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Full Length Article

Appraising the Potential of Integrated use of *Bacillus* Strains for Improving Wheat Growth

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Abstract

Plant growth-promoting rhizobacteria (PGPR) and endophytes form symbiotic associations with plants and improve growth through several mechanisms. In the present study, phosphate (P) solubilizing PGPR and endophytic bacteria (EB) were isolated from wheat roots and screened for plant growth-promoting features. Most of the P solubilizing isolates were capable of solubilizing zinc along with production of exopolysaccharides (EPS) and siderophores. A total of seven isolates from endophytic bacteria (EB) (ZE1, ZE5, ZE7, ZE15, ZE19, ZE32 and ZE38) and eight isolates from rhizosphere bacteria (ZR2, ZR3, ZR5, ZR7, ZR15, ZR18, ZR19 and ZR25) were chosen to evaluate their plant growth promoting prospective under axenic conditions. Results revealed that the maximum root colonization, and improvement in seedling vigor index (69%), root length (31%), shoot length (29%), root dry biomass (33%) and shoot dry biomass (36%) as compared to uninoculated control was found by co-inoculation of ZE32 and ZR19 followed by the combination ZE15 + ZR2, and ZE15 + ZR3. Strains from these best performing combinations were identified as *Bacillus subtilis* ZE15, *B. megaterium* ZE32, *B. subtilis* ZR2, *B. subtilis* ZR3 and *B. megaterium* ZR19 through 16S rRNA partial gene sequencing. These strains possess multiple plant growth promoting characteristics and can be recommended as potential bioinoculants for biofertilizer after extensive evaluation under field conditions. © 2020 Friends Science Publishers

Keywords: *Bacillus* spp.; Phosphate solubilization; *Triticum aestivum*; Growth; Root colonization

Introduction

Soil-plant-microbe interactions have significant role in plant growth, development and soil health even under a nutrient-deficient environment (Etesami and Maheshwari 2018). Numerous bacterial genera reside in rhizosphere and inside plant tissue are called as PGPR and endophytes, respectively (Kumar and Sharma 2017; Singh *et al.* 2017). These are diverse group of bacteria form a mutual association with host plants and improve plant growth and development (Hussain *et al.* 2016; Singh *et al.* 2017). Endophytic bacteria live inside the plant tissue without damaging them (Meela and Serepa-Dlamini 2019) while rhizobacteria live in rhizosphere and make symbiotic associations with plants (Khan *et al.* 2017). These bacteria also alleviate environmental stresses (Etesami and Maheshwari 2018; Danish *et al.* 2020). Recent studies about plant-microbe associations revealed that plants synergize with nutrient solubilizing PGPR and endophytic communities under nutrient-deficient conditions (Berendsen *et al.* 2012; Ahmad *et al.* 2019b). The symbiotic relationships between plant and bacteria exist in different ecosystems and have a significant role in nutrients acquisition (Luo *et al.* 2014).

Phosphorus (P) is the second most important macronutrient needed by plants for their growth and development (Minhas *et al.* 2020). The soil-inhabiting microbes are responsible for the P cycle continuation within the biosphere. The mechanisms concerned in P solubilization rely upon the nature of phosphate complexes (Nadeem *et al.* 2014). Although 0.05% (w/w) P is present in soil in the form of organic and inorganic P, however, its availability for plant uptake is restrictive and among total P, only 1% is available for immediate use by plants (Lambers and Plaxton 2015). Phosphate solubilizing bacteria (PSB) solubilize inorganic phosphate complexes through production of organic acids and discharge of proton (Rfaki *et al.* 2014). The extent of fixed P in soil depends on the soil pH, as in acidic pH, it forms complexes with iron and aluminum, while in alkaline pH, it forms complexes with calcium (Wei *et al.* 2018; Munir *et al.* 2019). Specialized microbial enzymes called phosphatases solubilize the organic P and release it for plant (Goswami *et al.* 2016). The calcareous soil has a high buffering capacity that challenges the effectiveness of PSB in liberating P (Stephen and Jisha 2008).

The application of P fertilizers is increasing day by day due to extensive crop cultivation. The dynamics of P in the soil are complicated as a large proportion of applied

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P fertilizers move into the immobile pool resulting in poor fertilizer use efficiency (FUE). However, numerous bacteria located in the rhizosphere have the ability to solubilize immobile and precipitated P contents making it available for plant uptake (Richardson and Simpson 2011). Therefore, the application of PSB as biofertilizer is a promising eco-friendly strategy to improve P uptake and reducing the use of chemical fertilizers. Mostly, PSB belong to heterotrophic bacteria that produce organic acids as their secondary metabolites, which solubilize the insoluble P (Glick 2014; Suleman *et al.* 2018). They are not only involved in P release in soil solution but these bacteria also assimilate P for their own use to expand their colonies thus help in improving soil microbial population (Khan and Joergensen 2009). The PGPR and endophytes are the broad groups of bacteria that offer huge diversity and potential to convert insoluble P into plant-available form (Chaves *et al.* 2019). Such PSB use the alkaline and acidic phosphatases which use insoluble compounds as a substrate and free up bioavailable inorganic P (Dodor and Tabatabai 2003). Application of multi-strain PSB biofertilizer is also helpful in improving FUE especially for P fertilizers through solubilizing non-labile or precipitated P (Perez *et al.* 2007).

The PGPR release organic acids in rhizosphere that solubilize unavailable source of P while some bacteria improve P availability enzymatically by releasing phosphatases which mineralize organic P (Nesme *et al.* 2018; Chen and Liu 2019). Scientists described the mechanisms adapted by bacteria for mineralization of organic P through release of phosphatase, phytase and C-P lyase (Sembiring *et al.* 2017). The P solubilization in rhizosphere is affected by soil pH, organic matter, nature of P complexes, environmental conditions and most prominently the interaction of P solubilizing bacteria with other microorganisms in rhizosphere (Billah *et al.* 2019). Solubilization of P by bacteria reduce the use of synthetic fertilizers which ultimately lessen the cost of production and improve soil health (Nazli *et al.* 2020). In addition to increase in P availability, PGPR promote plant growth by increasing germination, inducing resistance against environmental stresses, improving root morphology and working as biocontrol agent against pathogens (Ahmad *et al.* 2019b).

The EB live inside plant roots and promote plant growth by performing complex functions in regulating plant physiology (Lucava and Azevedo 2013). They are involved in biocontrol of plant-pathogens through their antagonistic nature against pathogens especially the fungi. The EB and PGPR are well-documented for symbiotic nitrogen fixation, solubilization of P, potassium (K), and zinc (Zn), production of phytohormones, siderophores, allelochemicals and interact with other useful soil-inhabiting microorganisms (Garcia-Frail *et al.* 2015; Naseer *et al.* 2019). It is evident from previous studies that soil-plant associate bacteria improved the growth of crop plant. However, biochemical processes involving in plant growth promotion were not clear yet. Therefore, present study was designed to isolate and characterized endophytes and rhizosphere bacteria for biochemical and growth promoting attributes. The selected strains were genetically identified through 16S rRNA sequencing. The individual and combined inoculation of selected P solubilizing EB and PGPR, was also evaluated for improving seedlings vigor index and growth attributes of wheat (*Triticum aestivum* L.) under axenic conditions.

Materials and Methods

Isolation of endophytic and rhizospheric bacteria

Wheat plants were randomly selected, uprooted and whole plants including the root system were carried to the laboratory in sterile plastic bags, and stored at 4ºC until further processing. For isolation of EB, the root system of collected plants was separated from shoots and washed under tap water to dispose of adhering soil debris. After that, surface sterilization of root was done by dipping them in 1% solution of NaClO for 3 min and in 70% ethanol for 5 min. During surface sterilization, roots were five times rinsed with sterile distilled water before and after dipping in ethanol (Hallmann *et al.* 2006). Surface sterilized root material then macerated and crushed in a sterilized mortar using phosphate-buffered saline solution to make a homogeneous mixture. The root extract was diluted up to 10^{-8} and 1 mL of aliquot was transferred on autoclaved Reasoner's 2A (R2A) agar media which was prepared by dissolving the following chemicals in 1L distilled water; protease peptone (0.5 g), casamino acids (0.5 g), yeast extract (0.5 g), dextrose (0.5 g), soluble starch (0.5 g), dipotassium phosphate (0.3 g), magnesium sulfate (0.05 g), sodium pyruvate (0.3 g), agar (15 g) and pH was adjusted to 7.0 ± 0.2 (Reasoner and Geldreich 1985). The agar plates were incubated at 30 ± 1ºC for 72 h. For isolation of RB, a suspension in sanitize distilled water was made with 1 g of wheat rhizosphere soil and serially diluted up to 10^{-8}. One mL of aliquot from every dilution was transferred on R2A agar plates and incubated at 30 ± 1ºC for 72h. The representative different morphological colonies resulted both from EB and RB isolates were purified through multiple streaking method and a total of 40 pure isolates each for EB and RB were kept in 50% glycerol stock at -20ºC till more experimentations.

Screening for *in vitro* plant growth-promoting characteristics

The bacterial isolates were screened for *in vitro* PGP characterization in terms of solubilization of P and Zn, and production of exopolysaccharides (EPS) and siderophores. Bacterial isolates were routinely grown on Dworkin and Foster (DF) salt agar (Dworkin and Foster 1958). The P solubilization was evaluated by spot inoculation of overnight grown isolates on Pikovskaya's (PVK) agar media (Pikovskaya 1948) and incubated at 30 ± 1ºC for 72 h. Zn solubilization by bacterial isolates was evaluated

through inoculating freshly grown isolates on tris-minimal salt media (Fasim *et al.* 2002) and incubated at 30 ± 1ºC for 72 h. After incubation of both P and Zn solubilization bioassays, the presence of the halo zones around the bacterial colonies was deliberated positive both for P and Zn solubilization, respectively. The halo zone diameter and colony diameter were measured to calculate solubilization index (SI) and solubilization efficiency (SE) of both for P and Zn by following the formula reported by Ahmad *et al.* (2019a). The quantitative solubilization of P by isolates was assessed by using the PVK broth medium. After incubation of inoculated PVK broth at 30 ± 1ºC for 72 h, were centrifuged. Available P concentration in the supernatant was measured as reported by Ryan *et al.* (2001). For the determination of solubilized Zn concentration, isolates were grown in tris-minimal salt broth modified with 0.1% ZnO and incubated for 72 h at 30 ± 1ºC. After incubation, culture supernatant was subjected to Atomic Absorption Spectrophotometry analysis and values were compared with a calibration curve drawn using working standards. Siderophores production was determined by streaking isolates on chrome azurol S (CAS) agar media (Schwyn and Neilands 1987) and plates were incubated at 30 ± 1ºC for 48 h. After incubation, orange halos around the colonies were considered siderophores positive. The isolates were screened for exopolysaccharides (EPS) production by propagating them on De Man, Rogosa and Sharpe (MRS) agar media at 30 ± 1ºC for 72 h. The isolates that produce mucoid colonies were recorded EPS positive.

The amount of indole-3-acetic acid (IAA) production by way of bacterial isolates both in the presence and absence of L-tryptophan (L-trp) become assessed through a colorimetric technique using Salkowski's method (Ehmann 1977). The 48h old bacterial cultures were inoculated in DF salt media in the presence and absence of L-trp and incubated for 48 h at 30 ± 1°C and 100 r.p.m. After incubation broth cultures were filtered through sterile Whatman # 1 filter paper. The IAA standards of various concentration *viz*., 25, 50, 100, 150, 200, 250, and 300 μg mL^{-1} was prepared. The 1 mL of culture aliquot and 2 mL of Salkowski's reagent was mixed and put in dark for 60 min. The optical density (OD) was measured at 530 nm and values were compared by plotting standard curve. The catalases, oxidase, and urease activities by selected EB and RB isolates were performed through following standard procedures reported by Cappuccino and Sherman (Cappuccino and Sherman 2002). Some miscellaneous plant growth promoting characterization tests in terms of chitinolytic activity, cellulose degrading ability, protease, and esterase activities were also carried out by following the standard protocols (Atlas 1946; Sierra 1957; Chernin *et al.* 1998; Patel and Desai 2015).

Effect of PSB isolates on growth of wheat seedlings in axenic conditions

The efficient PSB isolates were confirmed for their prospective to escalates growth of wheat seedlings under axenic conditions. These conditions were maintained in growth room as 70% humidity, 12 h day length (light intensity @ 1000 flux unit $area^{-1}$ unit $time^{-1}$ with artificial light) and day and night temperature was adjusted as 20°C and 15°C, respectively. Seven isolates from EB and eight isolates from PGPR were prepared through growing in R2A broth for 48 h in a shaking incubator at 30 ± 1°C. Wheat seeds were surface disinfected using ethanol (95%) and mercuric chloride (0.2%) solution as reported by Al-Adham *et al.* (2012). Surface sterilized wheat seeds were inoculated with EB and PGPR isolates through dipping in respective culture for 10 min. Plastic jars (20 cm height) filled with sand (600 g jar^{-1}) were moisturized with half-strength Hoagland solution and autoclaved. Inoculated wheat seeds were sown in each jar but control was maintained by sowing seeds soaked in a sterilized broth. Jars were arranged in completely randomized design in triplicate and incubated in alternative 12 h of light at 20 ± 2ºC and 12 h of the dark period at 15 ± 2ºC. To meet nutritional and irrigational requirements, jars were irrigated with a half strength Hoagland solution. Three plants in each jar were maintained by thinning after establishment of seedlings. The ability of the isolates to colonize wheat roots was estimated by taking seven days old root samples (0.2 g) in a sterilized mortar and crushed after adding sterilized water (5 mL). The crushed suspension was diluted up to 10^{-5} and 1 mL from every dilution was poured on R2A agar plates and incubated at 30 ± 1°C for 72 h. After incubation, total bacterial colonies were calculated by using digital colony counter and expressed as a colony-forming units (CFU) mL^{-1}.

After three-weeks of sowing, wheat seedlings were harvested and growth attributes in terms of shoot length, root length, shoot and root dry biomass were recorded. Four most efficient isolates each from EB and PGPR were selected on the basis of better tendency to improve wheat growth and were evaluated for compatibility of isolates to each other. Two isolates (one from EB and other from PGPR) were cross streaked on R2A agar plates in perpendicular direction. Similarly, sets of compatibility tests were conducted through cross-matching the rest of EB and RB isolates and incubated in triplicate at 30 ± 1°C for 48 h. The inhibition zone at the point of intersection was observed and results marked compatible for isolates having no inhibition zone at the point of intersection. The compatible EB and RB isolates were selected to assess for their growth promoting ability through co-inoculation. A jar trial was conducted through sowing wheat seeds co-inoculated with respective EB and PGPR isolates in a 1:1 ratio. All protocols of jar experiment were followed as reported above for sole inoculation. After three-weeks of emergence, seedling vigor index was calculated and growth attributes were measured as mentioned above for sole inoculation.

Identification of selected isolates

The best isolates on the basis of growth-promoting abilities

(two from EB; ZE15 and ZE32 and three from PGPR; ZR2, ZR3, and ZR19) were selected for identification through 16S rRNA partial gene sequencing. Crude DNA of selected isolates was extracted through treating with proteinase K (Cheneby *et al.* 2004). The PCR reactions were performed by using 2.5 μL crude DNA and PCR primers were 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Hussain *et al.* 2015). The length of amplified product was confirmed through setting apart on agarose gel (1%) along with GeneRuler. The purified PCR product was sequenced using commercial service of MACROGEN Seoul, Korea (http://macrogen.com/eng/) by using sequenced primers 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAATTCMTTTRAGTTT). Resulted sequences were blasted on NCBI servers and strains were identified through constructing the phylogenetic tree with selected closely related taxa using MEGA 7.0.14 (Roohi *et al.* 2012; Kumar *et al.* 2016).

Statistical analysis

For statistical analyses, the data were compared through one-way analysis of variance (ANOVA) technique by employing a liner model Completely Randomized Design (CRD). The means were compared through multiple comparisons (LSD at 5% level of probability) using Statistix v. 8.1 (Analytical Software, Tallahassee, FL, USA) (Steel *et al.* 1997).

Results

Isolation of P solubilizing EB and RB isolates

Forty isolates each for endophytic bacteria; coded as ZE1, ZE2… ZE40 and PGPR; coded as ZR1, ZR2, … ZR40 were isolated and screened for the solubilizing P qualitatively and quantitatively. Results of qualitative P solubilization showed that 11 EB and 12 PGPR isolates were able to solubilize P (Table 1). The PGPR isolates showed more promising results in terms of qualitative P solubilization, SE, SI and solubilized concentration than EB isolates. Isolate ZR3 showed the maximum P solubilization halo zone diameter (21.7 mm), SE (369%), and SI (4.7) (Table 2). The EB isolates were also better P solubilizers. Among them, isolate ZE32 showed the maximum P solubilization halo zone diameter (18 mm), SE (290%), and SI (3.9). Quantitative P solubilization revealed that RB isolates ZR3 and ZR19 showed the maximum concentration of solubilized P (32.7 mg L^{-1}) which were statistically similar to each other (Table 2). Among EB isolates, ZE32 showed the maximum P solubilization (32 mg L^{-1}) which was non-significant ($P \geq 0.05$) with RB isolates ZR3 and ZR19. The isolates ZE2, ZE10, ZE18, ZE28, ZR11, ZR22, ZR36, and ZR40 were weak P solubilizers, and were not selected for further evaluation.

Screening for *in vitro* plant growth-promoting characteristics

Phosphate solubilizing EB and PGPR isolates were screened for solubilization of Zn, and production of EPS and siderophores. Results (Table 1) indicated that most of the isolates were progressive for Zn solubilization except isolates ZE2, ZE28 and ZR11. The isolate ZE19 showed the maximum Zn halo zone diameter (24.3 mm), however the maximum SE of 244% and SI of 3.4 was observed by the isolate ZR19. Isolate ZR19 also showed the maximum concentration of solubilized Zn (27.7 L^{1}) that was statistically similar to isolates ZR2 and ZR3 ($P \leq 0.05$). Among EB, isolate ZE15 showed better results in terms of solubilized Zn (26 mg L^{-1}) which was non-significant ($P \geq 0.05$) to isolates ZR2, ZR3, and ZR19. The majority of tested PSB isolates have the ability to produce siderophores except the isolates ZE18, ZR11, ZR22, ZR36, and ZR40, while all isolates were able to produce exopolysaccharides. Seven EB and eight PGPR isolates positive for all tested traits *viz.*, P and Zn solubilization, and EPS and siderophores production were selected for further evaluation.

Effect of sole inoculation of EB and PGPR isolates on growth of wheat seedlings

Effectiveness of sole inoculation of EB and PGPR isolates to escalate growth of wheat seedlings was estimated in jar trial. The sole inoculation of P solubilizing EB and PGPR isolates significantly ($P \leq 0.05$) enhanced the shoot length, root length, shoot and root dry biomass of wheat seedlings as related to uninoculated control (Table 3). The mean values of growth attributes revealed better results due to inoculation with PGPR isolates over the EB isolates. The highest improvement in shoot length up to 22% was recorded due to application of isolate ZR3 followed by isolate ZR19 (21% increase over control). Results of these isolates were parallel to each other, while more than uninoculated control ($P \leq 0.05$). The isolate ZR3 reported the absolute increase in root length (29%) that was non-significant with the isolate ZE32. The bacterial isolates ZR2, ZR3, and ZR19 showed the highest improvement in dry biomass which were similar to each other. The highest improvement in root dry biomass was observed due to inoculation with ZR19 that showed 65% increase in dry biomass of root. The EB isolates inoculation also showed better increase in growth attributes of wheat seedlings. The EB isolate ZE15 showed better increase in shoot length (17%), root length (24%), shoot dry biomass (17%) and root dry biomass (39%) over un-inoculated control. The best plant growth promoting RB isolates *viz.*, ZR2, ZR3, ZR5, and ZR19 and EB isolates *viz.*, ZE15, ZE19 and ZE32 were further selected for co-inoculation compatibility assay.

Table 1: Evaluation of endophytic and rhizospheric bacterial strains for phosphate and zinc solubilization, and production of exopolysaccharides and siderophores

Bacterial isolates	Phosphate solubilization	Zinc solubilization	Production of EPS	Production of siderophores
ZE1	+	+	+	+
ZE2 *	+	-	+	+
ZE5	+	+	+	+
ZE7	+	+	+	+
ZE10 *	+	-	+	+
ZE15	+	+	+	+
ZE18 *	+	+	+	-
ZE19	+	+	+	+
ZE28 *	+	-	+	-
ZE32	+	+	+	+
ZE38	+	+	+	+
ZR2	+	+	+	+
ZR3	+	+	+	+
ZR5	+	+	+	+
ZR7	+	+	+	+
ZR11 *	+	-	+	-
ZR15	+	+	+	+
ZR18	+	+	+	+
ZR19	+	+	+	+
ZR22 *	+	+	+	-
ZR25	+	+	+	+
ZR36 *	+	+	+	-
ZR40 *	+	+	+	-

*Isolates were week phosphate and zinc solubilizers and were not selected for further experimentations (Results for solubilization of phosphate, zinc, and production of exopolysaccharides (EPS) and siderophores were confirmed by repeating the bioassays in three replication)
The sign, (+) express the accuracy of the tested attributes and the sign, (-) demonstrate the lack of the traits

Table 2: Qualitative and quantitative solubilization of phosphate and zinc by endophytic and rhizospheric bacterial isolates

Bacterial isolates	Phosphate solubilization				Zinc solubilization			
	HZD (mm)	SI	SE (%)	SC (mg L^{-1})	HZD (mm)	SI	SE (%)	SC (mg L^{-1})
ZE1	8.7 gh	3.3 d-f	203.3 d-f	27.3 de	11.0 g	3.1 bc	207.8 bc	22.7 d
ZE5	9.7 g	3.7 b-d	269.4 b-d	28.3 c-e	13.0 fg	3.1 bc	206.4 bc	23.0 cd
ZE7	7.3 h	2.4 f	137.8 f	26.0 de	18.7 bc	2.9 c	193.6 c	23.0 cd
ZE15	16.7 cd	3.7 b-d	265.9 b-d	31.7 a-c	17.7 b-d	3.0 bc	203.7 bc	26.0 ab
ZE19	13.7 ef	3.4 c-e	243.3 c-e	28.7 b-e	24.3 a	3.2 a-c	215.2 a-c	23.4 b-d
ZE32	18.3 c	3.9 bc	290.5 bc	32.0 ab	14.3 ef	3.1 bc	205.6 bc	25.2 a-d
ZE38	12.3 f	3.7 b-d	265.0 b-d	29.3 a-d	14.3 ef	3.1 bc	205.6 bc	23.2 b-d
ZR2	18.7 bc	3.8 bc	281.8 bc	29.3 a-d	19.0 b	3.1 a-d	211.5 bc	27.3 a
ZR3	21.7 a	4.7 a	368.9 a	32.7 a	16.0 c-e	3.3 ab	228.9 ab	27.0 a
ZR5	16.7 cd	4.1 ab	313.3 ab	27.0 de	14.3 ef	3.3 ab	233.3 ab	25.0 a-d
ZR7	15.0 de	3.4 c-e	238.9 c-e	26.3 de	13.3 e-g	2.9 c	190.58 c	25.0 a-d
ZR15	13.3 ef	2.9 ef	187.8 ef	25.3 e	15.3 d-f	2.9 c	192.1 c	24.0 b-d
ZR18	13.3 ef	2.6 ff	161.1 f	27.3 de	14.3 ef	2.9 c	186.8 c	25.0 a-d
ZR19	20.7 ab	4.0 bc	299.4 bc	32.3 a	18.7 bc	3.4 a	244.1 a	27.7 a
ZR25	12.7 f	2.9 ef	193.6 ef	26.0 de	13.0 fg	3.1 bc	205.6 bc	25.7 a-c
LSD ($P \leq 0.05$)	2.05	0.66	66.33	3.38	2.99	0.32	31.39	2.84

Means sharing same letter(s) within the column are similar to each other according to least significant difference (LSD) test at P 0.05
HZD= Halo zone diameter; SI= Solubilization index; SE= Solubilization efficiency; SC= Solubilized concentration; ZE and ZR, Z is taken from first letter of researchers' name (Zafar Iqbal) while E and R for endophytes and rhizospheric bacteria, respectively

Compatibility test and effect of co-inoculation of EB and PGPR isolates on growth of wheat

The selected PGP isolates of EB and PGPR were cross streaked on agar media for their compatibility test and results revealed compatibility of isolate ZE15 with isolates ZR2, ZR3, and ZR19 while isolate ZE19 was compatible with isolates ZR3 and ZR19 (Table 4). The isolate ZE32 was compatible with isolates ZR3, ZR5, and ZR19. All other EB and RB isolates were antagonistic to each other. The compatible isolates were co-inoculated on wheat seeds to assess their competence to stimulate growth of wheat.

Outcomes of the stimulus of co-inoculation with EB and PGPR isolates on wheat seedlings are presented in Table 5. The co-inoculation with EB and PGPR isolates exhibited considerable ($P \leq 0.05$) escalation in seedling vigor index, and growth of wheat seedlings as related to un-inoculated control. The co-inoculation treatment ZE32 + ZR19 was the best combination to improve seedlings growth of wheat then other co-inoculation treatments. It exhibited the maximum improvement in seedling vigor index (69%), root length (31%), shoot length (29%), root

Table 3: Effect of sole inoculation of endophytic and rhizospheric bacterial isolates on growth of wheat seedlings under axenic conditions

Bacterial isolates	SL (cm)	RL (cm)	SDB (g jar^{-1})	RDB (g jar^{-1})
Control	7.1 i	6.8 i	0.05 e	0.04 i
ZE1	7.4 gh	6.9 hi	0.05 e	0.04 f
ZE5	7.6 fg	7.1 gh	0.05 e	0.04 fg
ZE7	7.7 ef	7.3 f	0.05 e	0.04 h
ZE15	8.3 bc	8.4 b	0.06 bc	0.05 de
ZE19	7.6 fg	7.9 c	0.05 de	0.04 f
ZE32	8.2 cd	8.7 a	0.06 cd	0.05 e
ZE38	7.4 gh	8.0 c	0.05 de	0.04 gh
ZR2	8.0 de	8.3 b	0.07 a	0.06 b
ZR3	8.6 a	8.8 a	0.07 a	0.06 a
ZR5	7.3 hi	7.4 ef	0.06 bc	0.05 cd
ZR7	7.4 h	7.3 fg	0.06 bc	0.05 de
ZR15	7.5 f-h	7.7 d	0.06 b	0.05 c
ZR18	7.4 gh	7.9 c	0.06 b	0.05 c-e
ZR19	8.5 ab	8.4 b	0.07 a	0.06 a
ZR25	7.3 hi	7.6 de	0.06 b	0.05 c-e
LSD ($P \leq 0.05$)	0.26	0.21	0.004	0.002

Means sharing same letter(s), within the column are statistically similar to each other according to least significant difference (LSD) test at $P \leq 0.05$; Three plants were maintained in each jar

SL=Shoot length; RL = Root length; SDB = Shoot dry biomass; RDB = Root dry biomass

Table 4: Compatibility test of endophytic and rhizospheric bacterial isolates

Combination	Compatibility	Combination	Compatibility
ZE15 + ZR2	Compatible	ZE19 + ZR5	Not compatible
ZE15 + ZR3	Compatible	ZE19 + ZR19	Compatible
ZE15 + ZR5	Not compatible	ZE32 + ZR2	Not compatible
ZE15 + ZR19	Compatible	ZE32 + ZR3	Compatible
ZE19 + ZR2	Not compatible	ZE32 + ZR5	Compatible
ZE19 + ZR3	Compatible	ZE32 + ZR19	Compatible

Incompatible pairs of isolates were not selected for further evaluation (Compatibility of endophytic and rhizospheric bacterial isolates were confirmed by repeating the bioassays in three replications)

Isolates were cross streaked on a petri plate and incubated at 30°C ± 1 and observed the growth after 24, 48 and 72 hours. Growth inhibition at the point of intersection means isolates were incompatible and vice versa.

Table 5: Effect of co-inoculation of endophytic and rhizospheric bacterial isolates on growth of wheat seedlings under axenic conditions

Co-inoculation	SVI	SL (cm)	RL (cm)	SDB (g jar^{-1})	RDB (g jar^{-1})
Control	6.2 e	6.5 g	6.4 h	0.041 f	0.037 d
ZE15 + ZR2	9.4 b	7.0 de	7.5 de	0.053 b	0.048 a
ZE15 + ZR3	7.4 d	7.9 b	8.2 b	0.046 de	0.040 c
ZE15 + ZR19	8.3 cd	7.2 d	7.7 c	0.049 cd	0.044 b
ZE19 + ZR3	7.4 d	6.8 f	7.4 ef	0.045 e	0.044 b
ZE19 + ZR19	8.1 cd	7.5 c	7.7 cd	0.044 e	0.039 cd
ZE32 + ZR3	8.7 bc	6.8 f	6.8 g	0.049 cd	0.045 b
ZE32 + ZR5	8.4 b-d	6.9 ef	7.3 f	0.050 c	0.040 c
ZE32 + ZR19	10.5 a	8.4 a	8.4 a	0.056 a	0.049 a
LSD ($P \leq 0.05$)	1.06	0.24	0.18	0.003	0.003

Means sharing same letter(s) within the column are statistically same according to least significant difference (LSD) test at $P \leq 0.05$; Three plants were maintained in each jar

SVI= Seedling vigor index; SL= Shoot length; RL= Root length; SDB= Shoot dry biomass; RDB= Root dry biomass

dry biomass (33%) and shoot dry biomass (36%) as compared to uninoculated control. The co-inoculation combination of ZE15 + ZR2 also showed better root dry biomass of wheat seedlings as compared to un-inoculated control and was similar to the co-inoculation of ZE32 + ZR19.

Growth promoting attributes and biochemical features of selected isolates

The selected two isolates of EB (ZE15 and ZE32) and three isolates of PGPR (ZR2, ZR3, and ZR19) were tested for multiple plant growth promoting attributes. All of these isolates showed production of IAA in presence as well as absence of L-trp (Table 6). Accumulation of IAA by selected isolates in presence and absence of L-trp was similar ($P \leq 0.05$) to each other. Isolate ZE32 produced the maximum IAA without L-trp (3.0 μg mL^{-1}) and with L-trp (16.9 μg mL^{-1}) and was statistically non-significant to other tested isolates. The EB isolates were better in terms of root colonization over PGPR isolates (Table 6). The EB isolates ZE15 and ZE32 showed root colonization up to 3.0×10^6 CFU g^{-1} while all PGPR isolates ZR2, ZR3 and ZR19 showed 1.5×10^6 CFU g^{-1} root colonization. All the tested isolates have the ability to produce HCN except isolates ZE15 and ZR19. The enzymatic activities in terms of catalase, oxidase, protease, cellulase, urease, chitinase and esterase activities by EB and RB isolates were performed and their results are depicted in Table 6. All the tested isolates were positive for protease, cellulase, and esterase activities. Three isolates *e.g*. ZR2, ZR3, and ZE15 demonstrated oxidase activity while urease activity was only observed in isolate ZE32. Only PGPR isolates (ZR2, ZR3, and ZR19) were catalase positive while all the tested isolates were negative for chitinase activity.

Identification of selected isolates

The selected plant growth promoting EB and PGPR isolates *viz*., ZE15, ZE32, ZR2, ZR3, ZR19 were identified through 16S rRNA partial gene sequencing. The isolate ZE15, ZR2, and ZR3 showed resemblance with *Bacillus subtilis* up to 99.85, 99.55 and 99.71%, respectively. These isolates were identified as a *B. subtilis* ZE15 (Fig. 1), *B. subtilis* ZR2 (Fig. 2), and *B. subtilis* ZR3 (Fig. 3) and submitted to NCBI under accession number MN003400, MN007184 and MN007185, respectively. The isolates ZE32 and ZR19 showed similarity of 99.55 and 97.78%, respectively, with *B. megaterium* and identified as *B. megaterium* ZE32 (Fig. 4) and *B. megaterium* ZR19 (Fig. 5), respectively. These isolates were submitted to NCBI under accession numbers MN003401 and MN007186.

Discussion

The sole as well as co-inoculation of compatible EB and PGPR isolates significantly promoted the growth of wheat seedlings and the selected EB and PGPR isolates were identified as *Bacillus* spp. through bioinformatics analysis (Table 1–6).

In the present study, the EB and PGPR were isolated and selected on the basis of solubilization of insoluble source of P. Qualitative P solubilization revealed the solubilization index was in the range of 2.3 to 3.9 by EB and

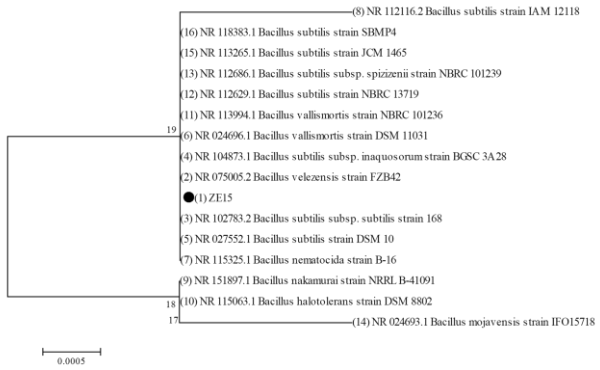

Fig. 1: Neighbor-joining phylogenetic evaluation consequent from the various associations of 16S rRNA gene sequence of *Bacillus subtilis* ZE15 with existences of different bacterial strains from Gene Bank database

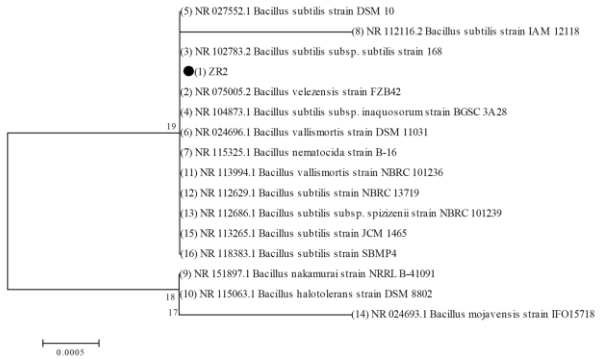

Fig. 2: Neighbor-joining phylogenetic evaluation consequent from the various associations of 16S rRNA gene sequence of *B. subtilis* ZR2 with existences of different bacterial strains from Gene Bank database

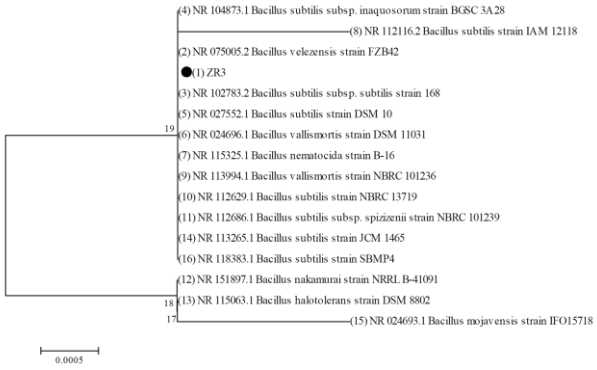

Fig. 3: Neighbor-joining phylogenetic evaluation consequent from the various associations of 16S rRNA gene sequence of *Bacillus subtilis* ZR3 with existences of different bacterial strains from Gene Bank database

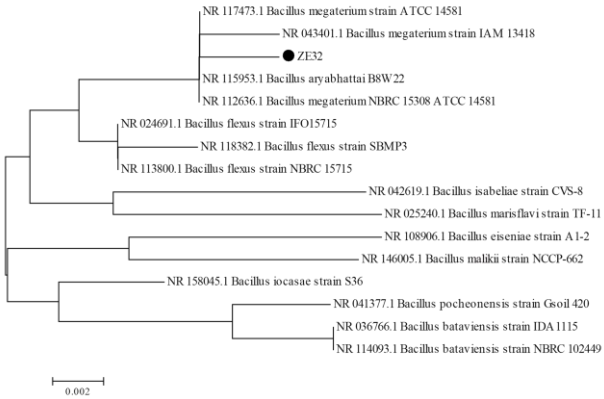

Fig. 4: Neighbor-joining phylogenetic evaluation consequent from the various associations of 16S rRNA gene sequence of *B. megateriam* ZE32 with existences of different bacterial strains from Gene Bank database

2.6 to 4.6 by PGPR isolates while solubilization efficiency ranged from 138 to 290% for EB and 161 to 369% for PGPR isolates. Inoculation of EB isolates solubilized P up to 26 μg mL^{-1} and PGPR isolates solubilized P up to 27 μg mL^{-1}. Solubilization of P by EB and PGPR isolates in the present study could be due to production of organic acids. One of the most common mechanism to increase P solubility from insoluble P source is the accumulation of organic acids (Mumtaz *et al.* 2019). For example, Saeid *et al.* (2018) informed the solubilization of P by *B. megatarium* through production of different organic acid like gluconic, lactic, acetic, succinic and propionic acids that solubilized insoluble source of P. Different *Bacillus* spp. (*B. cerus, B. subtillis, B. thurengenses, B. megatarium, etc*.) present in soil and improve the availability of P (Meena *et al.* 2017). Bacterial strains showed acid phosphatases activity which improved mineralization of P (Cheng *et al.* 2017). Acid phosphatase activity in rhizosphere stimulate plant roots to produce organic acids which boost solubilization of P (Eisenhaur *et al.* 2017). The mechanism adopted by bacterial strain for solubilization of phosphate might be helpful for the solubilization of insoluble zinc. It can be correlated with the release of organic acids by bacteria which react with insoluble zinc compounds and release plant available form of zinc (Mumtaz *et al*. 2019).

The EB isolates in the present study, solubilized insoluble Zn compound up to 26 μg mL^{-1} while PGPR isolates exhibited solubilization of Zn up to 27 μg mL^{-1}. Similarly, solubilization of Zn in agar and broth culture were reported by Ramesh *et al.* (2014). In another study, Mumtaz *et al.* (2019) described the possible mechanism of Zn solubilization by *B. cereus* and *Bacillus* spp. ZM20 through accomulation of lactic acid and acetic acid as major Zn solubilizing metabolites. They stated that lactic acid released by *B. cereus* up to 22 m*M* with an increase up to 691% under ZnO amended media as compared to lactic acid production in media without ZnO that was 0.035 m*M*. The solubilization of Zn could also be due to production of formic, isobutyric, isovaleric, citric and succinic acids identified as minor metabolites for release of Zn. In current study, inspected EB and PGPR isolates were also progressive for production of EPS and siderophores. Parallel

Table 6: Characterization for biochemical and plant growth promoting attributes of selected strains

Characteristics	ZE15	ZE32	ZR2	ZR3	ZR19
IAA without L-tryp (μg mL^{-1})	2.0 a	3.0 a	2.9 a	2.8 a	2.2 a
IAA with L-tryp (μg mL^{-1})	15.0 ab	16.9 a	15.3 ab	16.1 ab	14.0 b
Root colonization (CFU g^{-1})	3.00×10^6	3.03×10^6	1.53×10^6	1.47×10^6	1.50×10^6
Catalase activity	-	-	+	+	+
Oxidase activity	+	-	+	+	-
HCN production	-	+	+	+	-
Protease production	+	+	+	+	+
Cellulose degradation	+	+	+	+	+
Urease activity	-	+	-	-	-
Chitinase activity	-	-	-	-	-
Esterase activity	+	+	+	+	+

Indole-3-acetic acid (IAA) production values both in presences and absences of L-tryptophan (L-trp) are mean of three replicates and means followed by the same letter(s) are not significantly different according to the least significant difference test at 5% probability
The symbol, (+) represent the presence of traits and symbol, (-) represent the absence of traits; HCN= Hydrogen cyanide

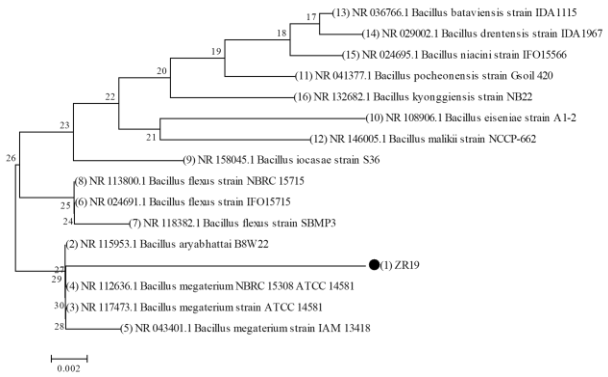

Fig. 5: Neighbor-joining phylogenetic evaluation consequent from the various associations of 16S rRNA gene sequence of *B. megateriam* ZR19 with existences of different bacterial strains from Gene Bank database

findings were stated by Mumtaz *et al.* (2017) who described that P and Zn solubilizing rhizobacteria strains could produce EPS and siderophores, and could promote plant growth through facilitation of iron availability for plant uptake. Siderophores production improve plant growth through supply of Fe or indirectly by controlling the uptake of Fe by other microbes (Ahmad *et al.* 2008). The ability of microbes to solubilize P and Zn, and production of exopolysacchrides and siderophore has been well documented to increase the nutrient use efficiency even under harsh environments (Ahmad *et al.* 2019b) that can be attributed to improvement in germination, root development and growth.

Results of the current study showed that co-inoculation of PSB isolates was better in improving the seedling vigor index, root and shoot length, fresh and dry biomass over uninoculated control as compared to sole inoculation. Improvement in growth of wheat seedlings would be due to direct and indirect plant growth promoting traits of the isolates. These mechanisms could be in terms of increase in nutrient availability *viz*. fixation of atmospheric nitrogen, solubilization of P and Zn (found positive in current study) and suppression of pathogenic microbes (Nazli *et al.* 2020), accumulation of plant hormones (Ahmad *et al.* 2019b) and siderophores (Wilson *et al.* 2006). Improvement in plant growth due to individual and combined application of PGPR, and endophytes was also described in former studies (Ahmad *et al.* 2019b; Hussain *et al.* 2020).

The two EB isolates (ZE15 and ZE32) and three PGPR isolates (ZR2, ZR3 and ZR19) showing better efficiency in improving wheat growth were evaluated for plant growth promoting traits. These isolates possess multiple plant growth promoting characteristics which support the increase in plant growth due to their inoculation in wheat. In the current study, selected isolates showed the production of IAA in presence as well as in absence of L-trp which is a valuable in PGP attributes (Patten and Glick 2002). IAA produced by bacteria act as a signaling compound for the expression of rubisco that promote the production of amino acids and organic acids (Defez *et al.* 2019). These biochemical characteristics of bacterial strains promote plant growth through creation of resistance in plants against different stresses and suppressing pathogens activity in rhizosphere as well (Ahmad *et al*. 2019b).

In the current study, the selected isolates possess more than three plant growth promoting attributes in terms of HCN production, catalase, oxidase, protease, cellulase, and esterase activities which might synergistically helped to increase plant growth. The selected isolates ZE15, ZE32, ZR2, ZR3 and ZR19 were identified as *Bacillus* spp. through 16S rRNA partial gene sequencing. In the present study, we identified P solubilizing EB and PGPR strains possessing PGP traits, however, these inoculants need to be evaluated for their effectiveness under natural conditions before recommendation to be applied as biofertilizer. Furthermore, before launching them as phosphate solubilizing biofertilizer, it is highly recommended that they should be evaluated for production of organic acids, and phytase gene expression and quantification is also required to find out as possible P solubilization mechanism.

Conclusion

Phosphorous solubilizing strains *viz*., *B. megaterium* ZE32, *B. subtilis* ZR3 and *B. megaterium* ZR19 have several plant

growths promoting features in terms of solubilization of zinc, production of exopolysaccharides, hydrogen cyanide, and siderophores and enzymatic activities. The sole as well as co-inoculation of endophytes and rhizosphere bacteria improved the vigor index and growth attributes of wheat seedlings. These strains could be potential bio-inoculants to overcome the problem of phosphorous deficiency; however, their extensive evaluation under natural conditions is required before their recommendation to be used as biofertilizer to improve nutrient use efficiency.

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Author Contributions

All authors contributed to the study commencement and design. The experiment plan, material preparation, data collection, analyses and preparation of figures and tables were performed by Zafar Iqbal, Maqshoof Ahmad, Moazzam Jamil and Muhammad Fakhar-U-Zaman Akhtar. The initial draft of manuscript was prepared by Zafar Iqbal and edited by Maqshoof Ahmad. All the Authors, reviewed drafts of the paper, commented and approved the final draft.

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