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# COMPARISON OF APPLICATION METHOD OF PGPR-BASED BIOFERTILIZER ON WHEAT (*TRITICUM AESTIVUM* L.) GROWTH UNDER CLIMATE CONTROL AND FIELD CONDITIONS

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**Abstract**

Plant growth-promoting rhizobacteria (PGPR) improve plant growth and their application with suitable delivery matrix is an eco-friendly approach to sustainable agriculture. In the current study, *Bacillus licheniformis* ARS8, *Bacillus marisflavi* ARS23, and *Pseudomonas fluorescens* SS14 strains were investigated for their performance as liquid inoculum, coated seeds, and Enriched Soil-Based Fertilizer on wheat growth under climate room and field conditions. Both *Bacillus* strains ARS23 and ARS8 showed phosphate solubilization of 170 and 150 μg/ml, respectively, while *Pseudomonas* strain SS14 showed 23.54 μg/ml. SS14 also showed nitrogenase activity 199 nmol ethylene/h/ mg/protein. The maximum increase in root and shoot lengths and weight was recorded in treatments inoculated with *Bacillus* strain ARS8 and *Pseudomonas* strain SS14 in Enriched Soil-Based Fertilizer compared to liquid inoculum, coated seeds with [Bacterial strains + 1% (w/v) CMC] and control in pot experiments. A significant increase was noted in root and shoot lengths, weight, and yield in *Bacillus* strain ARS23 using enriched soil based followed by liquid inoculum and coated seeds inoculated treatments in both pot and field experiments. These results indicated that *Bacillus* spp. and *Pseudomonas* sp. using enriched soil as a carrier can be used as biofertilizers.

**Key words:** Plant growth promoting rhizobacteria (PGPR); Carrier material; Biofertilizer; Enriched Soil-Based Fertilizer; Wheat.

## Introduction

Chemical fertilizers are used to increase plant growth and crop yield around the world. However, their excessive use causes soil erosion, disruption of biological processes, affects the indigenous microflora, and leads to the loss of soil biodiversity (Hinsinger *et al*., 2011; Jacobsen & Hjelmsø, 2014; Granada *et al*., 2018; Yadav & Sarkar, 2019). Biofertilizers offer an environmentally friendly and sustainable solution to enhance crop productivity while safeguarding the environment and soil microorganisms. Plant growth-promoting rhizobacteria (PGPR) has gained widespread use in sustainable agricultural practices worldwide for their role in supporting long-term agricultural production (Mehnaz *et al*., 2001; Kumar *et al*., 2014). These microorganisms contribute to plant growth by directly influencing various processes, such as the synthesis of phytohormones, nutrient mobilization, nitrogen fixation, and phosphate solubilization. Indirectly, they also play a role in plant health by producing compounds with antifungal and antibacterial properties to combat diverse plant pathogens (Yasmin & Bano, 2011; Goswami *et al*., 2016).

Nitrogen and phosphorus are essential macronutrients for plant growth. As the soil has a deficiency of available nitrogen and phosphorus, the nitrogen fixation and phosphorus solubilization driven by nitrogen fixing and phosphate solubilizing bacteria have a central role (Yevdokimov *et al*., 2008). Numerous rhizobacteria, such as *Azospirillum*, *Bacillus*, *Pseudomonas*, and *Streptomyces*, are widely recognized as beneficial nitrogen-fixing bacteria in the soil (Cakmakci *et al*., 1999). Bacterial genera including *Bacillus* and *Pseudomonas* are well documented for their ability to solubilize unavailable phosphates in soil and produce phytohormone-like auxin. The PGPR belonging to genera *Enterobacter*, *Bacillus*, *Azospirillum*, *Burkholderia*, *Serratia,* and *Pseudomonas* have been suggested for their promising influences as biofertilizers (Berninger *et al*., 2018).

A biofertilizer is a product consisting of living organisms that when inoculated to plants or soil, inhabits the rhizosphere and increases plant growth by providing sufficient nutrients to plants. The preparation of such a biofertilizer requires suitable carrier material that is appropriate for the multiplication of bacterial cells. Selected carrier materials should be eco-friendly, cost-effective, easily soluble in water, non-toxic, porous, and degradable (Goswami *et al*., 2016; Macik *et al*., 2020; Bhattacharyya *et al*., 2020). Bioformulations are of different types e.g., powdered, granules, or liquid. Various eco-friendly biofertilizers have been developed by combining different carrier materials, including biogas sludge, farmyard manure, soil, carboxy methyl cellulose (CMC), and polyvinyl pyrrolidone (PVP), with PGPR strains and an inorganic phosphate source (Mehnaz *et al*., 2001, 2010; Shrivastava & Kumar, 2015; Mukhtar *et al*., 2017). Soil enriched with PGPR is considered a cost-effective, non-toxic, easy-to-process, and easily available biofertilizer (Mukhtar *et al*., 2017).

Wheat (*Triticum aestivium* L.) is used worldwide as a staple food. It is important to reduce the cost of chemical fertilizers for the cultivation of wheat. PGPR strains have been used as biofertilizers for plant growth promotion and improve the yield of many crops, such as maize, rice, sugarcane, barley, wheat, and chickpea (Tilak & Reddy, 2006; Kumar *et al*., 2014; Mukhtar *et al*., 2017). Among the PGPRs, *Pseudomonas* and *Bacillus* are known to have the ability to improve seed germination, seedling growth, and wheat yield (Govindasamy *et al*., 2010). They are also effective colonizers of wheat roots (Cakmakci *et al*., 2007; Mukhtar *et al*., 2017).

The objective of this study was to assess the impact of solid and liquid biofertilizers containing *B. licheniformis*, *B. marisflavi*, and *P. fluorescens*. Additionally, it aimed to evaluate the survival and biological effectiveness of these formulations. Another focus of the study was to compare the effects of various inoculum application methods, including liquid inoculum, coated seeds, and Enriched Soil-based Biofertilizer (ESBF), on both potted wheat plants and field experiments.

## Material and Methods

**Source of strains:** Three previously isolated and identified halotolerant bacterial strains from the rhizosphere of cotton and cowpea nodules were collected from our lab (Mukhtar *et al*., 2016, 2020). These bacteria were revived on halophilic medium (HAP) (g/L: Tryptone 5, Yeast Extract 1, KCl 5, $MgSO_4$ 10, $K_2HPO_4$ 2; pH 7·2) supplemented with 5% (w/v) NaCl (Schneegurt, 2012 ).

**Screening of plant growth-promoting abilities:** The nitrogenase activity, indole acetic acid production, phosphate solubilization, hydrogen cyanide production, and siderophore production of the bacterial isolates ARS8, ARS23, and SS14 were assessed using the following assays.

**Acetylene reduction assay (ARA):** Selected bacterial strains were detected for their nitrogen-fixing ability by growing them on the semisolid combined carbon medium (CCM; 5 mL/vial) (Rennie, 1981) supplemented with 5% (w/v) NaCl. About 1 mL of acetylene (10% v/v) was added into the vials containing 48 h old bacterial culture and incubated at 30°C for 24 h. These samples were evaluated by using gas chromatography (GC). GC-2014 System (Shimadzu Corporation, Japan) fitted with a Porapak column and a flame ionization detector (FID) was used. Its temperature was maintained at 150°C, column temperature at 80°C, and equilibrium time 0.5 min. Helium and Hydrogen were used as carrier gases (Mehnaz & Lazarovits, 2006).

**Indole acetic acid (IAA) production assay:** Bacterial strains were quantified for IAA production as described by Gordon & Weber (1951). Bacterial cultures were grown in the presence of 100 mg/L L-tryptophan as IAA precursor and 5% (w/v) NaCl for 10 days at 30°C. The IAA content in the same culture was measured using high-performance liquid chromatography (HPLC) with a 2998 photodiode array (PDA) detector and a Nucleosil C18 column (4.6 × 250mm, 5µM; Macherey-Nagel, Germany) following the protocol outlined by Kamran *et al*., (2017).

**Phosphate solubilization assay:** The bacterial strains were assessed for their phosphate solubilization capability by cultivating them on modified National Botanical Research Institute's phosphate growth medium (NBRIP) supplemented with 5% NaCl, as described by Nautiyal (1999). The quantitative measurement of P-solubilization was performed using the molybdate blue color method with cell-free supernatants, following the procedure outlined by Watanabe & Olsen (1965). Solubilized phosphate was analyzed by a spectrophotometer (Camspec M350-Double Beam UV-Visible Spectrophotometer, UK) at 882 nm and values were generated by using a standard curve of $KH_2PO_4$ solution.

**Siderophore and hydrogen cyanide production assay:** The presence of siderophore production in bacterial strains was determined by inoculating them on chrome azurol S (CAS) blue agar plates, following the procedure outlined by Alexander & Zuberer (1991). The production of hydrogen cyanide (HCN) was detected using the method described by Sadasivam & Manickam (1992).

**Identification of bacterial isolates based on 16SrRNA sequence:** Genomic DNA was isolated from all bacterial strains (Winnepenninckx *et al*., 1993). 16SrRNA gene was amplified by using a universal forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and universal reverse primer (5'-ACGGACTTACCTTGTTACGACTT-3') (Tan *et al*., 1997), and profile described by Mukhtar *et al*., (2020).

**Phylogenetic analysis:** The obtained sequences were compared to reference strains using the Basic Local Alignment Search Tool (BLAST). The 16S rRNA gene sequences were analyzed using Chromas 2.6 software and aligned with Clustal W 2.2 software (Technelysium Pty Ltd., South Brisbane, Australia). Phylogenetic tree construction was performed using the neighbor-joining method in MEGA7 software (Tamura *et al*., 2004; Kumar *et al*., 2016). The sequences were deposited in the NCBI GenBank database. All parameters for phylogenetic tree construction were based on the methodology described by Mukhtar *et al*., (2016).

**Preparation of enriched soil-based fertilizer:** Each culture was prepared as described by Molina-Romero *et al*., (2017). The bacterial concentrations were adjusted at $1{\times}10^8$ CFU /ml for ARS8, ARS23, and SS14 based on $OD_{600}$ nm. For the preparation of Enriched Soil-Based Fertilizer (ESBF), the soil was collected from the field of FCCU (pH 8, electrical conductivity (EC) 2 dS/m, moisture 25%, available phosphorous 1.9 mg/kg, organic matter 0.5% and total nitrogen 0.06%), sterilized and mixed with 1% (w/v) glucose and 1% (w/v) rock phosphate (RP). Each bacteria was inoculated in triplicate, in one kg of sterilized soil by aseptically adding 100 mL of bacterial suspension, and non-inoculated sterilized soil was used as control. These bioformulations were air-dried at 30°C and then stored in the dark in polythene bags at 30°C for 180 days.

**Preparation of seed coating:** For seed coating, polymer compound carboxy methyl cellulose (CMC) was used. Wheat seeds of the local variety Galaxy-2013 were sterilized with 70% (v/v) ethanol for 1 minute and 1% sodium hypochlorite solution for 5 min, followed by washing with autoclaved distilled water (Rudolph *et al*., 2015). The sterilized seeds were incubated with individual bacterial cultures ($1{\times}10^8$ CFU/ml) and 1% CMC for 3 h. Seeds dipped in 1% CMC solution without bacterial inoculum were used as a control. Seeds were air dried, and stored at 30°C for 180 days.

**Preparation of liquid inoculum:** The liquid inoculum was prepared as described by Molina-Romero *et al*., (2017). The bacterial concentrations were adjusted at $1\times10^8$ CFU/ml for ARS8, ARS23, and SS14 based on $OD_{600\ nm}$, and these liquid inoculums were stored at 4°C for 180 days.

**Evaluation of the shelf life of the coated seeds and enriched soil-based fertilizer:** Survival of PGPRs in ESBF, liquid inoculum, and coated seeds was determined by using the serial dilution method as described by Somasegaran & Hoben (1994). The viability of the bioformulations was studied after their storage at different time intervals namely 15, 30, 60, 90, 120, 150, and 180 days.

In the case of ESBF, after each specific storage time, one gram of Enriched Soil-Based Fertilizer was added to 10 mL of sterile 0.85% saline, and different dilutions (from $10^{-2}$ to $10^{-6}$) were prepared. One hundred µl of each dilution was plated in triplicate on petri dishes having halophilic medium and incubated at 30°C for 48 h.

The viability of bacteria on coated seeds was determined as mentioned above, i.e., one gram of seeds was added to 10 mL of sterile 0.85% saline, and different dilutions were prepared. Triplicate petri dishes containing halophilic medium were inoculated with 100 µL of each dilution. The dishes were then incubated at 30°C for 48 hours, and the colony-forming units (CFUs) were subsequently counted.

The viability of liquid inoculum was studied by adding one mL broth into 9 mL sterile 0.85% saline. Then dilutions were prepared as mentioned above and CFU were counted.

**Effect of PGPR inoculation on seed germination:** Sterilized one-gram seeds of wheat were soaked with 50 ml ARS8, ARS23, and SS14 ($1 \times 10^8$ CFU/mL), separately for 3 h, and then placed on petri plated (9 cm diameter) having 0.5% (v/v) water agar incubated for 7 days at 30°C. Controlled experiments were conducted using a completely randomized design (CRD) with non-inoculated seeds serving as the control. Each treatment consisted of three replicates, and each replicate comprised 20 seeds. After 72 hours, the germination percentage of the seeds was recorded. Various seed germination parameters, including germination percentage and seedling vigor index, were calculated using the formulas described by Islam *et al*., (2016).

**Effect of PGPR inoculation on root morphology:** The same germination experiment was repeated using hydroponic culture to evaluate the effect of PGPR strains on root morphology by using the WinRhizo root scanning software system (Epson V800 Photo model J221B, Japan). The seeds were germinated as described above and one-week-old seedlings were transferred to glass jars filled with Hoagland solution (Hoagland & Arnon, 1950). In each glass jar, one seedling inoculated with PGPR strain was grown for four weeks in a climate room with 16 h of light at 25°C during the day (200 $\mu M/m^2/s$ at pot heights with fluorescent lights) and 8 h of darkened at 15°C during the night. The experiment was carried out in a completely randomized design (CRD) with three replications.

Plant roots were harvested after 4 weeks and fresh roots were processed on rhizome-scanner (Epson V800 Photo model J221B, Japan) to get data on root morphology e.g., length, area, root tips, root volume, and root projected area. The roots were scanned and data was noted using WinRhizo software.

**Effect of bioformulations on wheat growth**

**Pot experiment:** For all experiments wheat plants of variety Galaxy-2013 were grown in plastic pots of 9 cm diameter containing 1000 g of sandy loam unsterilized soil having electrical conductivity (EC) 2 dS/m, pH 8, available phosphorous 1.9 mg/kg, organic matter 0.5% and total nitrogen 0.06%. All strains ARS8, ASR23, and SS14 were selected for their effect on wheat growth under a climate control room with 16 h of light at 25°C during the day (200 $\mu M/m^2/s$ at pot heights with fluorescent lights) and 8 h of darkened at 15°C during the night.

The 1st set of experiments was treated with Enriched Soil-Based Fertilizer (ESBF); in each pot, three seeds were sown at 1 cm depth. Each seed was inoculated with one-gram ESBF containing $1\times10^7$-$10^8$ bacterial cells and non-inoculated soil was used as a control. In the 2nd set of experiments, seeds coated with bacterial suspension ($1\times10^6$ -$10^8$ CFU/mL) containing 1% (w/v) CMC were used, soaked for 2 h, and air-dried for 2 h before sowing, and non-inoculated seeds were used as control and about 10 g of rock phosphate/pot was also added before sowing. The liquid inoculum of individual strains was used as a biofertilizer in 3rd set of experiments. All plants were inoculated around roots at the rate of 1 ml of $1 \times 10^8$ cells CFU $ml^{-1}$ after germination of seeds on 3rd day. Control was kept without inoculation. Each treatment with 7 replicates was arranged in a completely randomized design (CRD). The recommended fertilizer dose for wheat is 150-100-60 kg/ha for Nitrogen, Phosphorus, and Potassium, respectively. Urea was used to provide Nitrogen at a rate of 100 kg/ha, while Diammonium Phosphate (DAP) was used for Phosphorus at a rate of 80 kg/ha. In treatments with bacterial inoculations, 80% of the recommended DAP dose and urea were added, while non-inoculated pots served as controls with 80% DAP and 80% urea. After 5 weeks, the plants were uprooted, and various growth parameters such as root and shoot lengths, as well as dry weights, were calculated. The plant roots and shoots were then dried in an oven at 70°C for 4 days, and their dry weights were recorded (Tahir *et al*., 2015).

**Field experiment:** The field experiment consisted of 36 plots, each measuring 7x4 feet, divided into 7 rows. A total of 21 rows were assigned to each treatment, and the experiment followed a randomized complete block design (RCBD) with 12 treatments. In each plot, 100 plants were grown, and the experiment was replicated 3 times for each treatment. The treatments used in the field experiment were identical to those employed in the pot experiment.

In the 1st experiment, plants were treated with ESBF, the 2nd set with coated seeds, and the 3rd set with liquid inoculum. Plants were provided with nitrogen and phosphorus as mentioned above for pots, a full dose of 80% DAP and half dose of urea were incorporated at the time of sowing and half a dose of urea was top-dressed at the time of the tillering stage. The plants underwent four irrigation cycles throughout their growth period. At the maturity stage, the plant growth parameters, including root and

shoot lengths, as well as dry weights, were measured after 6 weeks. To determine the dry weights, 10 plant roots, and shoots were dried in an oven at 70°C for 4 days and then weighed. The number of spikes, weight of 1000 grains, and yield were calculated at the harvesting stage, which occurred after 4 months.

**Colonization of inoculated bacteria in pot and field plants:** Colonization and viability of inoculated strains in roots and rhizosphere of inoculated wheat plants were studied after one month of sowing in pot and field experiments. The soil was collected from the roots of three replicate plants and a composite sample was made after a thorough mixing of the soil. The bacterial population was counted by serial dilution and recovered bacterial colonies were also identified based on morphological and biochemical characteristics compared to original bacterial cultures.

**Physicochemical analysis of soil before sowing and after harvesting:** Before sowing, 3 soil samples were collected from different plots, and after harvesting the wheat, 9 additional soil samples were collected. Each soil sample weighing 300g was mixed and passed through a 2 mm sieve to remove impurities. The moisture content, pH level, salinity, and temperature of the soil samples were determined. The electrical conductivity (EC) was measured following the method described by Adviento-Borbe *et al*., (2006). The pH was measured by making a 1:2.5 (w/v) soil-to-water suspension. The moisture percentage, temperature in degrees Celsius, and texture class were determined according to the method outlined by Anderson *et al*., (1993). The organic matter content was calculated using the Walkley & Black method (1934), while the concentrations of calcium and magnesium were measured using spectrometry. Phosphorus content was analyzed through extraction with sodium bicarbonate, following the procedure by Watanabe & Olsen (1965), and nitrate ions were determined as described by Rashid *et al*., (1994).

**Statistical analysis:** For statistical analysis, the data was subjected to ANOVA (analysis of variance), and significance was tested at a p-value of less than 0.05 using the least significant difference (LSD) test in IBM SPSS Statistics version 23 (SPSS Inc., USA). Mean values and standard errors of replicates for each treatment were calculated.

## Results

**Plant Growth promoting abilities of bacterial strains:** All three strains namely ARS8, ARS23, and SS14 were grown in NBRIP supplemented with glucose, and showed an increase in P-solubilization and a decrease in pH up to 7 days. Both *Bacillus* strains ARS23 and ARS8 showed maximum P solubilization (170 and 150 µg/ml) by using glucose after 7 days, respectively, while *Pseudomonas* strain SS14 showed P solubilization of 23.54 µg/mL (Table 1). *Bacillus* strain ARS23 produced 40 µg/ml of IAA in the presence of tryptophan, while *Bacillus* strain ARS8 and SS14 produced 6 µg/ml and 8.16 µg/ml of IAA using HPLC, respectively (Table 1). SS14 also showed nitrogenase activity as estimated by ARA 199 nmol ethylene/ h mg/protein (Table 1).

**Molecular characterization of bacterial isolates:** The phylogenetic analysis revealed that isolate ARS8 exhibited a high similarity of 99% with *Bacillus lichenoformis* (Accession no. LT797522). Similarly, isolate ARS23 showed a similarity of 99% with *Bacillus marisflavi* (Accession no. LT797524), while isolate SS14 displayed a similarity of 99% with *Pseudomonas fluorescens* (Accession no. MH491068) (Fig. 1).

**Survival of inoculated bacteria in bioformulations:** The shelf life of ARS8, ARS23, and SS14 was studied in ESBF, liquid inoculum, and coated seeds. The initial bacterial concentration was $1\times10^9$ CFU/g for all inoculated strains in enriched soil-based fertilizer (ESBF). Among all, ESBF proved the best, holding CFU $10\times10^7$, $7\times10^7$ for ARS8 and ARS23 and $2\times10^7$ CFU/g for SS14, respectively, after 180 days of incubation at 30°C temperature (Fig. 2a).

Coated seeds supported a high population up to 120 days of incubation at 30°C. After 180 days, population was decreased to $5\times10^2$, $2\times10^2$ for ARS23, ARS8 and $10^1$ CFU/g for SS14, respectively (Fig. 2b). Liquid inoculum also showed a decrease in population after 180 days of incubation and it was $4\times10^3$, $5\times10^2$ for ARS23, ARS8 and $2\times10^{2}$ CFU/ml for SS14, respectively (Fig. 2c).

**Effect of bioformulations on seed germination:** All inoculated bacterial strains showed a positive effect on the growth of wheat seedlings. A maximum germination percentage of 90% was recorded in ARS23 inoculated seeds followed by 85% in SS14 and 80% in ARS8. The seedling vigor index was calculated based on germination percentage and seedlings lengths. Maximum vigor index (1663) was observed in seedlings inoculated with *Bacillus* strain ARS23 followed by *Bacillus* strain ARS8 (1635) and *Pseudomonas* strain SS14 (1550).

To study the impact of inoculated bacteria on various parameters of root morphology, rhizoscanning was performed on wheat plants. The highest observed effects on root length, root volume, root surface area, and root diameter were noted on the plants inoculated with *Bacillus* strain ARS23 (refer to Table 2 and Fig. 3).

**Table 1. Plant growth promoting traits of bacterial strains used as inoculum.**

| Strains | P solubilization (µg/mL) | IAA production (µg/mL) | Nitrogen fixation (nmol ethylene/h/mg protein) | Siderophore production | HCN production |
|---|---|---|---|---|---|
| ARS8 | $170.01^b$ | $6.0^a$ | - | + | - |
| ARS23 | $150.0^c$ | $40.0^c$ | - | - | - |
| SS14 | $23.54^a$ | $8.16^b$ | 187 | + | + |

P, Phosphate; IAA, indole acetic acid; HCN, and Hydrogen cyanide were tested. Values for P solubilization are for glucose after 7 days. IAA values for HPLC. Data are the mean values of three replicates per treatment

The symbol, + represents the positive reaction, while the symbol, - shows the negative reaction

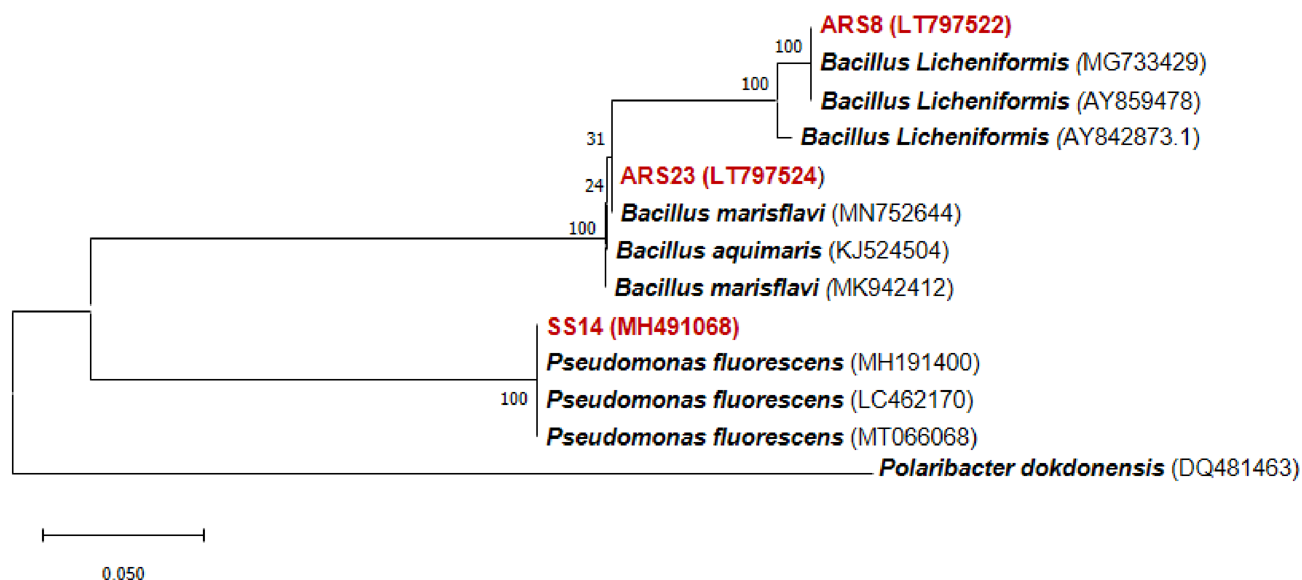


Fig. 1. The phylogenetic tree of three strains obtained from the rhizosphere of cotton and chickpea was constructed based on the 16S rRNA sequences. The analysis of these strains involved aligning their sequences with reference strains using the CLUSTALW method. The neighbor-joining method was then employed to generate the phylogenetic tree.

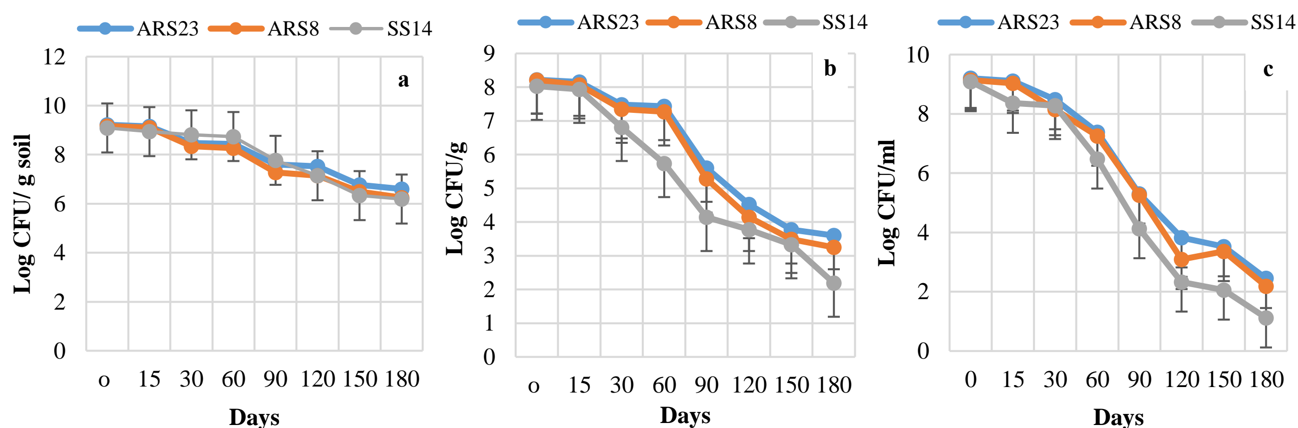


Fig. 2. Survival study of inoculated *Bacillus marisflavi* (ARS23), *Bacillus licheniformis* (ARS8), and *Pseudomonas fluorescens* (SS14) strains in (a) Enriched Soil-Based Fertilizer (ESBF) (b) Coated seeds with (Bacterial strains + 1% CMC) (c) Liquid inoculum after different time intervals 15, 30, 60, 90, 120, 150 and 180 days at 30°C and 4°C. Error bars are standard errors from 3 replicates that received the same treatment.

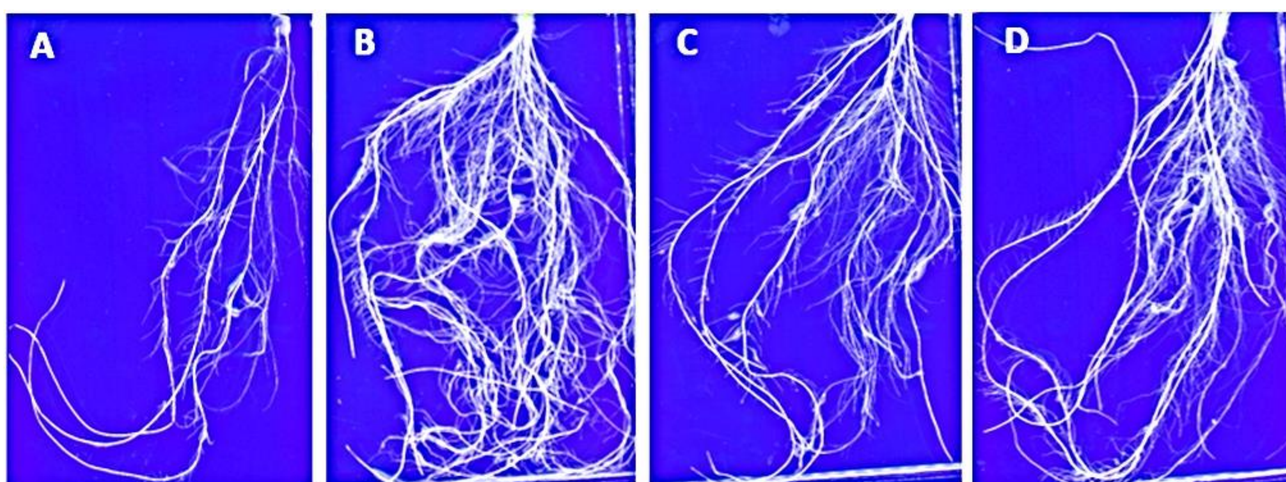


Fig. 3. Effect of inoculation of different PGPR on root morphology of wheat plants grown for 30 days in hydroponics condition. (a) Control, (b) Inoculated with *Bacillus marisflavi* ARS23 (c) Inoculate with *Bacillus licheniformis* ARS8 (d) Inoculated with *Pseudomonas fluorescens* SS14.

Table 2. Effect of *Bacillus marisflavi* ARS23, *Bacillus licheniformis* ARS8, and *Pseudomonas fluorescens* SS14 on root morphology after 30 days of inoculation.

| Treatments | Root length (cm) | Root surface area ($cm^2$) | Root diameter (mm) | Root volume ($cm^3$) | No of forks | Area of projections ($cm^2$) | No of root tips | No of crossings |
|---|---|---|---|---|---|---|---|---|
| Control | 861±15.19a | 1042±12.83a | 1.62±0.04a | 45.91±3.87a | 6893±8.01a | 374.25±12.17a | 1205±15.02a | 293±9.82a |
| ARS23 | 1017. 66 ±11.86c | 1202±13.91c | 5.62±0.07d | 111.78±5.26c | 8082±90.7d | 504.75±16.23d | 1844±31.59d | 440.33±19.03c |
| ARS8 | 959.33±8.96b | 1121±12.4b | 3.40±0.04b | 72.19±1.37b | 7311±80.67b | 447.36±10.13b | 1652±25.3b | 391±14.69b |
| SS14 | 974±10.19b | 1159±15.08b | 3.83±0.1c | 88.56±1.26b | 7637±64.78c | 471.7±8.41c | 1735±18.95c | 395.66±14.45b |

Data are the mean values of three replicates per treatment. This means carrying different letters are significantly different using LSD values at $p<0.05$

**Effect of bioformulations on wheat growth: Pot experiment: Root length:** Results presented in (Fig. 4a) illustrated the positive influence of inoculated treatments over un-inoculated control. The highest mean root lengths of plant, i.e., 27.14-30.33 cm were recorded in Enriched soil-based fertilizer ESBF inoculated treatments which showed a 35-50% increase over control while in liquid inoculum treatments, this increase was 13-29% and root length was 22.68-25.94 cm. The increase in root lengths of plants with coated seeds [Bacterial strains + 1% (w/v) CMC] was 14-24% and they had 23-25 cm root lengths. A non-significant variation was noticed between all bacterial strains, but ARS23 inoculation was found best with a 30.33 cm mean root length and 50% increase over its control while ARS8 and SS14 treated plants had 27.14 and 28 cm root length which indicated 35 and 39% increase over control.

**Shoot length:** The influence of different inoculated formulations on the shoot length of wheat plants is presented in (Fig. 4b). The highest mean of shoot lengths was 40.4-44 cm in enriched soil-based fertilizer ESBF inoculated treatments when compared to control the increase was 22-33%, followed by 8-14% increase and 36-38 cm height of liquid inoculum treated plants, while plants with coated seeds (Bacterial strains+1%CMC) had 33-37 cm height indicated 6-12% increase over control. All bacterial strains showed non-significant variation but ARS23 inoculated plants were found better with 44 cm mean length which had a 33% increase over its control while ARS8 and SS14 treated plants had 40.4 and 42 cm mean shoot lengths and 22 and 27% increases over the respective control. The overall results indicated that ESBF treatments showed more significant results than other formulations.

**Root dry weight:** The influence of inoculated formulations on root dry weight is presented in (Fig. 4c). Maximum mean root dry weight 0.18-0.21 g and increase of 50-75% against non-inoculated control was observed in inoculated ESBF treatments, followed by liquid inoculum treatments 0.15-0.17 g with 25-41% increase over control, while plants from seeds coated with bacterial strains+1% CMC showed 16-33% increase with dry weight 0.14-0.16 g over un-inoculated control. All bacterial strains showed non-significant variation, however, ARS23 inoculated plants were better with 0.21 g mean root dry weight which had a 75% an increase over its control, while ARS8 and SS14 treated plants had 0.19 and 0.18 g mean root dry weight, and 58 and 50% increase over the respective control. The overall result indicated that ESBF-inoculated treatments showed more significant results than other formulations.

**Shoot dry weight:** The response of all inoculated formulations on the dry shoot weight of wheat is presented in (Fig. 4d). The results presented a statistically significant variation between inoculated and non-inoculated control. The mean highest value of the dry weight of shoots was 0.39-0.42 g of treatments inoculated by ESBF and a 30-40% increase over control. In liquid inoculum-treated plants dry weight of shoot 0-36-0.38 g and an increase of 20-26% was noticed while this increase was 13-20% with 0.34-0.37 g of plants with coated seeds [bacterial strains+1% (w/v) CMC]. The analysis of variance and contrast analyses revealed a positive statistically significant increase of shoot dry weight for ESBF inoculated treatments against non-inoculated. All bacterial strains showed non-significant variation but ARS23 inoculated plants were found better with 0.42 g mean dry weight which had a 40% increase over its control while ARS8 and SS14 treated plants had 0.39 and 0.4 g mean shoot dry weight and 30 and 33% increase over the respective control. The overall results indicated that ESBF treatments showed more significant results than other formulations.

**Field experiment: Root length:** Results presented in (Fig. 5a) illustrated the positive influence of inoculated treatments over the un-inoculated control. The highest mean root length 16-19 cm was recorded in ESBF inoculated treatments showing an increase 41-68% over control while in liquid inoculum treatments, this increase was 22-45% and root length was 14-16 cm. The increase in plants with coated seeds [Bacterial strains + 1% (w/v) CMC] was 18-36% and they had 13-15 cm root lengths. A non-significant variation was noticed between all bacterial strains. ARS23 inoculation was found best with 19 cm mean root length and a 68% increase over its control while ARS8 and SS14 treated plants had 16 and 17 cm root lengths indicating 41 and 50% increases over control.

**Shoot length:** The influence of different inoculated formulations on the shoot lengths of plants of wheat is presented in (Fig. 5b). The highest mean of shoot lengths was 30-32 cm in ESBF inoculated treatments, when compared to the control the increase was 20-28%, followed by a 9-17% increase and 25.6-30 cm height of liquid inoculum treated plants while plants with seed coated [Bacterial strains + 1% (w/v) CMC] had 25-28 cm height showing 8-10.80% increase over control. All bacterial strains showed non-significant variation but ARS23 inoculated plants were found better with 32 cm mean root dry weight which had a 28% increase over its control, while SS14 and ARS8 treated plants had 31 and 30 cm mean shoot length and 24 and 20% increase over the respective control. The overall result indicated that ESBF treatments showed more significant results than other formulations.

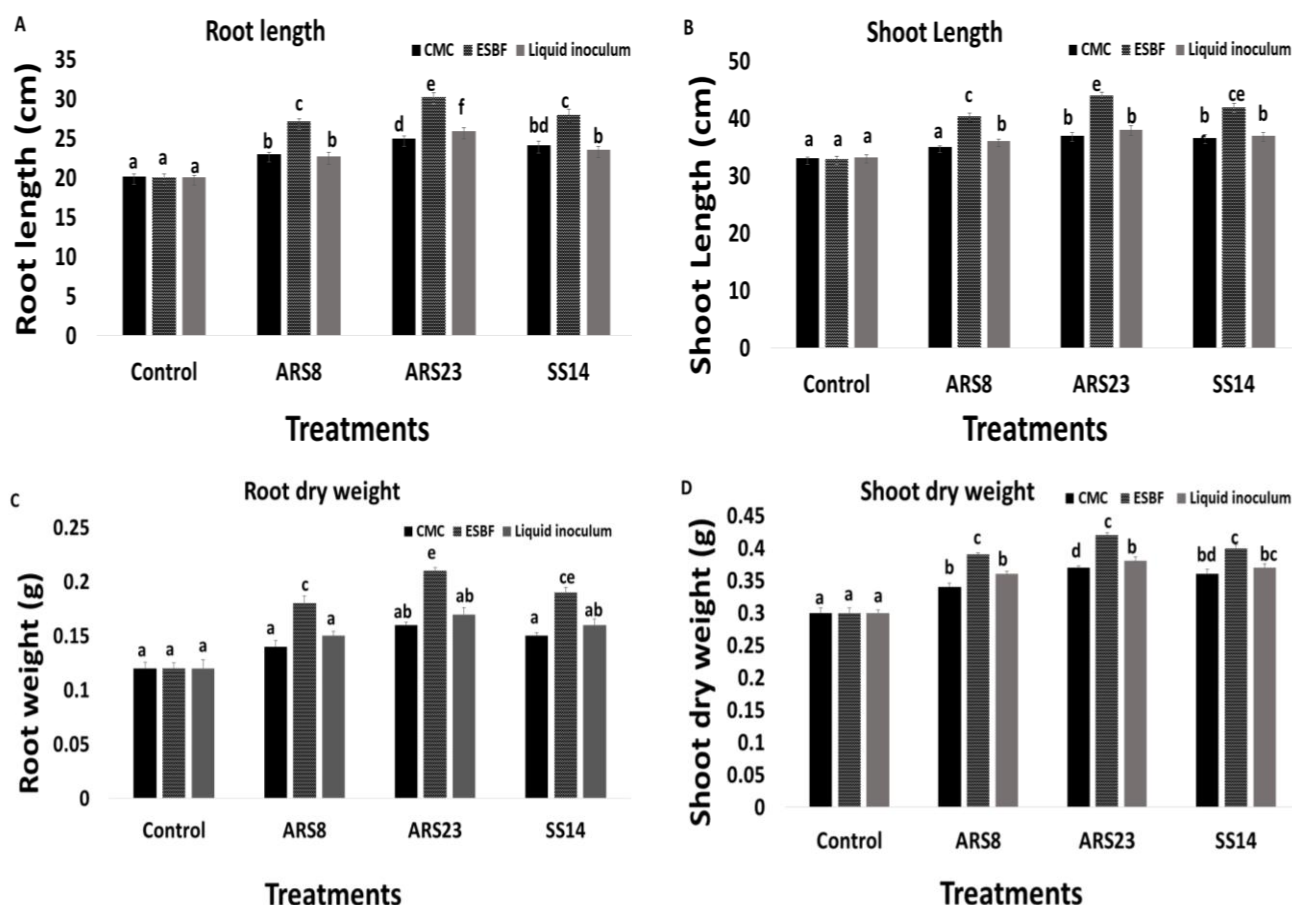


Fig. 4. Effect of coated seeds with [Bacterial strains + 1% (w/v) CMC], ESBF, and liquid inoculum on wheat growth (Pot experiment) and different parameters were recorded after 30 days (a) Root length, (b) Shoot length, (c) Root dry weight, (d) Shoot dry weight. This means carrying different letters is significantly different using (LSD) values at $p<0.05$. Error bars are standard errors from 10 replicates that received the same treatment.

**Root dry weight:** The influence of inoculated formulations on root dry weight is presented in (Fig. 5c). Maximum mean root dry weight 1.12-1.25 g and increase of 40-56.20% against non-inoculated control were observed in inoculated ESBF treatments, followed by liquid inoculum 0.93–0.99 g with 16- 23.70% increase over control while plants with coated seeds [Bacterial strains + 1% (w/v) CMC] showed an increase of 12.50-18% with dry weight 0.9-0.95 g over un-inoculated control. All bacterial strains showed non-significant variation but ARS23 inoculated plants were found better with 1.25 g mean root dry weight which had a 56.20% increase over its control, while SS14 and ARS8 treated plants had 1.12 and 1.17 g mean root dry weight and 46 and 40% increase over the respective control. The overall result indicated that ESBF treatments showed more significant results than other formulations.

**Shoot dry weight:** The response of all inoculated formulations on the dry shoot weight of wheat is shown in (Fig. 5d). The results presented a statistically significant variation between inoculated and non-inoculated control. The mean highest values of the dry weight of shoot 1.5-1.54 g of treatments inoculated by ESBF and increased 23.96-27.27% over control. In liquid inoculum treated plants' dry weight of shoot, 1.44-1.47 g and an increase of 18-20.40% was noticed while this increase was 23.96-27.27% with 1.38 -1.42 g of plants with coated seeds [Bacterial strains + 1% (w/v) CMC]. The analysis of variance and contrast analyses revealed a positive statistically significant increase of shoot dry weight for ESBF against non-inoculated. All bacterial strains showed non-significant variation but ARS23 inoculated plants were found better with 1.54 g mean root dry weight which had a 56.20% increase over its control, while SS14 and ARS8 treated plants had 1.52 and 1.5 g mean root dry weight and 25.60 and 23.96% increase over the respective control. The overall result indicated that ESBF treatments showed more significant results than other formulations.

**Number of spikes:** It is apparent from the data presented in (Fig. 5e) that different inoculated formulations positively influenced the number of spikes of wheat over the un-inoculated control. The highest mean number of spikes 10-11 was obtained in the ESBF inoculated treatments which showed a 42–57% increase over un-inoculated control, while liquid inoculum inoculated treatments and plants with coated seeds (Bacterial strains +1% CMC) had a 14–28% increase with an 8-9 mean number of spikes per plant. Non-significant variation was observed between different bacterial strains but plants inoculated with ARS23 were found better compared to other inoculated plants with the mean number of spikes 11 which was 57% more than its un-inoculated control, while in ARS8 and SS14 inoculated plants the increase over its control was 42 and 57%. The overall result indicated that treatments inoculated with ESBF showed the highest number of spikes.

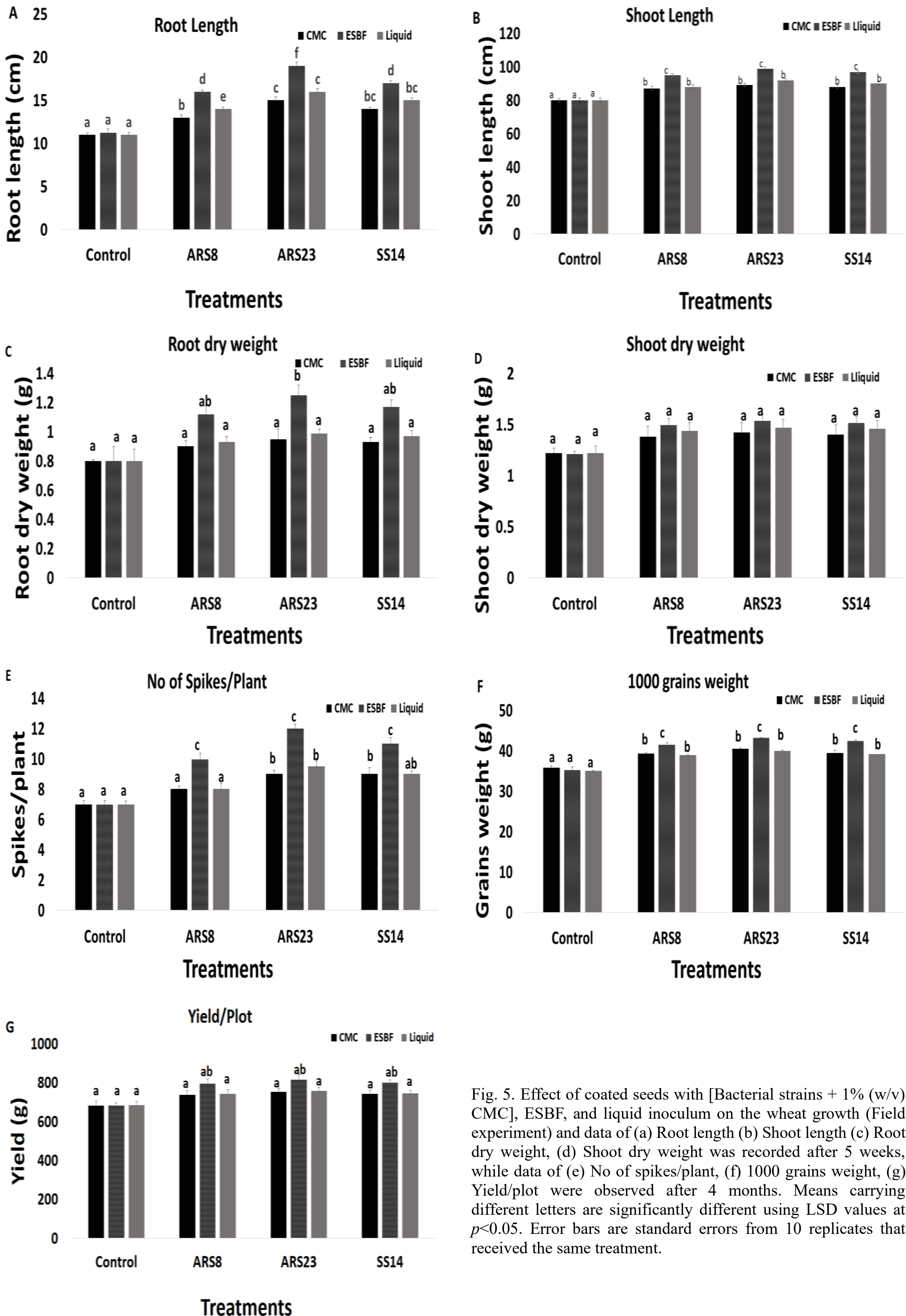


Fig. 5. Effect of coated seeds with [Bacterial strains + 1% (w/v) CMC], ESBF, and liquid inoculum on the wheat growth (Field experiment) and data of (a) Root length (b) Shoot length (c) Root dry weight, (d) Shoot dry weight was recorded after 5 weeks, while data of (e) No of spikes/plant, (f) 1000 grains weight, (g) Yield/plot were observed after 4 months. Means carrying different letters are significantly different using LSD values at *p*<0.05. Error bars are standard errors from 10 replicates that received the same treatment.

**1000 grains weight:** Improvement in 1000 grains weight 9-12 and 10-14% in coated seeds and inoculated liquid inoculum treatments while a significant increase was studied in ESBF inoculated treatment (Fig. 5f).

**Yield:** The data presented in (Fig. 5g) exhibited that different inoculated formulations positively influenced the yield of wheat over the un-inoculated control. The highest mean yield of 795-815 g was obtained in the treatments inoculated with ESBF which showed a 16-19% increase over un-inoculated control while liquid inoculum inoculated treatments had an 8-10.40% increase with 741-765 g mean yield. Plants with coated seeds [Bacterial strains + 1% (w/v) CMC] illustrated a 736.77-741 g mean yield of wheat which was 7.92–10.10% more than the control. Non-significant variation was observed between different bacterial strains but plants inoculated with ARS23 were found better compared to other inoculated plants with a mean yield of 815 g which was 19.60% more than its un-inoculated control while in ARS8 and SS14 inoculated plants the increase over its control was 16.70 and 17.60% as it had 795.22 and 801.44 g mean yield per plot. The overall result indicated that treatments inoculated with ESBF showed the highest yield.

**Root colonization effect:** Inoculated bacterial number (log CFU/g soil) in the rhizosphere of wheat was determined after one month of inoculation in pot and field experiments. Number of bacteria in the rhizosphere of plants with coated seeds ranged from $10^5$-$10^6$ and $10^5$ -$10^7$ CFU/g of soil in pot as well as in field experiments, respectively. Plants inoculated with ESBF showed several bacteria $10^6$-$10^8$ and $10^7$-$10^8$ CFU/g of soil in pot and field experiments, respectively. Plants inoculated with liquid inoculum varied from $10^5$-$10^7$ and $10^6$-$10^7$ CFU/g of soil in pot and field experiments (Fig. 6a and 6b).

**Unveiling the secrets of soil: a physiochemical analysis before and after harvesting:** The physicochemical properties of the soil are presented in (Table 3), revealing no significant differences in pH, salinity, texture, moisture content, and temperature before and after sowing. However, after harvesting, a remarkable increase in the availability of phosphorus (P) and nitrogen (N) was observed across all soil samples.

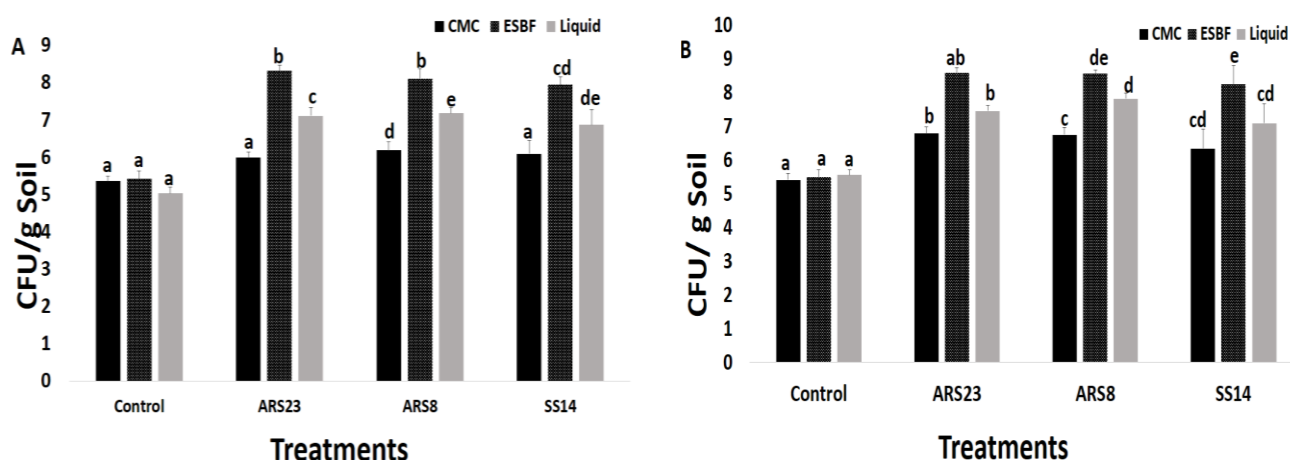


Fig. 6. Population density of different inoculated PGPR strains in wheat rhizosphere after 30 days old seedlings (a) CFU of samples collected from pot experiment (b) CFU of samples collected from a field experiment. This means carrying different letters are significantly different using LSD values at $p<0.05$. Error bars are standard errors from three replicates that received the same treatment.

**Table 3. Physiochemical properties of soil before sowing and after harvesting of wheat (Field experiment).**

| Parameters | Physiochemical properties of soil before sowing | Physicochemical properties of soil after harvesting | | |
|---|---|---|---|---|
| | | Treatment 1 (Enriched soil-based biofertilizer) | Treatment 2 (CMC coated seeds) | Treatment 3 (Liquid inoculum) |
| O.M (g/kg) | $28^a$ | $31^b$ | $31^b$ | $31^b$ |
| Available p (mg/kg) | $1.9^a$ | $2.57^c$ | $2.17^b$ | $2.27^b$ |
| $NO^{-3}$ (mg/kg) | $12.56^a$ | $13.56^a$ | $13.29^a$ | $13.32^a$ |
| Ca (mg/kg) | $1.3^a$ | $1.39^a$ | $1.32^a$ | $1.34^a$ |
| Mg (mg/kg) | $1^a$ | $1.13^a$ | $1.11^a$ | $1.12^a$ |
| pH | $7.70^a$ | $7.60^b$ | $7.68^b$ | $7.65^b$ |
| Temperature | $25^a$ | $35^b$ | $35^b$ | $35^b$ |
| Moisture | $29^a$ | $19^b$ | $19^b$ | $19^b$ |
| Texture | Silty loam | Silty loam | Silty loam | Silty loam |

Note: O.M (organic matter); P (Phosphorus); Ca (Calcium); Mg (Magnesium);g (gram);kg (Kilogram);mg (mili gram)

*Values are the mean of three samples (mean ± SE). Different letters represent statistically different values between treatments using LSD values at $p<0.05$

## Discussion

Biofertilizers are one of the most important approaches for sustainable r growth and development of plants. They provide nutrients to plants and improve the yield of many crops (Babalola & Igiehon, 2017; Basu *et al*., 2017). PGPR with plant growth properties has been reported from many plants. However, a limited number of isolates are available as a carrier-based product. Carriers are the best vehicle for the transportation of microbial inoculants in optimum numbers to the target crop because the viability of PGPR in bioformulations for long-term storage is a serious problem.

Bacterial strains ARS23, ARS8, and SS14 were identified as *B. lichenoformis*, *B. marisflavi,* and *P. fluorescence*. *Bacillus* and *Pseudomonas* are the most commonly reported genera isolated from different plants including mesophytes, halophytes, and xerophytes (Mehnaz *et al*., 2010; Tahir *et al*., 2015; Kruasuwan & Thamchaipenet, 2016). The PGPR belonging to genera *Enterobacter*, *Bacillus*, *Azospirillum*, *Burkholderia*, *Serratia,* and *Pseudomonas* have been suggested for their promising influences as biofertilizers (Berninger *et al*., 2018).

As nitrogen is a vital macro element for proper plant growth, it significantly improves crop yield and plays an important role in the biochemical and physiological activities of plants. Therefore, considering this importance, bacterial strains were screened for their nitrogen fixation ability. Based on the acetylene reduction assay, *P. flouresence* SS14 showed the ability to fix nitrogen. Biological Nitrogen fixation provides nitrogen to plants and it is also part of environmentally friendly agricultural practices (Cakmakci *et al*., 2007). A large number of free-living rhizobacteria, such as *Azospirillum*, *Bacillus*, *Pseudomonas*, *Serratia*, and *Streptomyces* are well-known $N_2$ fixing bacteria (Cakmakci *et al*., 1999; Aamir *et al*., 2020).

All three strains could produce IAA (Table 1). The ability of bacterial isolates to produce IAA showed their ability to be used as growth regulators for plants. Our results are in agreement with previous studies that *Enterobacter, Bacillus*, *Pseudomonas*, and *Azospirillum* strains produce IAA (Mehnaz *et al*., 2010; Suleman *et al*., 2018; Mukhtar *et al*., 2020). IAA-producing bacteria have been documented as a promising way to improve plant biomass, root length, and root surface area (Chen *et al*., 2014; Ali *et al*., 2017).

All three strains could solubilize insoluble phosphate into organic soluble phosphates (Table 2). The potential of PGPR strains to convert insoluble phosphate into soluble form is an important property under conditions where phosphorous is a limiting factor for crop growth. Phosphate-solubilizing bacteria increase the available form of phosphates in soil is helpful for sustainable agriculture (Majeed *et al*., 2015; Suleman *et al*., 2018).

For a successful bioformulation development, the survival of bacteria for a long time is very crucial. Based on shelf life during storage at room temperature ESBF proved the best storage carrier material for PGPR growth and survival. ESBF due to its optimum moisture content, pH, sufficient nutrients, and non-toxic nature when inoculated with PGPR strains *P. fluorescens* SS14, *B. marisflavi* ARS23, and *B. lichenoformis* ARS8 showed good shelf life and supported high bacterial population of inoculated strains up to 180 days of storage (Fig. 2A). Brockwell & Bottomley (1995), found that carriers with good aeration and neutral pH and adequate nutrients could support maximum microbial population. According to Macik *et al*., (2020), soil provides a large surface area for nutrient absorption and protection. It has a good cation exchange capacity which boosts up metabolic processes of bacteria leading to higher viability.

Inoculation of *Bacillus* and *Pseudomonas* strains improved the growth of wheat seedlings. Shen *et al*., (2013) also reported positive effects of inoculation of *Bacillus*, *Pseudomonas,* and *Enterobacter* strains on seedlings development. Different root growth parameters were significantly increased i.e., root length, diameter, and volume of inoculated plants with bacterial strains ARS23, ARS8, and SS14. It has found a positive effect on root growth of inoculated plants with bacterial strains ARS8, ARS23, and SS14 (Fig. 3). Many studies have stated root growth improvement by using PGPR, mainly at the early plant stages (Dal Cortivo *et al*., 2017, 2020).

Results of the pot experiment showed that these formulations could be further evaluated under field conditions. Data indicated that seed inoculation with all bioformulations was found to enhance the plant length, over non-inoculated plants. Plants inoculated with ESBF showed a significant increase of 42-57% in number of spikes and weight of 1000 grains (17-22%) compared to non-inoculated plants (Fig. 5E and 5F). Our findings also showed that plants inoculated with ESBF had a significant increase in yield of 16-19% (Fig. 5G). Being a good carrier material, ESBF with PGPR is considered a cost-effective, non-toxic, easy-to-process, and easily available biofertilizer. These findings were supported by a previous report that plants inoculated with ESBF inoculated with *Bacillus* strains PSB5 and PSB12 showed a significant increase in yield over to non-inoculated plants (Mukhtar *et al*., 2017). Inoculation effects of *Pseudomonas* and *Bacillus* strains on wheat growth, yield, and physiological attributes have also been documented by previous studies (Tabassum *et al*., 2017; Dal Cortivo *et al*., 2020). The findings of the present work also agreed with the study that the application of *Bacillus* and *Pseudomonas* bioformulations increased wheat yield by 15-25% over non-inoculated plants (Hassan & Bano, 2015).

In addition to the effect of the bioformulations on the plant's growth parameters, we also studied the persistence of each inoculated bacteria in the rhizosphere of wheat. Survival study of inoculated strains with different carrier materials in the rhizosphere competition with indigenous microflora is as important as the PGP trait and suitable carrier itself (Compant *et al*., 2019; Rilling *et al*., 2019). Roots of plants inoculated with ARS8, ARS23, and SS14 showed maximum bacterial count and are dominant colonizers (Fig. 6A and B). Many studies already reported the beneficial effects of these bacterial genera on different crops' growth and productivity. A great number of inoculated bacteria in the soil promote plant growth. Colonization of inoculated bacteria to the root surface

helps plant nutrition and growth (Lodewyckx *et al*., 2002; Smith & Read, 2008; Pagnani *et al*., 2020). However, this experiment proved that soil fertility was suitable for the inoculated bioformulations and preceded colonization in competition with the indigenous microbiome.

Plant growth-promoting bacteria play a vital role in enhancing soil fertility through the addition of essential nutrients such as phosphorus, nitrogen, potassium, and others. Therefore, an investigation of soil properties was conducted at the time of harvesting after inoculating these bacteria. No significant differences were observed in the physical properties of the soil, including pH, salinity, texture, and temperature, before and after harvesting. However, the availability of phosphate significantly increased in the inoculated soil samples following the harvest. Previous studies have reported the positive effects of inoculating rhizospheric microorganisms on crop yield and soil fertility in various plants, including potatoes and leguminous and non-leguminous species (Suri *et al*., 2011; Singh *et al*., 2013; Pagnani *et al*., 2020).

## Conclusion

The main objective of the present study was to develop efficient and cost-effective bioformulation for plant growth with minimum use of chemical fertilizers. The comparative effect of liquid inoculum, coated seeds, and ESBF on wheat plants in pot as well as in field experiment was analyzed. Formulation of *Bacillus* strains ARS23, ARS8, and *Pesudomonas* strain SS14 in ESBF stored at 30ºC showed better shelf life and increased CFU $g^{-1}$ of soil as compared to liquid inoculum and coated seeds. PGPR helped to improve wheat seed germination and seedling growth. ESBF significantly increased wheat growth and yield at pot scale as well as in field experiments. *Bacillus* strain ARS23 and *Pseudomonas* strain SS14 showed comparatively better results as compared to *Bacillus* strain ARS8 at both levels. Survival and colonization studies of these multifaceted PGPR strains indicated that they have the potential to be used as biofertilizers for different crops.

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