

Original Research

Exopolysaccharide and Siderophore Production Ability of Zn Solubilizing Bacterial Strains Improve Growth, Physiology and Antioxidant Status of Maize and Wheat

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Abstract

Zinc solubilizing bacteria can solubilize zinc oxide and make them available for plant uptake. Rhizobacteria were isolated and screened for exopolysaccharide (EPS) and siderophore production and plant growth promotion. The strains IUB2, IUB3, IUB6, IUB10 and IUB11 showed potential zinc solubilization and plant growth promotion. The maximum increase of 31% and 40% in shoot length was recorded by the strain IUB3 in maize and wheat, respectively. The strain IUB11 showed the maximum increase of wheat vs. maize; 29 vs. 32% and 40 vs. 39% in dry biomass and root length. Furthermore, maximum bacterial colonization of 53×10^5 CFU g⁻¹ root in wheat found by IUB2. While in maize (81×10^5 CFU g⁻¹ root) found in maize by IUB3. All the tested isolates showed positive results for the exopolysaccharides, siderophore, HCN, catalase, protease and cellulose-degrading activities. Only strains IUB2 and IUB10 showed positive results for phosphate solubilization. Moreover, these strains were identified as *Bacillus subtilis*-IUB2, *Bacillus velezensis*-IUB3, *Bacillus subtilis*-IUB6, *Bacillus vallismortis*-IUB10 and *Bacillus megaterium*-IUB1. Our study found that inoculation of the selected zinc solubilizing bacterial strains enhanced the growth of both maize and wheat crops. Hence these strains can be utilized as biofertilizers in future.

Keywords: Zinc solubilization, Exopolysaccharide, Siderophore, *Bacillus* sp.

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Introduction

Zinc is an essential micronutrient. Being a part of various metabolic activities, it plays a role in photosynthesis and respiration. Zinc is also responsible for the stimulation of many enzymes [1]. It is essential for the development of plant, humans as well as animals. Zinc is compulsory for obtaining maximum fruit size, yield and production of crop plants. It is involved in the functioning of carbonic anhydrase enzyme that is component of most of the photosynthetic materials and essential for chlorophyll formation [2]. Zinc is involved in the proteins formation, enzymes stimulation, metabolism and oxidation of biological compounds. It has been found that zinc and inorganic fertilizers enhance the crop value; however, the shortage of these components is the base for decreasing the protein, carbohydrates and ribonucleic acid formation which results in the reduction of crop growth and yield [3].

Zinc deficiency occurs mainly in females and children of 3-5 years old, leading to hidden hunger. It was found that over 2 billion people globally are suffering from hidden hunger of micronutrients, mainly Zn, Se, I, and Fe [4, 5]. The problem of micronutrient deficiency is also spreading in Pakistan due to arid to semi-arid climate, alkaline soils with low organic matter and high temperature [6]. The environmental conditions lead to a high evaporation rate from the surface, leaving salts behind, resulting in deficiency of micronutrients and high soil pH [7]. Other reasons for the deficiency of micronutrients are the cultivation of high yielding crops, intensive cropping system, poor recycling of crop residues, and excessive fertilization [8].

For proper growth and development, plants require 0.05 kg ha⁻¹ of zinc. Among applied zinc fertilizers, a large amount of Zn is not available to the crop plants [9]. Farmers usually spread high concentration of inorganic fertilizers, which becomes a great risk for human health and causes atmospheric pollution [10]. Inorganic fertilizers dissolve gradually in the soil solution at a level that is not enough to meet the adequate Zn concentration for the proper growth and development of crop plants [11]. Therefore, PGPR is considering best in the sustainable and biological farming system for effective utilization of natural resources of Zn present in the soil and to minimize the harmful participation of inorganic fertilizers in the atmosphere [12], to increase the growth and yield of crops [13]. So, PGPR are consider best in the sustainable and biological farming system for effective utilization of natural resources of Zn present in the soil and to minimize the harmful participation of inorganic fertilizers in the atmosphere [14] with the objective of increasing the growth and yield of crops [15]. Soil organic matter stabilize nutrients availability for plant uptake that resulted in enhanced nutrient use efficiency and high yield [16].

Plant growth-promoting rhizobacteria (PGPR) are the bacteria that can solubilize unavailable zinc present in soil which results ultimately in increased plant

growth [17]. PGPR converts the various inaccessible forms of zinc like zinc phosphate, zinc carbonate and zinc oxide that are extensively found in the soil to the accessible form for plant uptake [18]. Bacteria release various chelating metabolites in the rhizosphere of plants, including siderophore, which are considered an essential source of micronutrients readily available to plants like Zn and Fe [19]. By making complexes with Zn, microbial siderophore increases the available plant fraction of it [6].

Zinc solubilizing bacteria make the Zn available by different processes like organic acid production, the release of siderophore and protons [20]. These bacteria also release gluconic acid, which converts the insoluble Zn to a soluble form and enhances the growth and development of cereals [21]. The availability of micronutrients in the soil is highly dependent on soil pH [22]. Zinc availability can be increased by lowering the pH of soil. Zinc solubilizing bacteria (ZSB) produces organic acids that decrease soil pH and enhance the bioavailable zinc contents in the soil [17].

Application of Zn solubilizing bacteria with inorganic fertilizer is likely considered the best soil fertility management strategy that can improve agricultural output. Such benefits may not be achieved if we use organic or inorganic fertilizer alone. Hence, the present study hypothesized as the application of zinc solubilizing bacteria and enriched compost may enhance uptake of zinc and promote plant growth. Therefore, the present experiment has been planned with the objectives of isolation, characterization, identification and evaluation of the potential zinc solubilizing bacteria to promote seedling growth of maize and wheat. The crops maize and wheat have been selected to evaluate zinc solubilizing bacteria because these are important staple foods globally.

Experimental Procedures

Twenty-five zinc solubilizing bacterial isolates were isolated from wheat and maize rhizosphere in Bahawalpur region. Bahawalpur is main district of Cholistan desert lies 117 m above sea level. It is located on 29.41° latitude and 71.67° longitude. The mean annual temperature in Bahawalpur is 25.6°C with annual rainfall of 143 mm.

Zn Solubilization Assay

Zn solubilizing ability of bacterial strains was determined as defined by Fasim et al., [23]. The formation of a halo zone around the colony was an indication of a positive result. The halo zone diameter, as well as colony diameter, was measured. Solubilization efficiency and solubilization index was calculated. For the quantitative zinc solubilization test, tris minimal broth media amended with 0.1% ZnO was inoculated with respective strains in 100 mL conical

flask and incubate at $30 \pm 2^\circ\text{C}$ in a shaking incubator at 100 rpm. After 15 days of incubation, solubilized zinc content was measured through the atomic absorption spectrophotometer using standard protocol [24].

Screening of Zn Solubilizing Bacteria Based on Growth Promotion under Axenic Conditions

Twelve bacterial isolates having high zinc solubilizing potential were selected for further evaluation in jar trials. The isolates were screened for plant growth promoting ability in growth room. The conditions were adjusted as 12 hrs light (1000 flux) and 12 hrs dark. Maize crop was grown in the growth room at $14\text{--}28^\circ\text{C}$ temperature with 60-75% humidity. Wheat crop was sowed in the growth room at $15\text{--}20^\circ\text{C}$ temperature and 70-90% humidity. Jars were filled with sterilized sand. Irrigation was done with half strength Hoagland solution [25]. The experiment was carried out in a completely randomized design with 13 treatments (control, IUB2, IUB3, IUB4, IUB5, IUB6, IUB9, IUB10, IUB11, IUB12, IUB14, IUB17 and IUB23) and 6 replications. Seeds of wheat variety Johar 2016 and maize variety DK-9108 were inoculated with zinc solubilizing bacteria by dipping of seed for half hour in broth culture of respective strains. While in the case of control, uninoculated seeds were sown. After 35 days of sowing, seedlings were harvested. Root development, growth, physiological and biochemical parameters were recorded.

Root Colonization Assay

Seven days after sowing, seedlings were gently uprooted from three replication and washed with tap water. Surface sterilization of roots was done by dipping them in 2% mercuric chloride solution for the 30S followed by dipping in 95% ethanol for 1 minute and 6 time washing with sterilized distilled water. Two hundred mili gram (200 mg) of surface-sterilized roots macerated and ground in 5 mL sterilized distilled water with sanitized mortar and pestle. After shaking, the bacterial suspension was diluted from 10^{-1} to 10^{-6} . Add 1ml of each dilution into petri dishes containing sterilized general purpose media and incubated at 30°C for 48 hours. Then, the bacterial population was counted in terms of colony forming units (CFU) using digital colony counter (Digital S model, Selecta P) described by Iqbal et al. [26].

Characterization of Selected Isolates

The selected strains were characterized for growth-promoting and biochemical attributes. Phosphate solubilizing potential of the isolates was evaluated by forming halo zone in Pikovskya's agar medium after 3-5 days of incubation at 28°C which was considered positive [27]. Siderophore production potential of the isolated strains was examined as defined by Schwyn

and Neilands [28]. For this purpose, Chrome azurol S (CAS) blue agar plate was spot inoculated with actively growing strains and incubated for 3 days at 37°C . The appearance of the yellow-orange halo zone around the colony showed the formation of siderophore on the agar plate. Exopolysaccharide production (EPS) was determined by inoculating the strains on De Man Ragosa and Sharp (MRS) agar media and incubated at 30°C for 3 days. The mucoid growth of colonies considered as EPS positive. Following Patten and Glick [29], the production of indole-3-acetic acid (IAA) was in the presence and absence of L tryptophan. The biochemical activities such as cellulose degradation, oxidase, protease, catalase and urease activity HCN production was determined [26].

Identification of Selected Zinc Solubilizing Bacterial Isolates

The selected isolates were identified through 16S rRNA gene sequencing technique by the amplification sequencing and bioinformatics procedure. Adopting proteinase K treatment rough genetic material of selected bacterial isolates IUB2, IUB3, IUB6, IUB10 and IUB11 was isolated from the bacterial cells [30]. Then Identification of selected zinc solubilizing bacterial isolates was done by using the rough genetic material according to the method defined by Hussain et al. [17].

Statistical Analysis

The data obtained from the above tests was subjected to statistical model 8.1 and means were compared by least significant difference to see the significance of results [31].

Results and Discussion

Isolation of Zinc Solubilizing Rhizobacterial Strains

Twenty-five zinc solubilizing bacterial colonies were isolated from maize and wheat rhizosphere and coded as IUB1, IUB2, IUB3,, IUB25. The ability of zinc solubilizing bacteria to solubilize the insoluble source of zinc is presented in Table 1. Data depicted that all the zinc solubilizing bacterial isolates solubilized the insoluble zinc compound (ZnO). The formation of a clear halo zone indicates the solubilization of ZnO by ZSB. The strain IUB11 showed maximum halo zone diameter (2.5 cm) and halo zone area (12.6 cm^2). It was found that strain IUB10 gave maximum colony diameter (1.6 cm). The strains IUB9 and IUB11 were the following strains that showed better results for colony diameter. Both these strains showed 1.5 cm colony diameter. Data regarding zinc solubilization efficiency showed

Table 1. Zn solubilizing potential of rhizobacterial isolates.

Bacterial isolate	Halozone diameter (cm)	Colony diameter (cm)	Halozone area (cm ²)	ZSE	ZSI	Zn concentration (mg L ⁻¹)
Control	-	-	-	-	-	8.0 g
IUB1	0.6 m	0.3 ij	0.6 lm	217 de	2.70 a-c	8.9 a
IUB2*	1.6 d	1.0 de	5.2 f	157 f-i	2.57 a-c	8.6 b
IUB3*	1.9 c	0.9 g	5.9 e	216 de	3.10 a-c	8.4 de
IUB4*	1.8 c	1.2 c	7.4 d	149 g-i	2.59 a-c	8.3 e
IUB5*	1.3 f	1.0 d	4.4 g	129 hi	2.33 bc	8.5 cd
IUB6*	2.1 b	1.2 c	8.4 c	172 e-i	2.84 a-c	9.0 a
IUB7	0.8 ij	0.3 ij	1.0 l	328 ab	3.58 ab	9.0 a
IUB8	0.7 kl	0.2 j	0.6 lm	350 a	3.57 ab	8.7 b
IUB9*	1.9 c	1.5 b	8.7 c	127 hi	2.49 bc	8.1 f
IUB10*	2.0 b	1.6 a	10.4 b	127 hi	2.52 bc	8.9 a
IUB11*	2.5 a	1.5 ab	12.6 a	161 e-i	2.89 a-c	9.0 a
IUB12*	1.3 f	0.9 fg	3.9 h	148 g-i	2.43 bc	8.4 de
IUB13	0.6 lm	0.3 i	0.7 lm	192 e-g	2.57 a-c	8.3 e
IUB14*	1.5 de	0.9 e-g	4.6 g	161 e-i	2.56 a-c	8.5 c
IUB15	1.0 gh	0.6 h	1.9 jk	178 e-h	2.56 a-c	8.1 f
IUB16	1.1 g	0.6 h	2.4 j	174 e-h	2.49 bc	8.1 f
IUB17*	1.5 de	1.0 de	4.8 fg	147 g-i	2.47 bc	8.2 f
IUB18	0.9 hi	0.6 h	1.8 k	164 e-i	2.42 bc	8.1 f
IUB19	0.8 jk	0.2 j	0.8 lm	356 a	3.93 a	8.1 f
IUB20	0.6 lm	0.3 i	0.7 lm	194 e-g	2.64 a-c	8.1 f
IUB21	0.6 m	0.2 j	0.5 m	283 bc	3.03 a-c	8.1 f
IUB22	0.6 lm	0.2 j	0.5 lm	267 cd	2.87 a-c	8.1 f
IUB23*	1.4 ef	1.0 d-f	4.5 g	149 g-i	2.49 bc	8.1 fg
IUB24	0.5 m	0.3 ij	0.5 m	211 d-f	2.72 a-c	8.1 fg
IUB25	1.1 g	0.9 e-g	3.1 i	114 i	2.11 c	8.1 fg
LSD(p<0.05)	0.1002	0.0803	0.4342	59.237	1.3770	0.1056
CV	4.97	6.53	6.91	18.54	30.62	0.77

Data are shown as mean of five replicates. * strains showed better results and were selected for further evaluation. Means followed by same letter(s) within the column are not significantly different according to least significance difference (LSD) test at $p \leq 0.05$.

ZSE; Zinc solubilizing efficiency, ZSI; Zinc solubilizing index

that minimum zinc solubilization efficiency was given by the strain IUB25, which showed 114% zinc solubilization efficiency. Maximum zinc solubilization efficiency and Zn solubilization index was given by the strain IUB19 with 356% zinc solubilization efficiency and 3.93 Zn solubilization index. Other strains that gave better results for zinc solubilization index were IUB7, IUB8 and IUB3. All these strains expressed 3.58, 3.57 and 3.1 zinc solubilization index, respectively. These bacterial isolates were

further tested for quantitative zinc solubilization assay to calculate the quantity of solubilized zinc in the Tris minimal liquid broth culture amended with 0.1% ZnO. Maximum solubilized zinc concentration (9.0 mg L⁻¹) was observed by strain IUB11. While the strains IUB1 and IUB2 was next after IUB11 that showed 8.9 mg L⁻¹ solubilized zinc content. Based on zinc solubilization, twelve bacterial isolates were selected for further evaluation in jar trial for plant growth promotion attributes.

Table 2. Potential of zinc solubilizing rhizobacteria to improve growth parameters of maize in jar trial.

Treatments	Shoot length	Root length	Dry biomass	R/S Ratio	Root colonization
	(cm)	(cm)	(g jar ⁻¹)	-	CFU × 10 ⁵ mL ⁻¹
Control	29 g	8.6 h	1.46 j	0.33 g	18 j
IUB2	37 a	10.8 d	1.87 b	0.37 a	53 a
IUB3	38 a	11.3 c	1.82 c	0.36 b	27 e
IUB4	33 d-f	10.6 de	1.71 ef	0.34 ef	32 d
IUB5	32 f	8.8 h	1.72 e	0.34 ef	35 c
IUB6	35 bc	11.4 c	1.77 d	0.35 b	37 c
IUB9	34 c-e	10.4 e	1.69 f-h	0.34 d-f	22 gh
IUB10	35 b-d	11.6 b	1.94 a	0.37 a	20 hi
IUB11	36 ab	12.1 a	1.93 a	0.37 a	24 fg
IUB12	32 ef	9.9 f	1.70 e-g	0.34 de	45 b
IUB14	33 d-f	10.6 de	1.66 hi	0.33 fg	25 f
IUB17	29 g	9.8 f	1.67 g-I	0.35 bc	19 ij
IUB23	33 ef	9.4 g	1.66 I	0.35	52 a
LSD (p≤0.05)	1.683	0.231	0.027	0.008	2.029
CV	2.99	1.32	0.92	1.37	3.86

Data are shown as mean of five replicates. Means followed by same letter(s) within the column are not significantly different according to least significance difference (LSD) test at p≤0.05. R/S ratio; root to shoot ratio

Maize Trial

Growth Promoting Activity of Rhizobacterial Isolates in Maize Seedling

Healthy seeds of maize were procured from the local market. Seeds were surface sterilized and based on Zn solubilization efficiency; selected bacterial isolates were used for plant inoculation experiment. The selected bacterial isolates were used to study their influence on plant growth promotion of maize seedlings in terms of increase in shoot length, root length and dry biomass. The inoculated seedlings showed a tremendous increase in dry weight (33%) by strain IUB11 while the highest root/shoot ratio was given by the strain IUB2 (15%) in comparison to the uninoculated control (Table 2). The isolates IUB3, IUB6, IUB10 and IUB11 also showed significant increase upto 11, 9, 13 and 14% in root/shoot ratio, respectively.

The seed inoculation with zinc solubilizing rhizobacteria significantly enhanced root colonization under axenic conditions (Table 2). The maximum root colonization (53×10^4 cfu g⁻¹ root) was recorded by strain IUB2 followed by strains IUB23, IUB12 and IUB 6 that showed root colonization up to 52×10^4 cfu g⁻¹ root, 45×10^4 cfu g⁻¹ root and 37×10^4 cfu g⁻¹ root, respectively. Minimum root colonization was observed by strain IUB17 that showed 19×10^4 cfu g⁻¹ root among applied treatments.

Furthermore, selected strains improved leaf surface area and root growth such as average root diameter, root surface area and root volume. Data represented that inoculated seedlings with zinc solubilizing isolates significantly influenced the leaf and root growth (Fig. 1), which showed a tremendous increase in the leaf area (35%), root surface area (30%) and root volume (32%) by the strain IUB2 as compared to uninoculated control. The result depicted that all these strains showed significant results over uninoculated control and all the zinc solubilizing bacterial strains improved the growth of maize crop.

Effect of Zinc Solubilizing Bacterial Isolates on Physiology and Antioxidant Enzyme System of Maize Seedlings

Data in Table 3, demonstrated the effect of zinc solubilizing bacteria on physiological attributes and antioxidant enzyme system of maize. The results revealed that applying zinc solubilizing bacteria showed a greater impact on chlorophyll a, chlorophyll b, and carotenoids. The strain IUB2 showed the maximum increase in chlorophyll a (14%), chlorophyll b (13%), and carotenoids (12%) as compared to control. The strain IUB4 and IUB9 showed similar effect on chlorophyll a, where 6% increase was recorded. The chlorophyll b content of the strains IUB4, IUB5,

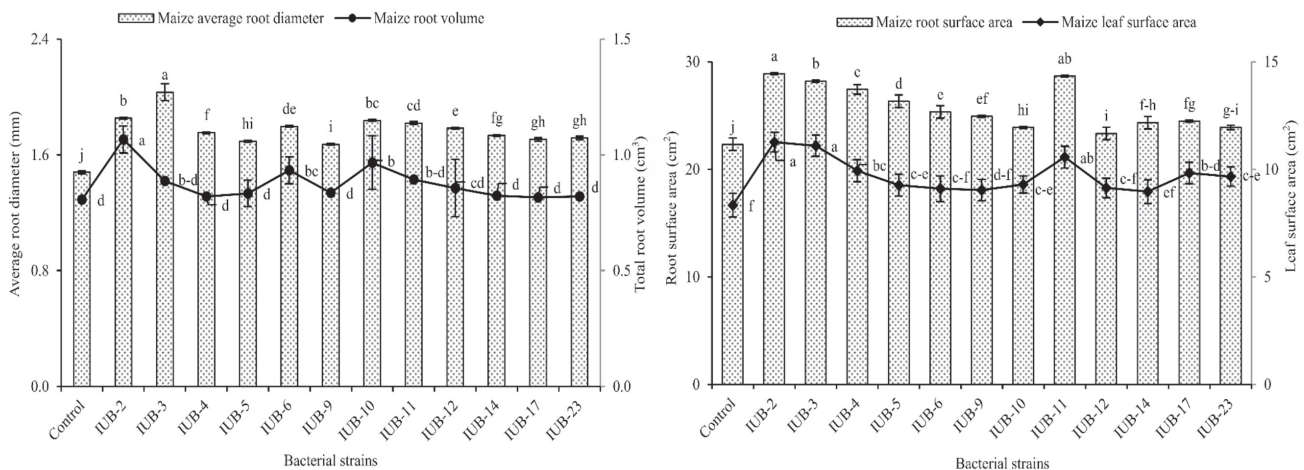


Fig. 1. Potential of zinc solubilizing rhizobacteria to improve root growth and shoot surface area in maize seedlings. Bars sharing same letter(s) does not differ significantly as per 5% level of probability. Bars represent average root diameter a) and root surface area b) while Line represent total root volume a) and leaf surface area b).

IUB9, and IUB17 was found to have increased by 1%, which was non-significant compared to the control. However, when compared to control, the strains IUB2 and IUB3 provided substantial results, with chlorophyll b contents increasing by 14% and 12%, respectively. The strains IUB5, IUB9, IUB12, and IUB23 provided similar results, with a 5% increase in carotenoid content

when compared to the control. However, strain IUB2 showed the maximum increase of 12% in carotenoid contents.

The production of antioxidant enzymes in the maize crop was also improved by zinc solubilizing bacteria (Table 3). The strain IUB2 performed best when compared with the uninoculated control in terms

Table 3. Potential of zinc solubilizing rhizobacteria to improve physiological parameters of maize in jar trial.

Isolates	Chlorophyll a	Chlorophyll b	Carotenoid	SOD	POD	CAT	APX	POX
	(µg g ⁻¹)			(Units g ⁻¹ FW)				
Control	0.62 i	0.62 c	0.98 d	124 j	0.91 e	7.4 h	117 m	0.25 e
IUB2	0.71 a	0.70 a	1.10 a	141 a	1.04 a	8.5 a	130 c	0.28 ab
IUB3	0.70 ab	0.67 ab	1.09 a	133 cd	1.00 a-c	8.5 a	129 d	0.27 a-c
IUB4	0.66 ef	0.62 c	1.00 cd	130 fg	0.97 b-e	7.6 fg	120 l	0.27 b-d
IUB5	0.63 hi	0.62 c	1.03 a-d	132 de	0.96 b-e	7.8 de	122 k	0.26 de
IUB6	0.69 bc	0.67 ab	1.07 ab	138 b	1.02 ab	8.1 bc	129 e	0.29 a
IUB9	0.66 ef	0.62 c	1.03 a-d	128 gh	0.97 b-e	7.6 fg	123 j	0.26 de
IUB10	0.68 cd	0.63 bc	1.06 a-c	134 c	1.01 a-c	8.2 b	133 b	0.27 b-d
IUB11	0.68 cd	0.66 a-c	1.05 a-d	139 ab	0.99 a-d	8.1 b	133 a	0.28 ab
IUB12	0.63 hi	0.63 bc	1.03 a-d	132 d	0.95 c-e	7.5 gh	124 l	0.26 c-e
IUB14	0.65 fg	0.63 bc	1.00 b-d	130 ef	0.93 de	7.5 gh	125 h	0.25 de
IUB17	0.64 gh	0.62 c	0.99 cd	127 hi	0.97 b-e	7.9 cd	127 f	0.26 c-e
IUB23	0.67 de	0.63 bc	1.03 a-d	126 i	0.93 e	7.7 ef	126 g	0.26 de
LSD (p≤0.05)	0.0157	0.0418	0.0668	1.7827	0.0615	0.1470	0.5829	0.0147
CV	1.41	3.90	3.84	0.81	3.76	1.11	0.28	3.31

Data are shown as mean of five replicates. Means followed by same letter(s) within the column are not significantly different according to least significance difference (LSD) test at p≤0.05. SOD; superoxide dismutase activity, POD; peroxidase dismutase activity, CAT; catalase activity, APX; ascorbate peroxidase activity, POX; peroxidase activity

of superoxide dismutase activity (14%), peroxidase dismutase activity (15%), and catalase activity (15%). The strains IUB10 and IUB11 expressed the highest ascorbate peroxidase activity that was 14% more than the control. The maximum peroxidase activity was recorded by strain IUB6 was 15% more than control. The strains IUB2, IUB3, IUB10 and IUB11 showed an increase of 12, 9, 7 and 11% in peroxidase activity, respectively.

Wheat Trial

Growth Promoting Activity of Rhizobacterial Isolates in Wheat Seedling

The selected strains were further evaluated for their growth promoting abilities in wheat crop. Seed inoculation with zinc solubilizing rhizobacteria significantly enhanced wheat growth under axenic conditions (Table 4). Statistical analysis showed that the strain IUB3 exhibited maximum shoot length, with 40% increase over the uninoculated control. The next strains that perform better were IUB11, IUB2, IUB6, and IUB10. These strains showed increase of 38, 35, 31, and 29% in shoot length, respectively. The maximum increase of 39% in root length was recorded by strain IUB11 followed by IUB3 and IUB2, which was 32 and 28% increased, respectively. The strain IUB17, IUB19 and IUB23 showed a similar effect on root length that

increased up to 18% compared to the control. According to statistical analysis, the strain IUB11 showed the highest root/shoot ratio, followed by the strains IUB10 over the uninoculated control that increased by 32% and 31%, respectively.

The results explored in (Table 4) showed that the inoculation of zinc-solubilizing rhizobacteria enhanced root colonization in wheat under axenic conditions. All applied strains showed a potential increase in root colonization as compared to the control. However, the strain IUB3 showed maximum root colonization (81×10^4 cfu g⁻¹ root) followed by strains IUB12, IUB10, and IUB5 that showed 62×10^4 cfu g⁻¹ root, 57×10^4 cfu g⁻¹ root and 55×10^4 cfu g⁻¹ root, respectively. Among applied treatments, minimum root colonization 16×10^4 cfu g⁻¹ root was observed by strain IUB17. However, all tested strains showed a significant increase in root colonization over control (9×10^4 cfu g⁻¹ root).

The results in Fig. 2 demonstrated the effectiveness of zinc-solubilizing rhizobacteria for increasing the leaf surface area, average root diameter, root surface area and root volume. The strain IUB11 showed maximum leaf area and average root diameter that was 31 and 33% more than control, respectively. The strain IUB2 showed the maximum increase of 28% in root surface area. While strain IUB4 showed a slight increase of 1% in root surface area that was non-significant as compared to control. The strain IUB6 showed the maximum increase of 37% in root volume followed

Table 4. Potential of zinc solubilizing rhizobacteria to improve growth parameters of wheat in jar trial.

Treatments	Shoot length	Root length	Dry biomass	R/S ratio	Root colonization
	cm		g jar ⁻¹	-	CFU $\times 10^5$ mL ⁻¹
Control	17.0 k	4.4 h	0.31 d	0.22 e	9 l
IUB2	22.8 c	5.7 bc	0.42 a	0.27 b	21 j
IUB3	23.8 a	5.8 c	0.41 a	0.28 ab	81 a
IUB4	21.2 fg	5.5 ce	0.37 bc	0.23 de	44 f
IUB5	21.2 f	5.3 fg	0.37 c	0.27 b	55 d
IUB6	22.2 d	5.6 cd	0.40 ab	0.28 ab	40 g
IUB9	21.0 g h	5.5 d-f	0.40 ab	0.24 cd	40 g
IUB10	21.8 e	5.6 cd	0.36 c	0.29 a	57 c
IUB11	23.5 b	6.2 a	0.40 ab	0.29 a	51 e
IUB12	20.7 i	5.4 e-g	0.37 c	0.24 cd	62 b
IUB14	20.0 j	5.2 g	0.37 c	0.24 cd	32 l
IUB17	21.0 g	5.3 g	0.37 bc	0.25 c	16 k
IUB23	20.8 hi	5.2 g	0.38 bc	0.27 ab	35 h
LSD(p \leq 0.05)	0.1842	0.1823	0.0274	0.0166	1.5438
CV	0.52	2	4.32	3.80	2.20

Data are shown as mean of five replicates. Means followed by same letter(s) within the column are not significantly different according to least significance difference (LSD) test at p \leq 0.05. R/S ratio; root to shoot ratio

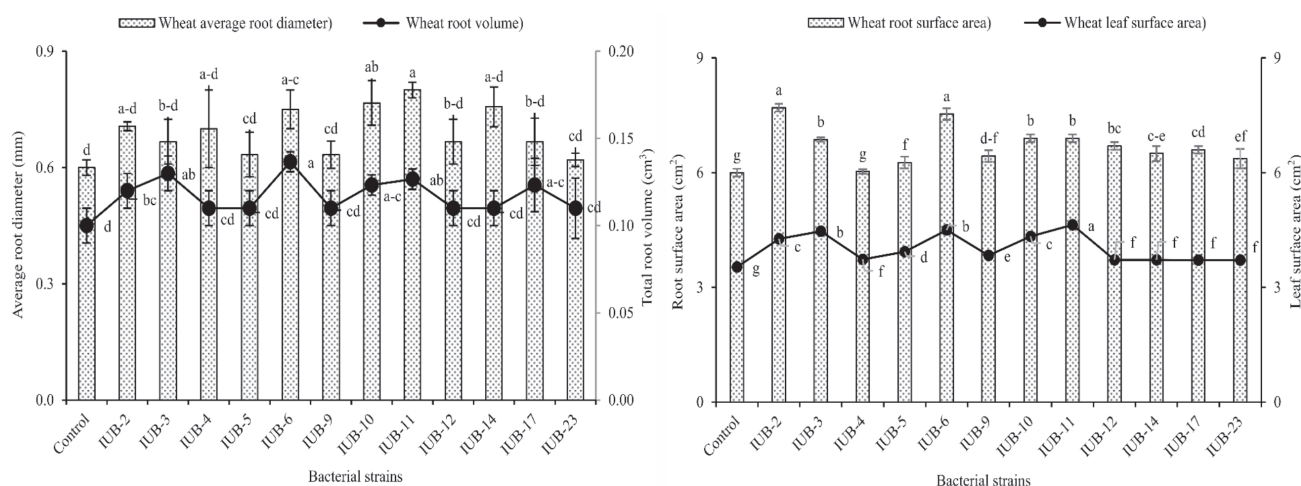


Fig. 2. Potential of zinc solubilizing rhizobacteria to improve root growth and leaf surface area in wheat seedling. Bars sharing same letter(s) does not differ significantly as per 5% level of probability. Bars represent average root diameter a) and root surface area b) while Line represent total root volume a) and leaf surface area b).

by 30, 27, 23 and 20% increase in root volume by strains IUB6, IUB11, IUB10 and IUB2, respectively.

Effect of Zinc Solubilizing Bacterial Isolates on Physiology and Antioxidant Enzyme System of Wheat Seedlings

Data in Table 5, showed the efficacy of zinc-solubilizing bacteria in increasing chlorophyll a, chlorophyll b, and carotenoid content. The strain IUB6 and IUB2 showed similar results for chlorophyll-a contents. These strains showed maximum increase of 18% in chlorophyll-a content as compared to the control. The next strain that showed a significant increase in chlorophyll-a content was IUB10 which showed 14% increase as compared to control. The strain IUB3, IUB9 and IUB23 showed a similar effect on chlorophyll-a content, where 12% increase was recorded. Results revealed that the strain IUB6 showed maximum increase of 17% in chlorophyll contents followed by strains IUB15 which showed 15% increase as compared to control. While the maximum increase of 16% in carotenoid content was recorded by strain IUB10. The strains IUB3, IUB2, and IUB6 were the following strains that perform better after IUB10. These strains showed similar results where 14% increase was recorded as compared to control.

All the applied treatments showed a significant effect on antioxidant enzymes such as superoxide dismutase activity (SOD), peroxidase dismutase activity (POD), catalase activity (CAT), ascorbate peroxidase activity (APX), and peroxidase activity (POX), in wheat seedlings under axenic conditions (Table 5). The data revealed that strain IUB2 showed the highest value for superoxide dismutase activity, with a 15% increase, followed by strains IUB3 and IUB6 that showed 13% increase in SOD as compared to control. The strain IUB10 was the subsequent treatment that

showed 11% increase in SOD. The strain IUB3 showed the highest increase of 13% in peroxidase dismutase activity and ascorbate peroxidase activity. While the highest catalase activity was found in strain IUB10 followed by the strains IUB11, IUB3, and IUB2, which showed a 13%, 10%, and 9% increase, respectively. The strain IUB11 showed the maximum increase of 15% in peroxidase activity. The subsequent strains that expressed better peroxidase activity were IUB10, IUB3, and IUB6 which showed 13, 12, and 9% increase, respectively.

Characterization of Zinc Solubilizing Bacteria

The five best performing rhizobacterial strains that showed maximum results to improve seedling growth of maize and wheat under axenic conditions were selected for further *in vitro* evaluation for plant growth promoting attributes. The results of *in vitro* characterization are presented in table 6. The selected strains were evaluated for exopolysaccharide production on agar plate assay. Appearance of mucoid growth around bacterial colony on RCV media indicated positive results for exopolysaccharides production. All the strains showed positive results for EPS production, siderophore production, hydrogen cyanide (HCN) production, protease activity, cellulose degradation ability and catalase activity. Catalase activity was considered positive if the strain form bubbles after pouring a drop of H_2O_2 bacterial colony. Among tested strains, only IUB2 and IUB10 showed solubilization of insoluble P in agar plate assay containing Pickovskay's nutrient media. All the tested strains were unable to produce ammonia (NH_3) in Christensen's urea broth and were reported as negative for NH_3 production. Oxidase activity were also not detected in any tested strains. The tested strains showed positive results for indole-3-acetic acid (IAA) production in the presence

Table 5. Potential of zinc solubilizing rhizobacteria to improve physiological parameters of wheat in jar trial.

Treatments	Chlorophyll a	Chlorophyll b	Carotenoid	SOD	POD	CAT	APX	POX
	$\mu\text{g g}^{-1}$			Units g^{-1} FW				
Control	0.52 e	0.58 d	0.21 d	128 g	19.7 j	15.6 g	177 i	0.30 e
IUB2	0.61 a	0.67 a	0.24 ab	147 a	22.1 b	16.9 cd	195 bc	0.32 b-d
IUB3	0.58 a-d	0.67 ab	0.24 ab	144 b	22.3 a	17.2 c	200 a	0.33 ab
IUB4	0.55 b-e	0.64 a-c	0.22 b-d	136 e	20.1 h	15.8 fg	183 h	0.30 e
IUB5	0.54 c-e	0.61 cd	0.22 b-d	131 f	20.5 g	16.4 e	191 de	0.30 de
IUB6	0.61 a	0.68 a	0.24 ab	143 bc	21.7 d	16.8 d	193 cd	0.32 a-c
IUB9	0.58 a-c	0.67 ab	0.22 b-d	129 g	20.0 hi	15.9 fg	189 ef	0.31 c-e
IUB10	0.59 ab	0.67 a	0.25 a	141 c	21.6 d	18.0 a	199 a	0.34 ab
IUB11	0.53 de	0.65 a-c	0.24 a-c	139 d	21.9 c	17.7 b	197 ab	0.34 a
IUB12	0.52 e	0.63 a-c	0.23 a-d	135 e	20.5 g	15.9 f	186 gh	0.31 c-e
IUB14	0.53 de	0.64 a-c	0.23 a-d	131 f	19.9 ij	16.1 f	187 fg	0.32 b-d
IUB17	0.53 e	0.62 b-d	0.22 cd	129 g	20.7 f	16.4 e	186 fg	0.31 c-e
IUB23	0.58 a-c	0.64 a-c	0.23 a-d	132 f	21.1 e	16.8 d	184 gh	0.31 c-e
LSD($p \leq 0.05$)	0.0466	0.0474	0.0227	1.6783	0.1567	0.2857	3.3900	0.0191
CV	4.97	4.38	5.91	0.74	0.45	1.03	1.06	3.59

Data are shown as mean of five replicates. Means followed by same letter(s) within the column are not significantly different according to least significance difference (LSD) test at $p \leq 0.05$. SOD; superoxide dismutase activity, POD; peroxidase dismutase activity, CAT; catalase activity, APX; ascorbate peroxidase activity, POX; peroxidase activity

and absence of L-tryptophane with a variable degree of IAA production. The strain IUB11 recorded the Maximum IAA production ($2.7 \mu\text{g mL}^{-1}$) in the absence of L-tryptophan. Strains IUB3 and IUB6 showed similar results for IAA production in the absence of L-tryptophane, where the recorded production of IAA was $2.4 \mu\text{g mL}^{-1}$. In the presence of L-tryptophan, the maximum IAA production was showed by the strain IUB10 followed by the strains IUB11 and IUB3 with $15.2 \mu\text{g mL}^{-1}$, $13.9 \mu\text{g mL}^{-1}$ and $13.6 \mu\text{g mL}^{-1}$ of indole acetic acid production, respectively.

Identification of Bacterial Strains

The five promising zinc solubilizing rhizobacterial isolates having manifold growth endorsing characters in maize and wheat were identified through 16S rRNA partial sequencing technique. On the bases of resemblance with different *Bacillus* species, these strains were identified as *Bacillus velezensis* (IUB3), *Bacillus subtilis* (IUB2 and IUB6), *Bacillus vallismortis* (IUB10) and *Bacillus megaterium* (IUB11) as shown in Fig. 3. The resulting sequences were also deposited in NCBI Genbank data base with the accession numbers MN696213, MN696212, MN696214, MN696215 and MN696216, respectively.

Discussion

The experiment was carried out for evaluating the plant growth promoting potential of zinc solubilizing rhizobacteria isolated from wheat and maize rhizosphere. The results expressed that inoculation of zinc solubilizing rhizobacteria significantly improved the growth of maize and wheat crops (Fig. 4). In our study strains were tested for the zinc solubilization (ZnO) qualitatively and quantitatively. Best strains for the qualitative and quantitative zinc solubilization were IUB2, IUB3 and IUB9 that is thought to be based on organic acid production. The solubilization of unavailable ZnO in the broth medium could be due to the release of organic acid by the zinc solubilizing rhizobacteria that decreases the pH of broth medium and solubilize the unavailable ZnO. This work is with the line of Hussain et al. [17], who reported that out of 52 bacterial isolates, only 14 bacterial strains showed a strong ability of zinc solubilization. Similar results were also reported by Sharma et al. [32], who reported that PGPR solubilized Zn through production of organic acids.

The results of the jar trial showed that inoculation of zinc solubilizing bacteria improved the shoot length, root length, root/shoot ratio and total dry biomass as compared to the uninoculated control in both wheat

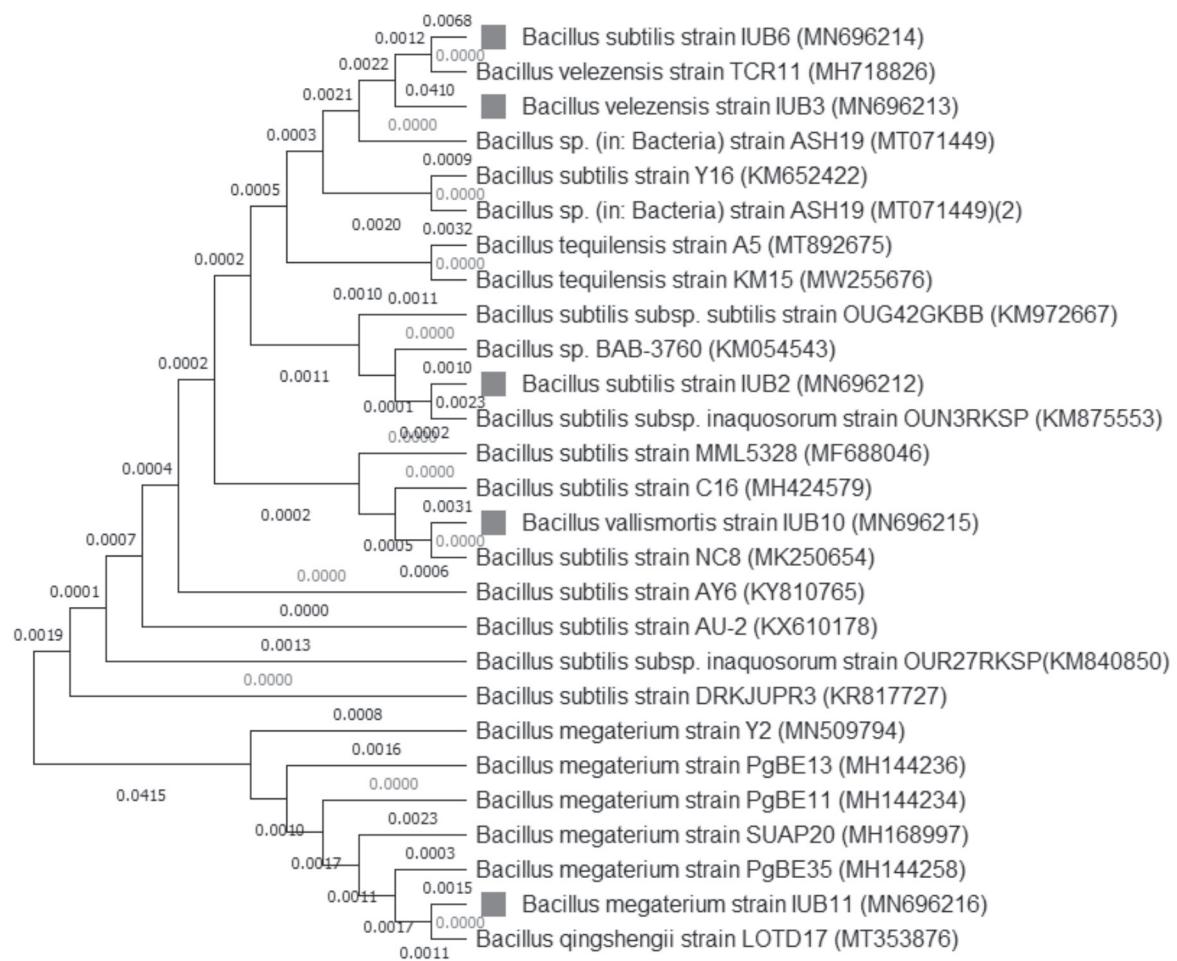


Fig. 3. Neighbor joining tree of *Bacillus subtilis* (IUB2 and IUB6), *Bacillus velezensis* (IUB3), *Bacillus vallismortis* (IUB10) and *Bacillus megaterium* (IUB11) constructed by closely related strains.

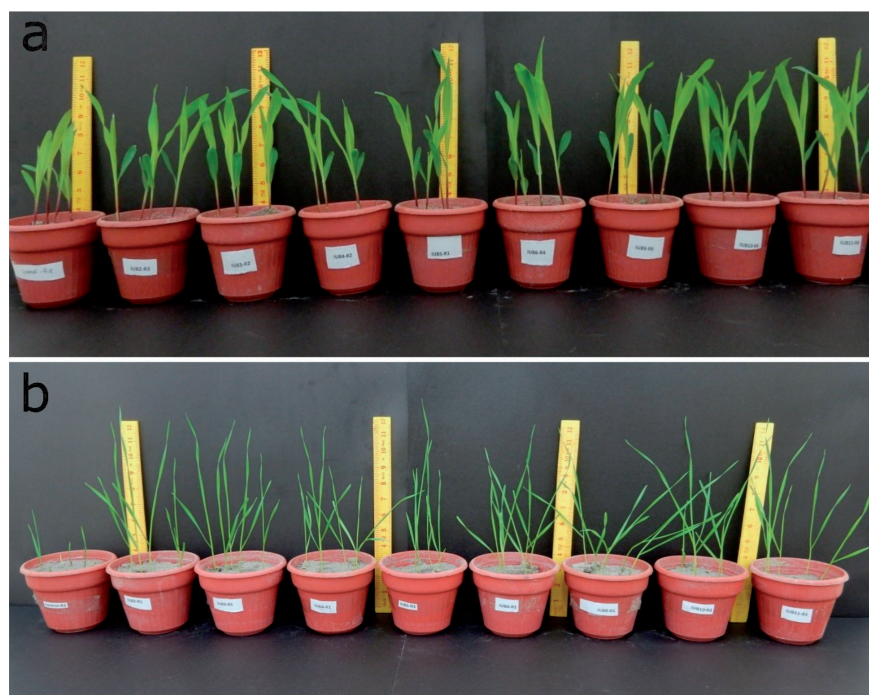


Fig. 4. Effect of zinc solubilizing rhizobacteria and enriched compost on growth of maize a) and wheat b) seedlings. The jars are arranged from left to right as; Control, IUB2, IUB3, IUB4, IUB5, IUB6, IUB9, IUB10 and IUB11.

and maize crops. The strain IUB3 showed maximum shoot length in both wheat and maize crop. However maximum root length was shown by the strain IUB11. The highest total dry weight was shown by the strains IUB11 and IUB10 in maize crop and zinc solubilizing bacterial strains (IUB9, IUB6 and IUB2) also increased these growth parameters in wheat crop. The increased shoot length, root length, and total dry biomass might be due to the solubilization of zinc, synthesis of various plant growth regulators, phosphorus solubilization and indole acetic acid production by the zinc solubilizing rhizobacteria. The improvement in growth parameters might be due increased availability of nutrients and production of plant growth regulators by plant growth promoting bacteria [33]. The results are similar as described by Goteti et al. [34] and Vaid et al. [35], where they described that inoculation of zinc solubilizing bacteria significantly improved the shoot length, root length and total dry biomass of rice, soybean as well as maize crops. These results are also supported by Hussain et al. [17], who described that use of zinc solubilizing bacteria enhanced shoots fresh biomass, fresh root biomass, shoot length, root length, shoot dry biomass, root dry biomass as well as total dry biomass of crop.

It was found that inoculation of zinc solubilizing rhizobacteria significantly increased the leaf surface area of both maize and wheat crop. This increase in the leaf area might be due to the enhanced chlorophyll content caused by increasing the solubilization of essential nutrients in the crop leaves by zinc solubilizing rhizobacteria, which increased the leaf area of crops. These results are in accordance with Nadeem et al. [36] and Ahmad et al. [37], who described that the application of zinc solubilizing rhizobacteria enhanced

the chlorophyll content in the mungbean and maize crops.

Root growth such as average root diameter, root surface area, and root volume were also improved by using zinc solubilizing rhizobacteria in the maize and wheat crop. This improvement in the root growth parameter might be due to increase in the zinc concentration in the plant roots by zinc solubilizing rhizobacteria. These results are similar to Fan et al. [38], who stated that root surface area and root hair are enhanced through the inoculation of zinc solubilizing bacteria. These results are also supported by Vaid et al. [35], who concluded that root growth in the rice plants enhanced by the application of zinc solubilizing bacteria.

Chlorophyll a, chlorophyll b, and carotenoid content also improved by applying zinc solubilizing rhizobacteria both in the maize and wheat crops. It could be due to the increase in the leaf area that significantly improved the chlorophyll content. These results are according to the results of Kang et al. [39]. Similar results are also reported by Hussain et al. [17] who stated that inoculation of zinc solubilizing bacterial isolates enhanced the chlorophyll content of maize crop.

The inoculation of plant growth promoting rhizobacteria also increased the superoxide dismutase activity (SOD), peroxidase activity (POX), peroxidase dismutase activity (POD), catalase activity (CAT) and ascorbate peroxidase activity (APX) in the leaves of both maize and wheat crops. The increase in the amount of antioxidants enzymes SOD, POX, POD, CAT and APX might be due to an increase in the amount of nutrients, especially zinc, for the crops by the zinc solubilizing rhizobacteria that improved amount

Table 6. NH_3 production, Siderophore production, HCN production, EPS production phosphate solubilization and enzymatic activity of zinc solubilizing rhizobacteria.

Biochemical characters		IUB2	IUB3	IUB6	IUB10	IUB11
IAA production	With L-trp	13.4	13.6	14.1	15.2	13.9
	Without L-trp	2.2	2.4	2.4	2.2	2.7
NH_3 production		-	-	-	-	-
Siderophore production		+	+	+	+	+
HCN production		+	+	+	+	+
EPS production		+	+	+	+	+
Phosphate solubilization		+	-	-	+	-
protease activity		+	+	+	+	+
Cellulose degradation activity		+	+	+	+	++
Catalase activity		+	+	+	+	+
Oxidase activity		-	-	-	-	-

Here: NH_3 = Ammonia, HCN = Hydrogen cyanide, EPS = Exopolysaccharide; Positive sign (+) indicates the presence of trait; double positive sign (++) indicate strong presence of trait; negative sign (-) indicates the absence of trait.

of antioxidants enzymes in the crop leaves. These results are in line with those of Chakraborty et al. [40], who reported that inoculation of bacillus species *B. safensis* enhanced the amount of antioxidant enzymes in the crop leaves.

For the testing of plant growth promoting abilities, only five best strains were selected. It was found that all the strains were positive for siderophore and HCN production. These results are in line with the work of Scagliola et al. [41], who stated that *Rhizobium* species and *Agrobacterium tumefaciens* strains synthesizes the siderophore. Shoukry et al. [42] described that zinc solubilizing bacterial strains can synthesize the siderophore and HCN that provides the resistance to the crops against the pathogens and increased the supply of iron to the crop plant. All the selected zinc solubilizing strains also showed positive results for exopolysaccharides production. According to Tewari and Arora [43], these results are reported that selected zinc solubilizing bacterial strains produce exopolysaccharides. All the tested strains gave positive results for IAA production both in the absence and presence of L-tryptophan. These results are in according to the results reported by Ramesh et al. [44]. Exopolysaccharides enhanced the ability of bacterial strains to colonize the roots. Strains IUB2 and IUB10 gave positive results for phosphate solubilization by forming clear zone in the plate assay. These results are in line with the work of Matse et al. [45] who described that *Pseudomonas* fluorescent strain showed the results for phosphate solubilization. The biochemical characteristics such as EPS, HCN, siderophore production promote plant growth through providing essential nutrients and suppressing pathogens [42, 46]. Plant growth regulators and IAA production mitigate the salt and drought stress and promote plant growth [47].

All the tested strains showed positive results for protease and catalase activity and cellulose degradation ability in agar plate assay in the current study. Similar results were found by Iqbal et al. [25], who reported positive results for catalase and protease activity of different rhizobacterial and endophytic strains. He also reported the cellulose degradation ability of *Bacillus* strains. These enzymes were comprehensively reported for their effectiveness in tolerating stress [48].

The data showed that all the zinc solubilizing bacteria have root colonization ability; however, the highest root colonization ability was given by the strain IUB2 in the maize crop and the strain IUB3 in the wheat crop. Rezaee-Niko et al. [49] supported these results and presented that seed inoculation enhanced root colonization, which ultimately resulted in improved growth. Root colonization enhanced uptake of nutrients and promote plant growth [26].

All the tested strains showed a negative value for ammonia production. Similar results have also been reported by Mumtaz et al. [24] in which four strains showed negative results for ammonia production.

All the zinc solubilizing bacterial strains also gave negative results for the oxidase activity. These results have also been supported by Dinesh et al. [50]. Similar results have also been described by Mumtaz et al. [24] who stated that from selected 6 bacterial strains 3 strains showed negative value for oxidase activity. These enzymes protect the crops from harmful pathogens and improve plant growth and development [42].

Conclusions

Zinc solubilizing bacterial strains and enriched compost improved the growth of maize and wheat crops. However, strains IUB2, IUB3, IUB6, IUB10 and IUB11 perform better to promote seedling growth. The tested strains possess several biochemical and enzymatic activities in laboratory studies. These strains also increased the availability of zinc through solubilization. Hence, combined application of zinc solubilizing rhizobacterial strains and enriched compost perform better than sole inoculation. Therefore, bioactive Zn-enriched compost may be formulated by using a consortium of these strains after field evaluation.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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