0.59(0.01)***	***0.66(0.003)***	***0.54(0.02)***	***0.68(0.002)***	*0.42(0.083)*
SOC	**0.50(0.03)**		***0.54(0.02)***	*0.23(0.365)*	*0.017(0.94)*	***0.56(0.016)***	*0.35(0.015)*	***0.53(0.024)***	*0.37(0.012)*	***0.65(0.004)***	*0.37(0.129)*
N	**0.60(0.007)**	**0.49(0.03)**		***0.83(0.001)***	***0.56(0.03)***	***0.68(0.002)***	***0.73(0.001)***	***0.51(0.02)***	***0.58(0.01)***	***0.85(0.001)***	***0.81(0.001)***
P	0.42(0.081)	**0.71(0.001)**	**0.55(0.016)**		***0.52(0.02)***	***0.68(0.002)***	***0.74(0.001)***	*0.44(0.065)*	***0.56(0.015)***	***0.73(0.001)***	***0.85(0.001)***
Ca	**0.47(0.04)**	**0.70(0.001)**	**0.52(0.025)**	**0.69 (0.001)**		***0.50(0.03)***	***0.66(0.003)***	***0.52(0.027)***	*0.43(0.075)*	***0.55(0.018)***	*0.31(0.21)*
Fe	**0.79(0.001)**	**0.66(0.002)**	**0.73(0.001)**	**0.67(0.002)**	**0.86(0.001)**		***0.70(0.001)***	***0.66(0.003)***	***0.54(0.020)***	***0.80(0.001)***	***0.61(0.001)***
Zn	0.35(0.15)	**0.67(0.002)**	**0.53(0.022)**	**0.89(0.001)**	**0.70(0.001)**	**0.65(0.003)**		***0.61(0.001)***	***0.62(0.001)***	***0.78(0.001)***	***0.58(0.01)***
Cu	**0.47(0.046)**	**0.66(0.003)**	**0.60(0.007)**	**0.68(0.002)**	**0.69(0.002)**	**0.70(0.001)**	**0.80(0.001)**		*0.41(0.093)*	***0.68(0.002)***	*0.37(0.13)*
B	**0.60(0.001)**	**0.57(0.013)**	0.45(0.056)	**0.71(0.001)**	**0.70(0.001)**	**0.76(0.00)**	**0.63(0.005)**	**0.72(0.001)**		***0.66(0.001)***	*0.27(0.28)*
TGW	**0.72(0.001)**	**0.62(0.005)**	**0.62(0.006)**	**0.80(0.001)**	**0.77(0.00)**	**0.89(0.001)**	**0.73(0.001)**	**0.57(0.013)**	**0.75(0.001)**		***0.63(0.001)***
TTP	**0.70(0.001)**	**0.59(0.009)**	**0.69(0.001)**	**0.77(0.001)**	**0.56(0.015)**	**0.72(0.001)**	**0.72(0.001)**	**0.76(0.001)**	**0.72(0.001)**	**0.70(0.001)**	

Values in bold are different from 0 with a significance level alpha = 0.05; p-values are shown in parenthesis; TPF: Triphenylformazan levels indicative of soil dehdrogenase activity; TTP: Total tillers per plant; Values for HD-2967 are shown in italics.

PGPB, and AM fungi (Zhang et al., 2014). PGPB and AM fungi combination are also likely to confer better protection against pathogens, stress alleviation, and weed control (Mustafa et al., 2019; Khan et al., 2020).

The major obstacle for most of the commercially available PGPB products is their very low survival rate, probably because these non-native PGPB microbes are replaced by indigenous native microbial species (Kari et al., 2019; Santos et al., 2019). Some studies suggested that these native PGPB are better adapted to their specific natural conditions and have better fitness and higher survival rates compared to the non-native PGPB. Further, spore-producing bacteria such as *Bacillus* sp. tend to survive for a longer period in soil. Considering the above issue, we used bacterial isolates, which are native and compared their effect on wheat growth with some of the non-native isolates in open greenhouse study with un-sterilized soil. Although most of the bacteria used in the present study belonged to *Bacillus* sp., one isolate belonged to the genus, *Staphylococcus*. Although bacteria belonging to the genus, *Staphylococcus*, are generally considered to be pathogenic, several isolates belonging to *Staphylococcus*, in general and *S. epidermidis*, in particular, have been shown to be normal constituents of plant microbiome (Chaudhry and Patil, 2016). Previous studies reported the role of *S. epidermidis* in plant protection, growth, and development (Biswas et al., 2018; Alibrandi et al., 2018) suggesting that all bacteria belonging to the genus *Staphylococcus* are not pathogenic unless proven by multiple tests to prove their pathogenicity. The bacterial isolate CP6 used in the present study did not show beta type of hemolysis when grown on sheep blood agar, and test for the presence of three genes, *lasB* (encoding elastase protein), *ecfX* (encoding a protein with extra-cytoplasmic function) and *ybtQ* (encoding ATPase/ABC transporter) were negative suggesting that CP6 is non-pathogenic (Sharma, 2019, unpublished data).

Yield-related parameters (thousand grain weight, average number of grains/spike, total tillers/plant) showed maximal levels in CP4 + VAM treatment based on normalized data. The increase in thousand grain weight and other related yield parameters are comparable with other studies, where the application of PGPB with different amendments was evaluated for their effect on plant growth (Rana et al., 2015; Ijaz et al., 2019). The use of biochar with a consortium of three bacterial isolates (two *Pseudomonas* sp. and one *Bacillus* sp.) and half dose of fertilizer gave the best results with grain yield increasing about 50% compared to the use of only half dose of fertilizer (Ijaz et al., 2019). The grain yield of the above treatment was equivalent to the use of full dose of fertilizer with significant cost benefit. In another study, the application of *Providencia* sp. with half dose fertilizer enhanced

grain yield approximately 25% control with only half dose fertilizer (Rana et al., 2015). Higher chlorophyll content as a result of PGPB and VAM inoculation is likely to enhance photosynthesis and plant growth and ameliorate multiple stresses (Ma et al., 2019). This may be linked to siderophore producing PGPB such as *Pseudomonas* spp., *Enterobacter* spp., and *Bacillus sporothernodurans*, which provide iron to plants, thereby enhancing chlorophyll content due to the involvement of iron in the formation of aminolevulinic acid, the precursor of chlorophyll (Pourbabaee et al., 2018).

Wheat, like many other staple cereals, contains low levels of essential micronutrients, iron and zinc, which is a serious health concern in regions where cereal-based diet is predominant (Niyigaba et al., 2019). Therefore, wheat is targeted along with other cereals in major biofortification programs to mitigate micronutrient deficiency (Wakeel et al., 2018; Zaman et al., 2018). Microbiome based bio-formulations are gaining importance in recent years in improving soil fertility, enhancement of crop yield, and bio-fortification of grains of agriculturally important crops such as wheat, maize, and rice (Prasanna et al., 2016; Garg et al., 2018). Phytoavailability of macro and micronutrients can be enhanced by beneficial microbes associated with soil and plants. Some bacteria can alter the root system in plants where the roots are thinner with more root hairs and proliferating lateral roots, which can improve the nutrient availability to plants (Verbon and Liberman, 2016). PGPB inoculants make the nutrients readily available to the plants most likely via solubilization and mineralization mechanisms (Ramakrishna et al., 2019). Different combinations of PGPB have been shown to significantly increase N, P, Fe and Zn content in wheat, which are comparable to our results (Rana et al., 2015; Dhuldhaj and Pandya, 2017). HD-3086 and HD-2967 used in this study are wheat varieties with high grain yield and are popular among farmers in North-Western India (Singh et al., 2014; Kaur, 2017). However, HD-2967 and HD-3086 are low iron accumulators, whereas HD-3086 is a low accumulator of Zn, and HD-2967 is a high accumulator of Zn (Singh et al., 2018). In our experimental conditions, the two cultivars showed different responses with reference to macro and micronutrient levels in grains. Iron levels increased to about 25 mg/kg in CP4 + VAM treated plants from much lower levels in HD-3086 than HD-2967. Although HD-2967 has been reported to be a high accumulator of Zn under field conditions with different soil and environmental conditions, our study showed similar levels of zinc in control plants of both HD-2967 and HD-3086 with approximately 10% higher levels in CP4 + VAM treated HD-2967 compared to HD-3086. A recent study has shown that *Bacillus* sp. could be potential Zn solubilizers and can enhance plant growth, yield, and Zn uptake in rice cultivars (Shakeel et al., 2015). The biological roles of most of the macro and micronutrients are well known and discussed by several authors (Maathuis, 2009; Pandey, 2018; Dogra et al., 2019). The role of boron as an essential micronutrient was highlighted by a recent report, supported by the fact that it is a constituent of plant cell wall and boron transporters exist in Arabidopsis and other plants (González-Fontes, 2019). Boron level in HD-3086 was close to 2-fold compared to HD-2967 in CP4 + VAM treated plants, which may be beneficial for plants. With reference to macronutrients, nitrogen and phosphorus are major components of chemical fertilizers applied by farmers to enhance wheat growth and yield. Wheat seeds with higher phosphorus concentration and content showed better growth with large leaves and long roots, which enhance nutrient uptake (Bilal et al., 2018; Deng et al., 2018). Higher nitrogen levels generally translate into elevated protein content in seeds. In this study, the protein concentration of grains from control plants (8.5%) was much lower than the protein concentration of 12.5% reported for HD-3086 under field conditions (Singh et al., 2014). This value reached close to 20% in grains HD-3086 plants inoculated with CP4 and VAM. As the levels of macro and micronutrients and protein concentration are based on normalized data, their total amount would be much higher when the calculations are based on per plant or per kg of wheat grain.

Soil enzymatic activity, organic carbon, pH, and microbial communities are important indicators of soil health because of their close relationship with soil biology, i.e., soil nutrient and nutrient availability to the plants and decomposition of soil organic matter and crop yield (Barone et al., 2019; Gu et al., 2019; Sekaran et al., 2019). Soil organic matter increased from 0.74% to 1% due to PGPB and farmyard manure addition in degraded soil (Kausar et al., 2018). In comparison, in the present study, soil organic matter increased by about 50%, reaching a maximum of 2%. Soil organic carbon increase mediated by PGPB contributes to carbon sequestration (Ramakrishna et al., 2020). Soil enzyme (dehydrogenase) activity more than doubled in most of the PGPB treatments indicating a significant improvement in soil health, which is an important aspect for minimizing or eliminating the use of chemical fertilizers. Stubble burning is carried out in some parts of India after harvesting paddy in order to sow wheat. This would severely affect soil health, killing most of the microbial communities close to the soil surface. The use of PGPB would enable restoration of soil health and enhance yield and biofortification of wheat plants, which are grown immediately after stubble burning. A significant correlation was observed among various grain yield parameters, soil health parameters, macro and micronutrients, especially in HD-3086, which indicates that they are working in tandem and there may be a cause and effect relationship.

Studying plant-microbe interactions using genomic, proteomic and metabolomic approaches would reveal biochemical and molecular processes underlying three-way interactions among plant, PGPB and AMF (Imam et al., 2016, 2017). Several bioinformatic tools are available, which enhance our understanding of the above processes and help in devising strategies for increasing the efficiency of biofertilizers (Chaudhary and Shukla, 2019b). Soil microbial volatiles regulate plant growth, induce systemic resistance and confer protection to biotic and abiotic stress through MAP kinases, transcription factors and hormones (Tyagi et al., 2018). Once the target genes are identified using omics approaches, gene editing tools can be used for the genetic manipulation of the microbes for enhancing the capabilities of bacteria for biological control, phytoremediation, and bioinoculation (Chaudhary and Shukla, 2019a). However, a comprehensive understanding of the effects of genetically modified bacteria on soil microbial communities is required before introducing them in the field (Ramakrishna et al., 2019).

5. Conclusion

The combined application of native PGPB and AM fungi showed superior yield-related parameters and biofortification in wheat grains compared to non-native bacteria. This data is being used for the evaluation of native PGPB and AM fungi under field conditions which are showing similar trend and will help in optimizing these microbial formulations specific to native soil microbial communities and agricultural crops, thereby reducing the intensive use of harmful chemical fertilizers by local farmers. The characterization of native PGPB for abiotic (drought, heat and salinity) and biotic (bacteria, fungi, virus and insects) stress resistance will enhance their utility for enhancing crop productivity by increasing plant growth and management of diseases and climate-related changes. The avenue to achieve this goal in future is through an integrated systems biology approach by employing omics technologies complemented with agronomical, physiological and biochemical data. The use of multitasking and efficient PGPB as part of biofertilizers to replace chemical fertilizers will be beneficial from an economic and environmental perspective.

Contributions

Radheshyam Yadav, Pankaj Ror and Parikshita Rathore performed the experiments. Radheshyam Yadav and Wusirika Ramakrishna analyzed the data and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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