Effect of Plant Beneficial Rhizobacteria on Growth and Yield of Wheat (*Triticum aestivum L.)* Grown in a sandy clay loam soil under Pot Experiment

ABSTRACT

Excessive and injudicious application of agrochemicals to optimize crop production has shown detrimental effects on microbial composition and function, soil-plant ecosystems, and human health via food chain. The microbiological strategies (phosphorus solubilization and ammonium production etc.), reduce the chemical dependency and mitigate environmental and human health risks. This study aimed to isolate and characterize plant beneficial bacteria from maize rhizospheres and asses their impact on wheat performance in pot trials. Cultures were characterized by morphological and biochemical assays. From these, two optimal cultures designated BSK1 and PS1, were selected for further investigation into their plant-growth promoting traits. The bacterial strains have ability to produce indole-3-acetic acid (IAA), siderophore, hydrogen cyanogenic and ammonium compounds along with phosphorus solubilization. Meanwhile, BSK1 demonstrated superior production levels of Indole-3-acetic acid (IAA), (203.61±2.08 µg ml^{-1}), phosphate solubilization (15 mm) and siderophores (17 mm). Furthermore, both cultures exhibited positive qualitative traits for hydrogen cyanogenic and ammonium compounds. Later identified by 16S rRNA gene sequencing as *B. subtilis* (BSK1) and *P. fluorescens* (PS1). Respectively. Subsequently, in a two-year trial, the co-inoculation of the wheat plants by *B. subtilis* with *P. fluorescens* maximally enhanced the whole plant biomass, total chlorophyll content (7.9 mg/g), grain yields (33.5 g/plant), and protein content (96.2 mg/g) at harvest. A heavy bacterial colonization onto the root rhizosphere was revealed under scanning electron microscopy (SEM) image which was significantly dense for mixed cultures of *B. subtilis* with *P. fluorescens*. The results suggest that *B. subtilis* and *P. fluorescens* expressing multiple plant growth-promoting activity can be used to produce composite bioinoculants for enhancing wheat production while reducing the use of fertilizers.

Keywords: Plant beneficial bacteria, wheat, grain yield, IAA, SEM, protein content.

INTRODUCTION

Recently, the agriculture sector is under tremendous pressure to optimize crop production to fulfil food demands of consistently growing human populations, expected to be approximately 9.7 billion by 2050 (UNDESA, 2015). The application of excessive agrochemicals in extensive

farming practices has however, led to the reduced chemical effectiveness, emergence of resistance in phytopathogens towards pesticides, and risk to human health. Moreover, the abrupt application of chemicals has shown detrimental effects on the soil-plant ecosystems, and microbial diversity also (Dincă *et al*., 2022; Sengupta *et al*., 2024). Recent research has increasingly focused on the application of cost-effective, natural resources, particularly Plant Growth Promoting Rhizobacteria (PGPR). These beneficial soil microbes colonize the root systems of plants following seed inoculation, thereby stimulating growth. The use of PGPR has the potential to diminish the dependence on chemical inputs within agricultural practices as well as produce secondary metabolites/antimicrobial compounds, which have the potential to combat phytopathogens. (Santoyo et al., 2021). Plant growth-promoting rhizobacteria (PGPR) are a group of free-living bacteria that enhance plant growth and crop yield through various mechanisms. These bacteria can produce hormones that promote plant development, facilitate nutrient availability, fix atmospheric nitrogen for example, biological nitrogen fixation (BNF) and improve soil fertility. (Sheirdil *et al.,* 2019). In addition Bioinoculants, when applied under real field conditions support plants in nutrient acquisition particularly N and P, reducing the reliance on synthetic fertilizers (Maitra *et al*., 2021). Also, the plant beneficial bacteria (PBB) belonging to genera *Bacillus, Pseudomonas, Azotobacter,* and *Burkholderia* etc. facilitates the growth and development of plants. Also, soil/plant microbiome make plants able to endure different biotic and abiotic challenges, maintain health, and consequently enhance crop yields under changing climate (Rizvi *et al*., 2021). Wheat (*Triticum aestivum* L.) is one of the most important cereal crops grown widely, accounting for 35% of the world's staple foods (Laino *et al*., 2015). India is the second largest producer of wheat worldwide, cultivated in approximately 30 million hectares, representing 14% of the world's wheat-growing area. This has led to a record-high production of 99.70 million tonnes, accounting for 13.64% of global wheat output, with a record average productivity of 3,371 kg/ha (Ramadas *et al*., 2019). Wheat stands out as the most crucial, nutrient-rich, and heavily consumed cereal globally (Arzani & Ashraf, 2017). Nutritionally, wheat supplies 1.8% fibre, 9.4% protein, 69% carbohydrates, and 2.5% fat (Ahmad *et al*., 2022). Realizing the importance of soil microbiome in wheat production, the present study was aimed at- (i) isolation, characterization, and selection of plant beneficial bacteria (ii) evaluation of plant growth promoting potentials of selected rhizosphere bacteria (iii) assessing the inoculation effect of PBB on growth, grain yield and nutrient content of wheat and (iv) determining the internalization/colonization efficiency of PBB using scanning electron microscopy (SEM) plays a vital role in elucidating plant-microbe interactions, providing high-resolution images

of microbial colonization and enables detailed imaging of microbial structures on root surfaces (Czymmek *et al* ., 2023). Plant beneficial bacteria produce auxin, ethylene, cytokinin, abscisic acid, and gibberellic acid, which significantly influence various plant physiological processes (Chauhan *et al*., 2021) . The mechanisms of plant growth promotion by various PGPR have been well documented by both direct (sequestering iron, phosphate solubilization, nitrogen fixation and production of plant hormones) and indirect mechanisms (production of HCN, induced resistance and lytic enzymes) (Sehrawat *et al*., 2022; Vocciante *et al*., 2022). Some potential bacterial candidates for biofertilizer include genera such as *Azospirillium, Pseudomonas, Bacillus, Azotobacter, Enterobacter, Burkholderia, Acinetobacter, Rhizobium, Erwinia, Flavobacterium, and Jeotgalicoccus*, etc. Another study included that inoculated maize with the bacterial strains *Bacillus polymyxa*, *Pseudomonas alcaligenes*, and *Mycobacterium phlei* reported a significant increase in root dry weight (19–52%) and increased maize total biomass by up to 38 percent (Sheirdil *et al.,* 2019). In addition, Seed coating technique has the potential to be a cost competitive and time-saving approach for crop production, reducing application efforts and providing desirable characteristics to the seeds (Yapa et al., 2021). However, we hypothesized that a promising PGPR exhibiting strong ability and effective root/rhizosphere colonizer. It is well known that plant beneficial rhizobacteria connected to plant root surfaces increase in yields of plants (Khan *et al*., 2021). The study plays an importance role in promoting food and nutrition security through its multifaceted approach. Given the vital role of the soil microbiome in enhancing wheat production.

MATERIALS AND METHODS

Isolation and characterization of plant-beneficial bacteria

The microbial cultures were isolated from maize rhizosphere, grown at the agricultural fields of the Faculty of Agricultural Sciences, Aligarh Muslim University (AMU), Aligarh, India (Latitude. 27°52′ 53″ N Longitude. 78°04′ 28″ E)**.** The soil samples were serially diluted and spread-plated on nutrient agar medium and King's B (KB) medium. The plates were incubated at 28±30 °C for 5 days and colonies differing in pigmentation were selected and streaked several times on the same medium to ensure the purity of isolates BSK1 and PS1.

Plant Growth Promoting traits

The method Bric *et al*. (1991) was used to quantitatively assess the amount of indole-3-acetic acid (IAA) produced by bacterial strains. This was accomplished by BSK1 and PS1 bacterial strains were grown in LB broth, inoculating 25 ml of LB broth containing 500 µg/ml tryptophan

with 100 µL of overnight-grown cultures (10^8 cells mL^{-1}), and then incubated for 48 hours at 28±2°C with shaking at 125 rpm. five millilitres of culture were centrifuged at 10,000 g for 15 minutes, and the two millilitres of supernatant were mixed with four millilitres of Salkowsky reagent and two to three drops of orthophosphoric acid. The samples were incubated for one hour at 28±2°C in the darkness. The amount of indole-3-acetic acid (IAA) was quantifying the absorbance of the pink colour at 530 nm using a UV-Vis spectrophotometer (UV-2450. Siderophores was qualitatively estimated by the method of (Reeves *et al*.,1983) while phosphate solubilization was determined by observing zone of solubilization (halo) around bacterial growth on solid Pikovskaya medium plates (Pikovskaya, 1948). The HCN synthesized by bacterial strains was determined the method of Bakker and Schipper (Bakker & Schippers, 1987). Whereas, the bacterial strains were grown in peptone water and incubated at 28±2 °C for four days. 1 ml of Nessler's reagent was added to each tube and the formation of a yellow color that indicated the synthesis of ammonia was using the method (Dye, 1962).

Molecular identification by 16S rRNA gene sequence analysis

The selected PBB strains, BSK1 and PS1, were identified to genus level employing different microbiological, biochemical tests, and PGP traits, and were later identified to species level by 16S rRNA gene sequence analysis. DNA Sequencing Service, Biokart India Pvt Ltd, Karnataka, India, conducted a commercial analysis of the 16S rRNA gene sequences of strains BSK1 and PS1 using universal primers, 23F (5′GGATGAGCCCGCGGCCTA3′) and 149R (5′ CGGTGTGTACAAGGCCCGG 3′). The NCBI Gen-Bank sequencing database later received nucleotide sequence data. CLUSTAL-W and MEGA11 software were used to align the 16S rRNA gene sequences for phylogenetic and molecular evolutionary analysis.

Bacterial preparation, Seed treatment, and Planting

The undamaged (healthy) wheat seeds (var. PBW343) were cleaned and surface disinfected with 70% ethanol for one minute followed by treatment with sodium hypochlorite (3%) for three minute to eliminate surface contaminants. The surface sterilized seeds were thoroughly rinsed many times with double distilled water (DDW) to remove residual surface disinfectants and shade-dried. The *B. subtilis* and *P. fluorescens* were grown in Luria broth and liquid KB medium, respectively, to a cell density of 1×10^6 and 1×10^8 cells/ml at 28±30 °C with gentle agitation for 48 h. Surface-sterilized seeds were dipped in a liquid culture medium containing an equal number of dual bacterial strains for 2 h using 10% gum Arabic as a sticker solution. Sterile and uncoated seeds soaked in sterile water served as control. The inoculated and un-

inoculated seeds (6 seeds/pot) were sown in earthen pots (22 cm high and 25 cm internal diameter) containing 4 kg unsterilized sandy clay soils pH-7.4, electrical conductivity (EC) 1.38 mmol cm^{-2}, water holding capacity (ml/g) 0.64, organic C 0.74%, (available N) 153.81 kg/ha, available, K 260 kg/ha, iron (Fe) 2.12 ppm and manganese (Mn) 0.38 ppm. Urea @ 130 kg/ha and diammonium phosphate (DAP) @ 60 kg/ha were applied to soil one day before sowing as basal dose. Wheat was sown on 15th November 2022 in the first year and then on 21st November 2023 in the second year. After germination, the seedlings were thinned and three uniform healthy wheat seedlings were retained and maintained in each pot. Three plants were maintained 15 days after emergence in each pot until harvest. The pots were watered regularly with tap water and were maintained in open field conditions (8 h light/16 h dark photoperiod for wheat) and irrigated regularly. There were 9 treatments and each treatment was replicated three times.

Plant biomass, total chlorophyll content and grain protein

All plants in nine pots for each treatment were removed at harvest (120 DAS). Roots was carefully washed and plants were dried at 80^0C in a ventilated oven (Yorco, York Scientific Industries, Pvt. Ltd. India) for 30 min. to remove moisture and whole plant biomass (gm/plant) was weighed using an electronic scale balance (BL-220 H, Shimadzu, Japan). The chlorophyll content in fresh leaves of inoculated and uninoculated wheat plants was measured before harvesting by Arnon's method (1949). The absorbance of the final solution was measured at wavelengths of 663, 645, and 652 nm, and total chlorophyll content was estimated using the formula-

$$\text{Total chlorophyll} = \frac{[20.2 \times D_{645} + 8.02 \times D_{663}] \times V}{1000 \times W}$$

The grain protein was determined using Lowry method (1951). The protein content was determined by quantifying the absorbance of the blue colour at 660 nm using a UV-Vis spectrophotometer (UV-2450, for grain protein estimation.

Root colonization assessment by scanning electron microscopy (SEM)

The colonization of wheat plant root surface by *B. subtilis*, *P. fluorescens* was examined by the SEM-EDX-elemental mapping system. The detached roots were cut 2 cm away from the root tip and fixed with 2.5% glutaraldehyde, made in PBS (pH 7.2) for 4 h at 4 ˚C. The prepared

specimen was dehydrated using a series of graded ethanol, 30–90%, for 10 min. in each solution and viewed under SEM (JEOL JSM-6510 Tokyo, Japan). **Statistical analysis**

The experimental data of wheat plants grown for two consecutive year seasons was statistically analysed employing one-way analysis of variance (ANOVA) was analysed by Duncan's multiple range test (DMRT) using SPSS 16.0 software at $P \leq 0.05$. and graph Origin2024b (10.15) software.

RESULTS AND DISCUSSION

Morphological and Molecular Characterization of PBB

Bacterial strains, BSK1, and PS1, isolated from maize rhizosphere exhibited variable morphological, biochemical, and plant growth-promoting activities (Table 1). Strains BSK1(Gram positive) and PS1(Gram negative) were long rods and showed positive reactions to citrate, indole and starch hydrolysis. Additionally, Quantitative estimation of IAA production has beneficial effects on root morphogenesis of plants, influencing growth and development. Of the two bacterial strains grown in LB medium treated with tryptophan, strain BSK1 secreted the greatest amount of IAA (203.61±2.08 µg/ml) followed by strain PS1 (182.72±1.89 µg/ml) and synthesized IAA, a useful plant hormone that has beneficial effects on root morphogenesis of plants, influencing growth and development. Both strains also demonstrated P solubilizing efficiency in a plate assay experiment, producing P solubilization zone (halo) of variable sizes, 15 mm by BSK1 and 13 mm by PS1. On CAS agar plates, the orange halo indicating siderophore activity ranged between 17 mm strain (BSK1) and 12 mm (PS1) (Table 1). Siderophore is a key component of cytochrome, heme, and non-heme proteins, and serves as a cofactor for various enzymes (Ranadev *et al.*, 2023). The release of plant growth promoting substances by PBB, observed in this study is crucial for the growth and development of plants. *Bacillus* and *pseudomonas* strains also generated HCN and NH_3 which is considered an important microbiological trait that may help protect plants from the destructive impact of phytopathogens by acting as a biological control agent. Based on the phenotypic characterization and biochemical reactions, strains BSK1 and PS1 were identified as *Bacillus* and *Pseudomonas*, respectively, which were later identified by 16S rRNA gene sequencing as *Bacillus subtilis* (BSK1) and *P. fluorescens* (PS1). The phylogenetic analysis (Fig. 1 A and B) showed a close relationship with the type strains of *Bacillus subtilis* (PQ315887) with the highest percent similarity of 93% and *Bacillus subtilis* (PP581788) exhibiting 92% similarity. The identification of bacterial strains to the species level expressing different plant growth

enhancing abilities are important because they can be used as microbiological formulations to optimize wheat production in agricultural practices.

Table: -1 Morphological, biochemical and plant growth promoting (PGP) activities of rhizosphere bacteria

Characteristics	**Bacterial strains**	
	BSK1	**PS1**
Colonial features		
Shape	Irregular	Regular
Pigmentation	Off white or pale-yellow	Fluorescent green
Margin	Dry rough	Smooth
Morphological		
Cell shape	Long rods	Long rods
Gram reaction	Positive	Negative
Biochemical properties		
Citrate utilisation	+	+
Indole	+	+
Starch hydrolysis	+	+
Plant growth promoting activities		
IAA (µg/ml)	203.61±2.08	182.72±1.89
Halo size (mm) on Pikovskaya plate	15	13
Orange halo size (mm) on CAS agar plate	17	12
HCN	+	+
Ammonium	+	+
Most significant alignment	*Bacillus* sp.	*Pseudomonas* sp.

Values are mean (±S.D.) of three replicates; IAA indicates indole acetic acid; + and - indicates positive and negative reactions, respectively.

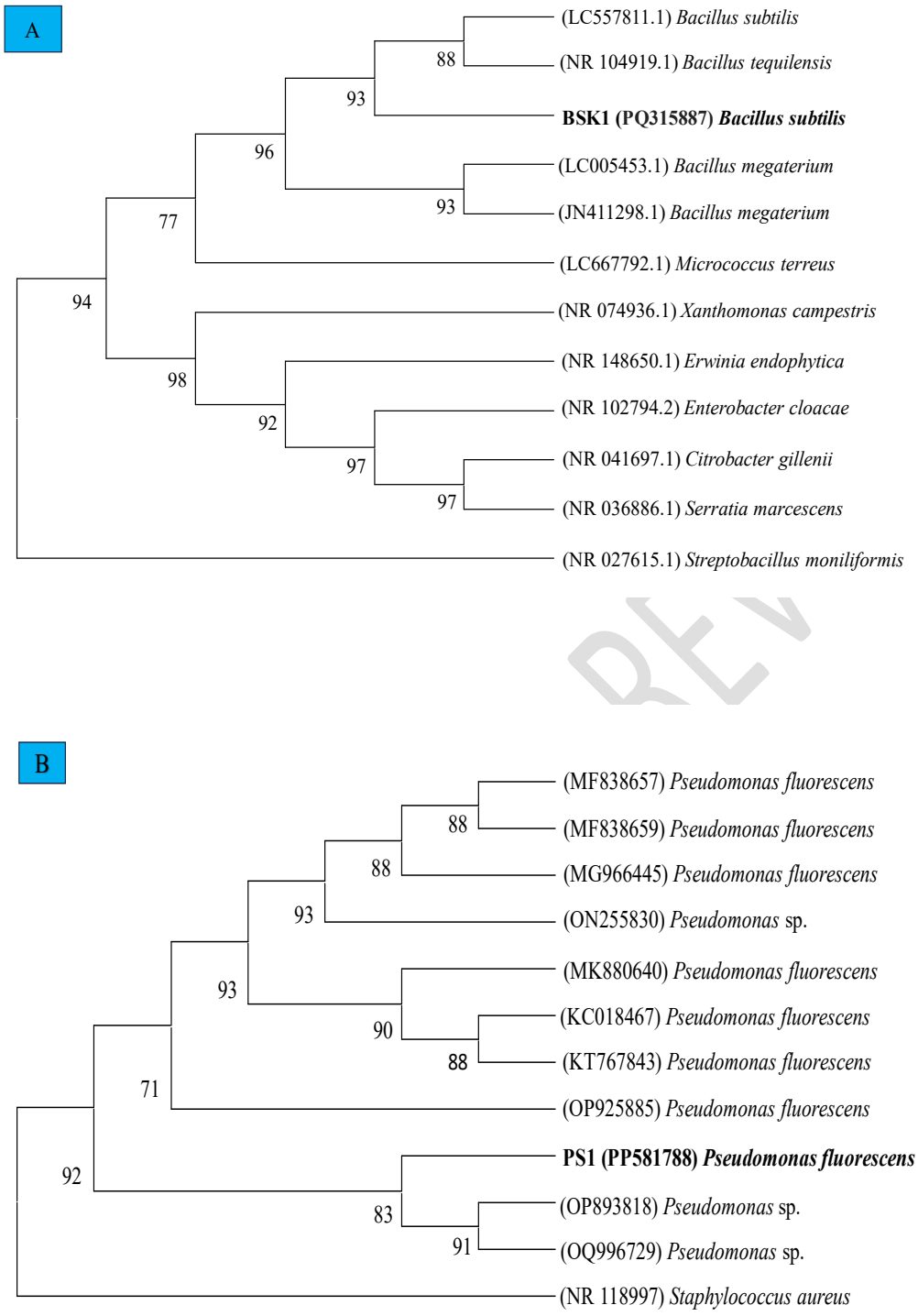


Fig. 1. Phylogenetic tree constructed from 16s rRNA sequence of *B. subtilis* (A) and *P. fluorescens* (B) (indicated by bold letters) by using Muscle alignment and MEGA 11 software

Impact of PBB on plant biomass and chlorophyll content

Due to pressure on agriculture sector to produce enough food to fulfil human food demands and the harmful impact of repeated and injudicious application of chemical fertilizers in the same fields to optimize crop production, researcher are looking for alternative to chemical fertilizers. In this regard, the soil microbiota provides solution to the expensive agrochemicals without any hazardous impact on the environment. In the present study, the impact of wheat rhizosphere PBB, *Bacillus* and *Pseudomonas,* used either alone or in combination both in the presence and absence of nitrogenous (urea) and phosphatic (DAP) fertilizers on the growth, photosynthetic pigments, and yield of wheat, raised in sandy clay loam soil was variable (Table 2). In general, the dry matter accumulation in *Bacillus* with *Pseudomonas* inoculated wheat plants, measured at harvest (120 DAS) was significantly ($P \leq 0.05$) greater than other treatments, during both cropping seasons. Among all treatments, the stimulatory effect on plant biomass was observed maximum for dual application of *B. subtilis* with *P. fluorescens* both in 2022 (21.3 g/plant) and 2023 (25.2 g/plant) which could be due to IAA secretion by the PBB. The IAA promotes biomass production, root exudation, and root elongation (Ansari *et al*., 2023). Even-though the nitrogenous and phosphatic fertilizers used alone enhanced the whole plant biomass but it was comparatively lower than those observed for inoculated plants but was higher than uninoculated plants. The composite culture of *B. subtilis* and *P. fluorescens* significantly enhanced the whole plant biomass by 22 % (2022) and 32 % (2023) relative to the mixed application of urea and DAP. Additionally, Chlorophyll formation in plants is an important physiological process for biomass production, supporting growth and development of plants. The application of PBB has been reported to increase the photosynthetic pigments in many crops (Michalak *et al*., 2021; Jalal *et al*., 2023). With this background, the effect of selected PBB on chlorophyll formation in wheat foliage was assessed. The effects of bacterial inoculation or fertilization on the photosynthetic pigments of wheat plants differed considerably. In general, the application of bacterial strains used either alone or as dual cultures, mitigated the negative impact of chemical fertilizers, enhancing chlorophyll formation in wheat foliage. The chlorophyll content was increased from 0.8 mg/g (control) to 3.2 and 2.6 mg/g in 2022 and from 1.7 mg/g (control) to 4.2 in 2022 and 3.6 mg/g in 2023 following *Bacillus* and *Pseudomonas* inoculation, respectively in fresh wheat foliage. Significantly ($p \leq 0.05$) increase chlorophyll formation (6.6 and 7.9 mg/g) in the 2022 and 2023 seasons was recorded with the dual culture of *B. subtilis* and *P. fluorescens* as compare to (urea 130 kg + DAP 60 kg/ha) showed in (Fig. 2. A). The chlorophyll content in fresh foliage of wheat plants

was further increased by 5.0 and 5.9 mg/g when plants were grown in soils treated with mixture of urea (130 kg/ha) and DAP (60 kg/ha) as compare to during both cropping seasons. *Bacillus* in the presence of 130 kg urea/ha enhanced the chlorophyll content from 0.8 mg/g (control) to 4.6 mg/g in 2022 while it was 5.4 mg/g in 2023. Among all treatments, the dual inoculation of B. *subtilis* and *P. fluorescens* was found superior (6.6 mg/g in 2022 and 7.9 mg/g in 2023) compared to other single cultures, culture with fertilizers or the mixture of urea and DAP. Similarly impact of microbial applications even in the presence of chemical fertilizers on chlorophyll synthesis and photosynthetic rates leading to overall improvement in many plants have been reported (Chandra *et al*., 2021; Rafique *et al*., 2022).

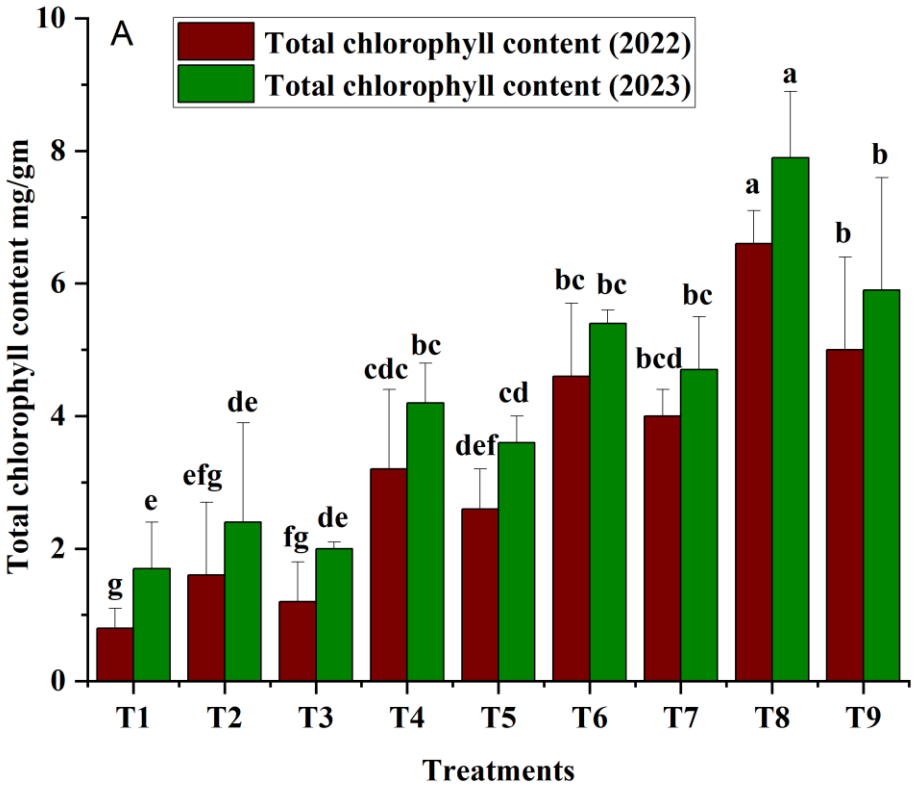


Fig. 2 (A) Impact of bacterial inoculations in the presence of chemical fertilizers on chlorophyll content in wheat foliage detected at 60 DAS.

Table: -2. Effect of bacterial formulations in the presence or absence of chemical fertilizers on biological growth and grain attributes of wheat plants raised in sandy clay loam soils

Treatment designation	Treatments	Whole biomass (g/plant)		120 DAS (2022)		120 DAS (2023)	
				Grain attributes			
		2022	2023	Grain yield (g/plant)	Grain protein (mg/g)	Grain yield (g/plant)	Grain protein (mg/g)
T1	Un-inoculated control	5.1g±0.8	6.4h±0.6	16.5h±1.2	61.6g±2.2	17.5h±0.8	63.1h±1.8
T2	Urea (130 kg/ha)	7.3ef±0.4	9.6f±0.3	19.2fg±0.5	68.2f±1.0	21.9f±0.8	74.4f±0.7
T3	DAP (60 kg/ha)	6.4fg±0.6	8.1g±0.4	18.1g±0.6	65.5f±1.4	19.7g±0.7	70.3g±1.7
T4	*B. subtilis*	9.8d±1.9	13.9de±1.0	21.8e±1.1	77.1d±0.9	24.6d±0.3	81.9e±1.4
T5	*P. fluorescens*	8.9de±0.1	12.5e±0.9	20.5ef±1.0	72.8e±1.5	23.4e±0.2	77.5e±1.3
T6	*B. subtilis* with 130 kg/ha urea	15.0c±0.4	16.2c±0.9	26.2c±0.5	82.9c±2.6	27.7c±0.8	86.8c±0.7
T7	*P. fluorescens* + 60 kg/ha DAP	13.2c±0.9	15.3cd±1.1	23.8d±0.6	79.1d±2.0	26.9c±0.5	83.1d±1.5
T8	*B. subtilis* + *P. fluorescens*	21.3a±1.4	25.2a±1.1	29.7a±0.4	90.1a±2.7	33.5a±0.5	96.2a±0.9
T9	Urea + DAP (130 +60 kg/ha)	17.5b±1.3	19.1b±0.95	28.1b±0.5	86.8b±1.3	30.6b±1.0	89.1b±1.6

Values are the mean of three independent replicates where each replicate constituted three plants per pot; Mean values followed by different letters are significantly different within a column at $P \leq 0.05$ according to Duncan's multiple range test (DMRT)

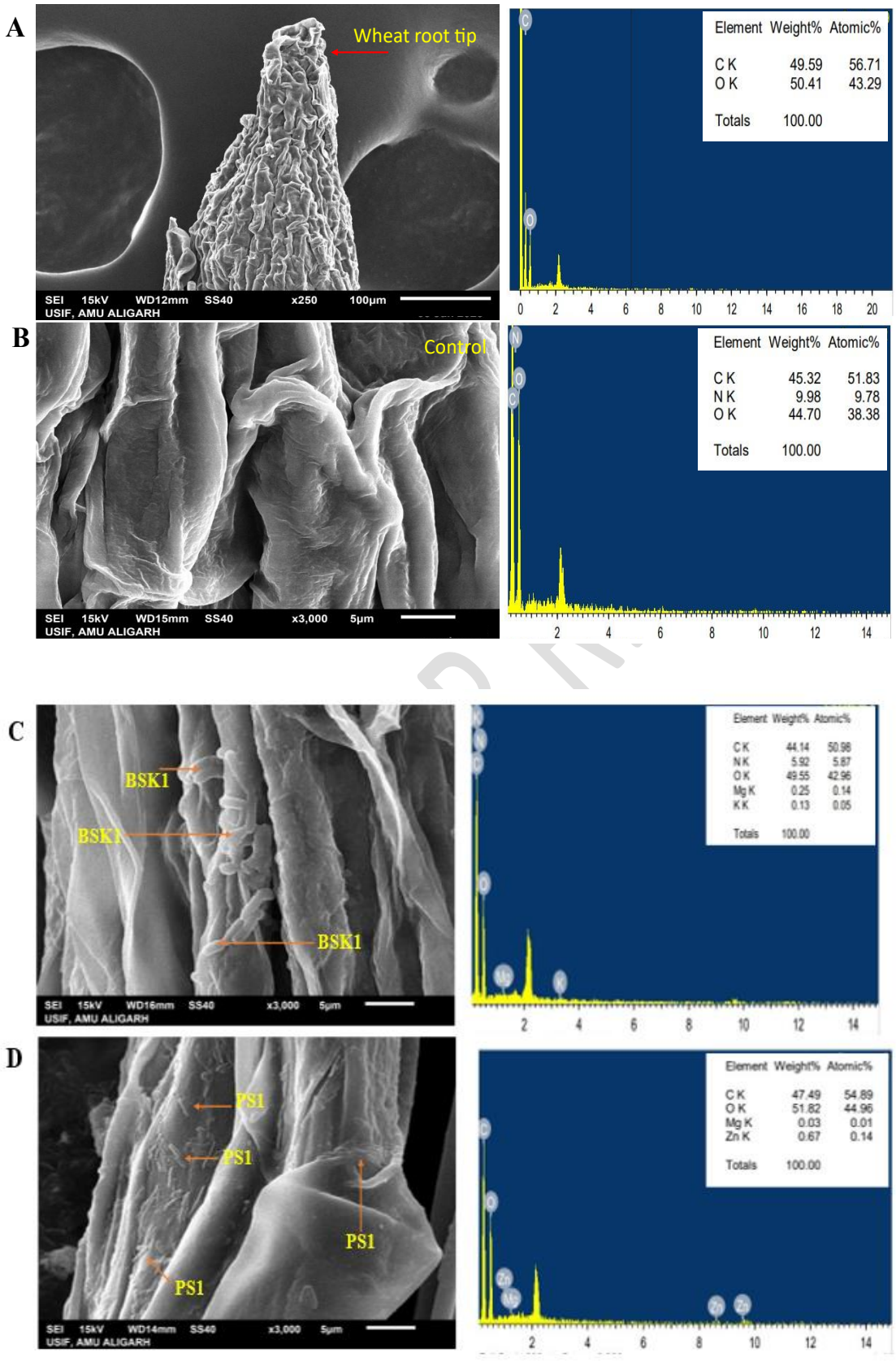
Grain yield and grain protein

Bacterial inoculation improved the growth and consequently enhanced the grain attributes of wheat plants grown in unsterilized soils fertilized with or without nitrogenous and phosphatic fertilizers during both years compared to uninoculated plants (Table 2). Generally, the grain yield (GY) in all treatments was higher in 2023 than in 2022. *B. subtilis* in the presence of 130 urea kg/ha showed maximum increased in GY (27.7 g/plant) in 2023 while compare to P. *fluorescens* in the presence of DAP 60 kg/ha increase in the GY (26.9 g/plant) in 2023 over urea, DAP, and sole bacterial cultures or uninoculated control. The co-inoculation of *Bacillus* with *Pseudomonas* significantly ($p \leq 0.05$) increased the GY during both years. For example, the dual inoculation of *B. subtilis* with *P. fluorescens* enhanced the GY significantly (29.7 g/plant) in 2022 and (33.5 g/plant) in the year 2023, respectively relative to control (16.5:17.5 g/plant in 2022: 2023) following the mixture of urea 130 kg/ha and DAP 60 kg/ha (28.1 g/plant) in 2022 and (30.6 g/plant) in 2023 (Table 2). The enhancement in grain yield following PBB were more profound compared with those of the control/fertilized plants. Similar enhancement in vigor, nutrient uptake and grain yields due to biofertilizers application such as *Azotobacter*, and other PGPR strains used either alone or in combination have been reported, confirming our data (Poveda & Eugui, 2022). Such increment in growth and yields of wheat in this and other related studies could be due to supply of plant growth promoting substances including nutrients and phytohormones. This finding is important because use of PBB in cultivation practices is cheap, easy to culture and apply and produce no detrimental impact on soil-plant ecosystems. Furthermore, studies demonstrate that combining organic and fertilizers, along with beneficial microbes, can lead to substantial increases in grain (Nadia *et al*., 2023). Grain protein (GP) content in wheat seeds even though was almost at par among treatments but in general it was maximum in bio-primed plants relative to uninoculated plants. Sole application of *B. subtilis* or *Pseudomonas* showed a considerable increase in GP over 130 kg/ha urea or 60 kg/ha DAP. Among all applications, the co-inoculation of *B. subtilis* with *P. fluorescens* displayed the highest GP (90.1: 96.2 mg/g in 2022:2023) followed by the mixed fertilization containing 130 kg/ha urea with 60 kg/ha DAP (86.8 in 2022 and 89.1 mg/g in 2023). Moreover, composite application of *Bacillus* with 130 kg/ha urea showed maximum GP (82.9 mg/g) in 2022 while it was 86.8 mg/g in 2023. *P. fluorescens* + 60 kg/ha DAP produced 79.1 mg/g GP in 2022 and 83.1 mg/g in 2023 (Table 2). The production of good quality wheat is critically important to fulfil nutritious human food demand. In these two-year studies, bacterial inoculation and their interactions within soils is highly significant due to greater nutrients and P supply to wheat

plants by *B. subtilis* and *Pseudomonas*. This increased availability and nutrient acquisition of N contribute directly to higher GP in wheat grains. Results by other researchers have shown improvement in wheat GP following combined application of PBB and chemical fertilizers (Arfan-Ul-haq *et al*., 2021; Yadav *et al*., 2022; Kaur *et al*., 2024).

Colonization of wheat rhizosphere

The use of bioinoculants for promoting plant growth and protecting plant health is an integral part of sustainable crop production. The efficacy of bacterial inoculants depends upon the complex process of plant-microbe interactions and environmental factors**.** In this study, time-course colonization of PBB, *B. subtilis* (BS1) and *P. fluorescens* (PS1) including adhesion to the root hair surface and the localization within intercellular spaces/cortex of the plants was recorded using SEM. The SEM image revealed heavy colonization of bacterial cells along the entire root hair surface suggesting the colonization efficiency of selected bacteria onto wheat plants. Interestingly, the bacterial cells were found as micro-colonies located either at the surface of the roots or onto root hairs. The SEM and EDX data also indicated an enrichment of the essential elements C, N, O, Mg, Fe and Zn detected in EDX (Fig. 3. E and F) which could be due to better nutritional status of co-inoculation (*B. subtilis* + *P. fluorescens.* Through accelerated nutrient availability and increased microbial activity, PBB benefits the host plants (Chandra *et al*., 2021). The present findings suggest that *Bacillus* and *Pseudomonas* can colonize successfully in the rhizosphere of wheat and therefore can have a beneficial influence on wheat plants confirming the colonizing efficiency of test bacterial strains


A
Wheat root tip
SEI 15kV WD12mm SS40 x250 100µm
USIF, AMU ALIGARH
Element Weight% Atomic%
C K 49.59 56.71
O K 50.41 43.29
Totals 100.00
B
Control
SEI 15kV WD15mm SS40 x3,000 5µm
USIF, AMU ALIGARH
Element Weight% Atomic%
C K 45.32 51.83
N K 9.98 9.78
O K 44.70 38.38
Totals 100.00
C
BSK1
BSK1
BSK1
SEI 15kV WD16mm SS40 x3,000 5µm
USIF, AMU ALIGARH
Element Weight% Atomic%
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N K 5.92 5.87
O K 49.55 42.96
Mg K 0.25 0.14
K K 0.13 0.05
Totals 100.00
D
PS1
PS1
PS1
PS1
SEI 15kV WD14mm SS40 x3,000 5µm
USIF, AMU ALIGARH
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O K 51.82 44.96
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Zn K 0.67 0.14
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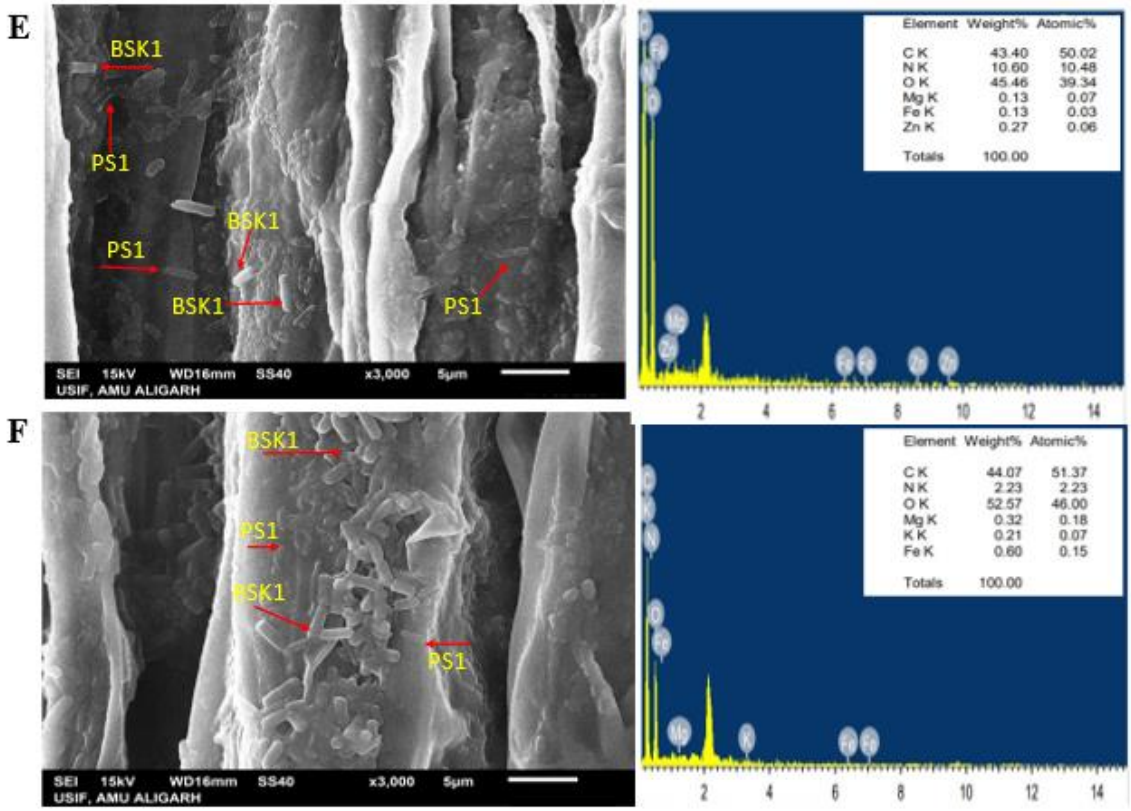

Fig. 3. SEM and EDX, (A and B) uninoculated Control, (C and D) root colonization of *B. subtilis* and *P. fluorescens,* and (E and F) co-culture of *B. subtilis* with *P. fluorescens* represented by arrows.

Conclusion

The PBB, *B. subtilis* and *P. fluorescens* exhibited variable plant growth-promoting activity. The combined inoculation of *B. subtilis* and *P. fluorescens* in the presence or absence of fertilizers was found superior and enhanced whole plant biomass, chlorophyll content, grain yields, and grain protein considerably. Moreover, the effects of co-cultures of *B. subtilis* and *P. fluorescens* were reasonably greater than the sum of the individual inoculation effects suggesting interactions that go beyond just additive effects. In addition, the mixture of *B. subtilis* and *P. fluorescens* colonizing root surface presents a complex microbial interplay enhancing nutrient uptake and plant fitness. Overall, our results indicated that the single or composite application of PBB could serve as potential plant growth regulator and may substitute costly chemicals in wheat production, reducing reliance on chemical fertilizers.

Conflict of Interest declaration

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Disclaimer (Artificial intelligence)

Option 1:

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