



**Full Length Article**

## **Plant Growth Promoting Rhizobacteria: An Alternate Way to Improve Yield and Quality of Wheat (*Triticum aestivum*)**

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### **Abstract**

In this research 198 isolates were collected from native soils of Pakistan and *in vitro* testing was done for Zn mobilizing activity. Three promising Zn solubilizers namely FA-2, FA-3, FA-4 and their consortium were tested under field conditions with four commercial wheat (*Triticum aestivum* L.) cultivars viz. Inqlab 91, Chakwal-50, Lasani-08 and SH-2002. A significant increase of 54, 68, 57 and 46% in wheat Zn content over chemical Zn fertilizer was observed under all PGPR treatments. Low increase in grain Zn concentration of 6.5, 7.0, 15.2 and 12.5% was noticed over control by Zn fertilizer treatment with all four wheat genotypes. Various wheat genotypes showed different response with PGPR applications. Similarly, all three strains and their consortium increased wheat grain yield by 2.4, 0.7, 2.2 and 8.6% over chemical Zn fertilizer, respectively. The strains identified by 16S rRNA, *gyrB* and *gyrA* gene analysis were *Serratia liquefaciens*, *S. marcescens* and *Bacillus thuringiensis*. The present findings show that enhanced rate of PGPR colonization can improve grain yield and Zn content of wheat as compared to chemical Zn fertilizer. Co-inoculation of PGPR proved to have more potential of Zn mobilization towards grain. Maintaining suitable density of Zn mobilizers in the soil through field inoculation might be a promising strategy to enhance grain yield and Zn content of wheat. Commercial field application of this approach among farmers is recommended. © 2015 Friends Science Publishers

**Keywords:** Zn solubilizing PGPR; Zn-biofortification; Wheat; Endophyte; Survivability

### **Introduction**

Zinc (Zn) is an essential element involved in many physiological and metabolic activities of plants, humans and microorganisms (Broadley *et al.*, 2007). This mineral nutrient is becoming scarce in the soils due to reasons like less organic matter, excessive fertilization, poor recycling of crop residues, high yielding crop cultivars and intensive cropping pattern (Hafeez *et al.*, 2013). The scarcity of Zn in the soil ultimately results in the deficiency of Zn in the crop produce.

Zn is among top five nutrient deficient elements in human and negatively influence about 1/3<sup>rd</sup> of the world's populations especially women and preschool children (Hotz and Brown, 2004; Stein, 2010; Zhang *et al.*, 2012). Wheat is a good source to supplement Zn in human diet. It is staple food in many developing countries of the world and can provide about 45 mg kg<sup>-1</sup> of grain Zn (Cakmak, 2008; Stein, 2010; Zhang *et al.*, 2012; Zou *et al.*, 2012). Improvement of Zn content in wheat can be a good option to overcome Zn deficiency.

Some breeding and transgenic approaches have been developed in the past to improve the Zn content in cereals. However long time span, socio-economic and political issues hindered these practices to adopt. Fertilizer application also appears to be a quick way to enhance the Zn content in grain but it is expensive and non-sustainable (Bahrani *et al.*, 2010; Cakmak *et al.*, 2010; White and Broadley, 2011; Bulut, 2013; Hafeez *et al.*, 2013). Development of suitable strategies on sustainable basis that would be handy, economically viable and socially acceptable is needed for Zn fortified cereals.

Presently bio-fortification approach is getting much attention to increase the availability of micronutrients especially Zn and Fe in the major food crops (Stein, 2010). Use of Plant Growth Promoting Rhizobacteria (PGPR) is becoming an effective approach to substitute synthetic fertilizers, pesticides, and supplements. PGPR mobilize the nutrients by various mechanisms such as acidification, chelation, exchange reactions, and release of organic acids (Chung *et al.*, 2005; Hafeez *et al.*, 2005).

The root colonization of native PGPR might show better effects on crop plants (Baig *et al.*, 2014). Their effectiveness has been found to be dependent upon population density (Smyth *et al.*, 2011). Moreover, the endophytic bacteria might appear more effective for plant growth and nutrition as compared to rhizospheric bacteria due to more close contact with plant tissues. The dual beneficial effects of PGPR on nutrient mobilization and bio-fortification of crop plants is also well documented (Hafeez *et al.*, 2006; Hayat *et al.*, 2010; Metin *et al.*, 2010; Rana *et al.*, 2012). Similarly native PGPR might respond differently with different genotypes of a crop species. To the best of our knowledge, very little information is available on this aspect. Hence, we designed a study to use locally isolated Zn-solubilizing rhizobacteria as model strains to see their effects on wheat yield and Zn contents under lab and field conditions. The Zn mobilizing rhizobacterial strains were inoculated on four commercial wheat varieties to observe colonization ability and Zn mobilization in wheat grain. The overall objective of the proposed study was to gain in depth knowledge of response of Zn-solubilizers towards growth, yield and quality of various wheat genotypes under field conditions.

## Materials and Methods

### Microbes, Source and Culture Conditions

In this study 198 bacterial strains were isolated. About 100 bacteria were isolated from the wheat rhizosphere growing at different climatic conditions of Pakistan and 99 strains were obtained from Pakistan Collection of Microbial cell Culture (PCMC) previously collected from wheat fields. The bacteria were routinely grown on LB agar and stored at -80°C in 20% glycerol. Different characteristics of isolates were detected on specified medium.

### Screening of Zn Solubilizing Rhizobacteria

The isolates were inoculated into Bunt and Rovira agar medium (Bunt and Rovira, 1955) containing 0.1% of the insoluble zinc compounds; zinc oxide (ZnO), zinc sulphide (ZnS), zinc phosphate [ $\text{Zn}_3(\text{PO}_4)_2$ ] and zinc carbonate ( $\text{ZnCO}_3$ ) incubated at 30°C for 36 to 96 h. Appearance of halo zone around the colonies indicated their potential to solubilize Zn which was estimated by measuring the zone diameter at different time intervals.

The Zn solubilizing ability of the strains was quantified by growing them in Bunt and Rovira broth at  $30 \pm 2^\circ\text{C}$  for 48 h with shaking at 160 rpm. The cell free supernatant was obtained by centrifuging the culture at 7000 rpm for 15 min followed by passing through a  $0.22 \mu\text{m}$  filter and analyzed on Atomic Absorption Spectrophotometer (AA-6300, Shimadzu). Amount of Zn solubilized by strains was quantified by comparing with the uninoculated broth. Experiment was conducted in three replicates.

### Morphological and Biochemical Characterization of Zn Solubilizing Rhizobacteria

The Zn solubilizing rhizobacteria were characterized for Gram reaction (Hassan *et al.*, 2010) and inherent resistance to various antibiotics namely ampicillin, amikacin, tetracycline and kanamycin (Alvarez and Brodbelt, 1995).

Exopolysaccharide (EPS) activity of strains was assessed qualitatively by growing them on Weaver mineral medium supplemented with glucose. The EPS production was observed visually as fluffy material on the plates after 48 h of incubation at  $28 \pm 2^\circ\text{C}$  (modified from (Weaver *et al.*, 1975).

### Phosphorus Solubilization and ACC-deaminase Activity

Phosphorous mobilization ability of strains was tested on Pikovskaya agar medium (Pikovskaya, 1948) and semi-quantified by measuring the zone diameter. Production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase was detected by observing the growth of strains on Brown and Dilworth (BD) minimal medium (Brown and Dilworth, 1975) containing  $0.7 \text{ g L}^{-1}$  ACC as a sole nitrogen source. The BD petri plates containing  $0.7 \text{ g L}^{-1} \text{ NH}_4\text{Cl}$  were kept as positive control, and BD plates containing no nitrogen source were used as negative control. Alternatively ACC-deaminase activity was verified by amplifying *acdS* gene using set of primers as mentioned in Table 1.

### Antagonism and Siderophore Production

The antagonistic activity of Zn solubilizing rhizobacteria was tested on potato dextrose agar (PDA) and yeast malt agar (YMA) against phytopathogenic fungi; *Fusarium caulimons*, *F. graminearum*, *F. oxysporum* and *F. solani* were obtained from the AIT strain collection by the dual culture technique (Tambong and Höfte, 2001). A 5 mm disk of each fungus was positioned in the midpoint of petri plates and  $10 \mu\text{L}$  of bacterial culture grown for overnight in tryptone soy agar (TSA) was speckled 2 cm away from the fungus. Petri dishes were incubated at  $27 \pm 2^\circ\text{C}$  for 12 days and inhibition of fungal mycelium was observed. The antagonistic activity of the strains was scored according to Perneel *et al.* (2007) and Hassan *et al.* (2010). Growth of fungal mycelium over bacterial colony = 0, growth of fungal mycelium near edge of bacterial colony = 1, Growth of fungal mycelium away from bacterial colony = 2. Ability of strains to produce siderophores was detected by growing them on the Chrome azurol S (CAS) agar and observing the change in color as exemplified by (Schwyn and Neilands, 1987).

### Auxin Production and Analysis of *nifH* Gene

Auxin production was assessed both in the presence and absence of L-tryptophan as described by Sarwar *et al.* (1992). IAA was determined by bacterial isolates grown in

LB broth ( $28 \pm 2^\circ\text{C}$ , 180 rpm) supplemented with 1% L-TRP. The cells were harvested by centrifugation ( $10,000\times g$  for 10 min) and three milliliters of the supernatants were mixed with 2 mL Salkowski's reagent ( $12 \text{ g L}^{-1} \text{ FeCl}_3$  in 429 mL of  $\text{H}_2\text{SO}_4$ ). The mixture was incubated at room temperature for 30 min for color development and absorbance at  $\text{OD}_{535 \text{ nm}}$  was measured using microplate reader. Auxin quantity produced by bacterial cells was determined using standard curve for IAA prepared from serial dilutions of  $10 - 100 \mu\text{g mL}^{-1}$ . The nitrogen fixing ability was assessed by amplifying *nifH* with set of primers given in Table 1.

### Identification of Zn Mobilizing Rhizobacteria

Zn mobilizing rhizobacteria were identified by 16S rRNA, *gyrB* and *gyrA* genes analysis. The genomic DNA was extracted using the FastDNA® Spin kit (MP Biomedicals, Solon, OH, USA). DNA was quantified using Nanodrop ND-1000 (Nano Drop Technologies, Wilmington, DE). PCR product was detected on 1% gel electrophoresis at Bio-resource lab, AIT and sequenced by LGC genomics (LGC Genomics GmbH, 12459 Berlin-Germany). The sequences were analyzed on BLAST and identified on the basis of homologous gene. The percent (%) identity of each strain with the gene bank entries is given in the Table 2.

### 16S rRNA Gene Analysis

The 16S rRNA gene was amplified by using primers (Table 1). PCR reaction mixture ( $20 \mu\text{L}$ ) was prepared by mixing  $15 \mu\text{M}$  of each primers (forward and reverse),  $10\times$  buffer BD, 2 mM deoxynucleoside triphosphate (dNTP), 25 mM  $\text{MgCl}_2$ , 5  $\mu\text{L}$  FIREPol® DNA Polymerase (Solis Biodyne) and 1  $\mu\text{L}$  of genomic DNA and amplified in thermocycler following conditions mentioned in Table 1.

### *gyrB* and *gyrA* Gene Analysis

PCR amplification of *gyrB* gene was done by using primers (Table 1) (Yamamoto and Harayama, 1995) while *gyrA* gene was PCR amplified using oligonucleotides (Table 1) (Roberts *et al.*, 1994).

### Field Experiments

The Zn mobilizing strains were evaluated for their ability to colonize the roots of different wheat varieties to enhance growth, yield and zinc content of grains under the field conditions. The experiment was conducted at the fields of Ayub Agriculture Research Institute (AARI), Faisalabad (situated between  $31^\circ - 25^\circ \text{ N}$  latitude,  $73^\circ - 06^\circ \text{ E}$  longitudes and 214 m altitude)-Pakistan.

The experiment was laid out as a completely randomized split-plot design with four blocks as replicates. There were seven treatments *viz.* Zn solubilizing strains FA-2 (T1), FA-3 (T2), FA-4 (T3), the combination of strains FA-2, FA-3 and FA-4 (T4), positive control with chemical

Zn ( $20\% \text{ ZnSO}_4 @ 30 \text{ kg ha}^{-1}$ ) (T5), negative control-1 with the non-Zn solubilizing strain FA-1 (T6), negative control-2 without the amendment of bacteria (T7). Treatments assigned in the main-plot and the four wheat varieties Inqlab 91, Chakwal-50, Lasani-08 and SH-2002 were allocated in the sub-plots. Size of main-plot and sub-plot was ( $6.9 \text{ m}^2$ ) and ( $1.7 \text{ m}^2$ ) respectively. The soil samples were collected from 0-30 cm depth and analyzed.

Soil was ploughed at desirable field condition followed by fertilizer NPK application @  $90:60:60 \text{ kg ha}^{-1}$ . All the P, K and  $1/4^{\text{th}}$  N was applied at the time of sowing and remaining N was applied in 2-3 split doses at tillering and dough stages. The seeds treated with bacteria were sown on  $15^{\text{th}}$  October, 2010 with Row-to-Row distance = 0.30 m and Plant-to-Plant distance = 0.8 m. The plants were irrigated five times as the earliest irrigation was done 30 days after crop emergence and later irrigations were applied at specific crop stages *i.e.*, tillering, booting, anthesis and grain development (Wajid *et al.*, 2002).

### Preparation of Bacterial Inocula

The Zn solubilizing rhizobacteria were grown in LB broth in a 250 mL Erlenmeyer flask on a shaking incubator at  $100 \text{ rev min}^{-1}$ ,  $28 \pm 1^\circ\text{C}$  for 48 h until a cell population reached up to  $10^9 \text{ CFU mL}^{-1}$  ( $\text{OD}_{595 \text{ nm}}$ ) (Hassan *et al.*, 2010). Five mL of this bacterial culture was poured into polythene zipper bag containing 20 g of wheat seed, 2 g of soil and mixed well to maintain the cell population of  $10^8 \text{ CFU mL}^{-1}$ .

### Root Colonization of Inoculated Zn Rhizobacteria on Wheat Roots

Roots of plants were sampled at the germination and harvesting of wheat to determine the bacterial colonization by the method of He *et al.* (2009). Root-adhered soil was collected by shaking the roots gently and mixed well. For endophytic bacterial population, the roots of plants were mixed, sterilized and blended in a mortar pestle. The rhizobacteria were isolated by making serial dilution method and inoculated strains were identified on the basis of their morphological and biochemical markers such as colony color, Zn solubilizing ability, antagonistic activity and antibiotic resistance. Average number of bacterial colony forming units was calculated as mean colony-forming unit  $\text{mL}^{-1}$  of root rhizosphere and root endosphere. The percent (%) survivability was calculated by the formulae given below:

$$\% \text{ survivability} = \frac{\text{CFU mL}^{-1} \text{ at harvesting}}{\text{CFU mL}^{-1} \text{ at germination}} \times 100$$

### Growth Parameters, Yield and Zn Analysis of Grain

Different growth parameters such as days to heading, grains spike $^{-1}$  and tillers plant $^{-1}$  were measured. Total biomass, grain yield and dry straw weight were recorded.

**Table 1:** Sequences of oligonucleotides used as primers for amplification of various genes

Primer	Sequence (5'-3')	Target gene (s)	PCR profile	Reference
8f	AGAGTTTGATCCTGGCTCAG	16S	95°C for 5 min, 39 cycles of 95°C for 30 s, (Weisburg <i>et al.</i> , 1991)	(Edwards <i>et al.</i> , 1989)
1520rev	AAGGAGGTGATCCAGCCGCA		55°C for 45s, 72°C for 90s, elongation at 72°C for 10 min	
UP1_S	GAAGTCATCATGACCGTTCTGCA	gyrB	95°C for 5 min, 35 cycles of 95°C for 60 s, (Yamamoto and	Harayama 1995)
UP2r_S	AGCAGGGTACGGATGTGCGAGCC		58°C for 60s, 72°C for 120s, elongation at 72°C for 10 min	
GyrAF	CAGTCAGGAAATGCGTACGTC	gyrA	95°C for 3 min, 35 cycles of 95°C for 30 s, (Roberts <i>et al.</i> , 1994)	
gyrAR	CAAGGTAATGCTCCAGGCATT		56°C for 45s, 72°C for 60s, elongation at 72°C for 10 min	
PolF	TGCGAYCCSAARGCBGACTC	nifH	94°C for 4 min, 30 cycles of 94°C for 60 s, (Poly <i>et al.</i> , 2001)	
PolR	ATSGCCATCATYTCCRCCGGA		55°C for 60s, 72°C for 120s, elongation at 72°C for 10 min	
DegACCF	GGBGGVAAYAARMYVMGSAAGCTYGA	acdS	95°C for 3 min, 30 cycles of 95°C for 30 s, (Nikolic <i>et al.</i> , 2011)	
DegACCr	TTDCCHKYRTANACBGGRTC		46°C for 60s, 72°C for 60s, elongation at 72°C for 5 min	

**Table 2:** Various gene analysis based percent (%) identity of Zn solubilizer with diverse entries of GENBANK

Strains	(%) Homology		
	16S rRNA gene	gyrB gene	gyrA gene
FA-2	<i>Serratia grimesii</i> , <i>S. liquefaciens</i> (99 %)	<i>S. liquefaciens</i> (99%)	
FA-3	<i>Bacillus thuringiensis</i> (100 %)	<i>B. thuringiensis</i> (99%)	<i>B. thuringiensis</i> (99%)
FA-4	<i>S. marcescens</i> (99 %)	<i>S. marcescens</i> (99%)	

After harvesting, the wheat grains were analyzed for Zn analysis by tri-acid (a mixture of  $\text{HClO}_4$ ,  $\text{HNO}_3$ ,  $\text{H}_2\text{SO}_4$  at 1:2:5) digestion (Allen *et al.*, 1986) using Atomic absorption spectrophotometer (AAS).

### Statistical Analysis

The Genstat 9.2 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK) statistical package was used for ANOVA on the data of plate assay, AAS analysis, wheat growth and yield, and grain Zn concentration parameters, followed by a post hoc least-significant difference (LSD) test for comparison of means.

## Results

### Screening of Zn Solubilizing Rhizobacteria

All the selected isolates FA-4, FA-3, FA-2 and FA-8 showed considerably diverse zone for Zn solubilization (59.50 mm, 51.17 mm, 27.5 mm and 17.17 mm) gradually on  $\text{ZnCO}_3$  (Fig. 1). Less difference was found between FA-3 and FA-4 for the zone formation on ZnO but both (FA-3 and FA-4) isolates showed distinctly higher zone as compared to FA-2 and FA-8. A similar trend was observed on  $\text{Zn}_3(\text{PO}_4)_2$ . The isolates FA-2, FA-3, FA-4 and FA-8 notably formed no zone on ZnS. The bacterial strains FA-4 as well as FA-3 appeared best and efficient in zone formation than other strains. The strain FA-8 showed less effectiveness so it was skipped in next study. The isolates showed no visible activity on ZnS ore till 18<sup>th</sup> day of inoculation. The solubilization activity of the strains was maximum on 3<sup>rd</sup> day, which remained almost constant until

**Table 3:** *In vitro* quantification of Zn through AAS mobilized by Zn-solubilizing rhizobacteria on different Zn ores

Strains	$\text{ZnCO}_3$ (mg $\text{kg}^{-1}$ )	ZnO (mg $\text{kg}^{-1}$ )	ZnS (mg $\text{kg}^{-1}$ )
Control	10 <sup>D</sup>	9.6 <sup>E</sup>	0.14 <sup>C</sup>
<i>S. liquefaciens</i> FA-2	135 <sup>C</sup>	18 <sup>D</sup>	0.20 <sup>C</sup>
<i>B. thuringiensis</i> FA-3	152 <sup>B</sup>	126 <sup>A</sup>	1.17 <sup>A</sup>
<i>S. marcescens</i> FA-4	164 <sup>A</sup>	93 <sup>C</sup>	0.35 <sup>B</sup>
LSD	11.93	5.99	0.41

Mean values (n=3); Means values with different letters in the same column differ significantly at  $P < 0.05$  based on LSD

18<sup>th</sup> day of inoculation except on  $\text{Zn}_3(\text{PO}_4)_2$  ore which significantly increased between 4<sup>th</sup> day to 18<sup>th</sup> day (Fig. 1).

### Quantification of Zn Solubilizing Ability of Rhizobacteria

Activity of three strains with maximum Zn-solubilization on agar plates based on zone diameter was further quantified by atomic absorption spectrophotometer (AAS). In AAS analysis, the strain FA-3 solubilized maximum Zn from ZnO ore (Table 3), while FA-4 solubilized maximum Zn from  $\text{ZnCO}_3$ . Amongst the strains, FA-4, FA-3 and FA-2 showed less potential to solubilize Zn from the ZnS ore (0.35  $\mu\text{g mL}^{-1}$ , 0.46  $\mu\text{g mL}^{-1}$  and 1.17  $\mu\text{g mL}^{-1}$ ). A significant correlation was found between the zone diameter and AAS quantification of Zn-solubilizing rhizobacteria.

### ACC-deaminase Production, Exopolysaccharide Activity (EPS) and Phosphorus (P) Solubilization

All the selected strains were positive for ACC-deaminase activity on plate assay and confirmed by amplifying *acdS*

gene by PCR (Table 3a). The strains FA-2, FA-3 and FA-4 showed positive EPS activity (Table 4). The strain FA-3 (14.66 mm) produced the clearest zone diameter for P mobilization followed by the strains FA-4 (13.33 mm) and FA-2 (13 mm), respectively (Table 5).

### Antifungal Activities and Siderophore Production

The rhizobacterial strains produced siderophore and suppressed the growth of *Fusarium* species, a fungal phytopathogen. Greater antagonistic activity of strain FA-3 (scored = 3) was against *F. graminearum*, *F. caulimons* and *F. solani* while it was less (scored = 1) against *Fusarium oxysporum* (Table 4). The efficiency of FA-4 was found to be more antagonistic (scored = 3) against *F. graminearum* as compare to *F. oxysporum*, *F. caulimons* and *F. solani* (scored = 2). The moderate antagonistic activity of FA-2 was observed against all *Fusarium* species.

### Nitrogen (N<sub>2</sub>) Fixing Activity and Production of IAA

The strains were positive for N<sub>2</sub> fixation *nif* gene amplification except FA-3 (Table 4). Moreover, all the strains also showed diverse pattern of IAA production with L-TRP (ranging from 2.57 to 4.20 µg/mL) and without L-TRP. Maximum IAA production was observed for FA-2 with L-TRP (4.20±0.01) and without L-TRP (1.23±0.01) followed by FA-3 (2.62±0.01) and FA-4 (2.57±0.01) with L-TRP and FA-3 (1.09±0.01) and FA-4 (1.08±0.01) without L-TRP (Table 5).

### 16S rRNA, gyrB and gyrA Genes Analysis of Strains

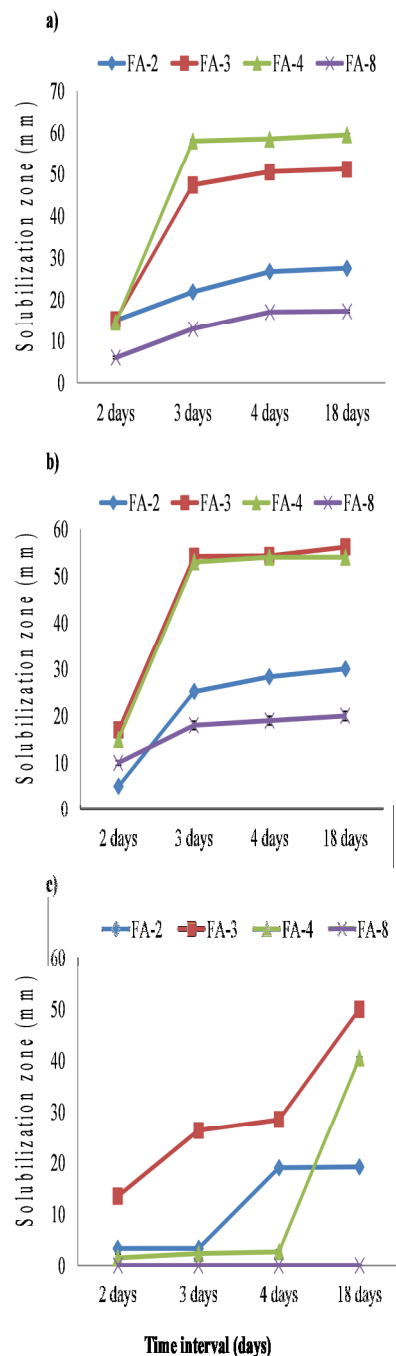
The 16S rRNA and gyr B genes analysis of the strains FA-2 and FA-4 revealed their identities as *Serratia liquefaciens* and *S. marcescens*, respectively. *gyrA* and *B* gene analysis of FA-3 was confirmed as *Bacillus thuringiensis* (Table 2).

### Field Efficacy of Selected Zn Rhizobacteria on Wheat Genotypes

Three Zn solubilizing strains were selected for field trials based upon *in vivo* plant growth promoting activities. Characterization for root colonization ability showed that the strain FA-4 exhibited maximum value of survivability (72.9%) followed by FA-2 (69%) and FA-3 (63%) in rhizosphere (Fig. 3). All three strains showed significant variation for endophytic survivability. Our results revealed that strain FA-2 had highest survivability (99%) followed by the strain FA-3 (97%) and strain FA-2 (81%) (Fig. 3).

### Effect of PGPR on Growth, Yield and Yield Components of Wheat

Data pertaining to the days to headings, tillers plant<sup>-1</sup> and yield as affected by different treatments are given in Table 6. Data for days to headings is found to be statistically significant. Maximum days to anthesis (106) were recorded



**Fig. 1:** Solubilization potential of Zn mobilizing rhizobacteria on the basis of halo zone formation with different insoluble Zn ores (a) Zn carbonate (b) Zinc oxide (c) Zinc phosphate at different intervals of time; Data are mean values of three replicates; error bars indicate the standard error

with absolute control, while minimum days (104) were observed with treatments FA-2, FA-4 and Zn fertilizer respectively.

**Table 4:** Plant growth promoting and biocontrol attributes of Zn-solubilizing rhizobacteria

Strains	Gram reaction	*ACC-deaminase activity		N <sub>2</sub> fixation		Exo-polysaccharide activity	Siderophore activity	Antifungal activity			
		Plate assay	<i>acdS</i> amplification	gene <i>nif</i> amplification	gene	Plate assay	Plate assay	<i>F. caulimons</i>	<i>F. graminearum</i>	<i>F. oxysporum</i>	<i>F. solani</i>
Control FA-1	-	-	-	+	+	+	-	-	-	-	-
<i>S. liquefaciens</i> FA-2	-	+	✓	+	+	+	+	++	+	++	++
<i>B. thuringiensis</i> FA-3	+	+	✓	--	+	+	+	+++	+++	+	+++
<i>S. marcescens</i> FA-4	-	+	✓	+	+	+	+	++	+++	++	++

\*ACC = 1-aminocyclopropane-1-carboxylic acid Readings are average of three replicates, +++ = Very good activity, ++ = Good activity, + = Fair activity, = No activity

**Table 5:** Phosphate solubilization and Indol Acetic Acid production of Zn-solubilizing rhizobacteria

Strains	P solubilizing activity		*IAA production	
	Plate assay (mm)	With L-TRP (µg mL <sup>-1</sup> )	Without L-TRP (µg mL <sup>-1</sup> )	
Control FA-1	-	-	-	
<i>S. liquefaciens</i> FA-2	13.00±0.2	4.20±0.01	1.23±0.01	
<i>B. thuringiensis</i> FA-3	14.66±0.2	2.62±0.01	1.09±0.01	
<i>S. marcescens</i> FA-4	13.33±0.2	2.57±0.01	1.08±0.01	

\*IAA = Indole acetic acid; values are average of three replicates ± standard error

**Table 6:** Growth and yield parameters of wheat crop inoculated with Zn-mobilizing rhizobacteria at Ayub Agriculture Research Institute (AARI), Faisalabad

Characteristics	Days to headings (No.)	Grains spike <sup>-1</sup> (No.)	Tiller plant <sup>-1</sup> (No.)	Grain yield (t ha <sup>-1</sup> )	Biomass (t ha <sup>-1</sup> )
<b>Treatments (average over varieties)</b>					
Control	106 <sup>B</sup>	50 <sup>C</sup>	11 <sup>D</sup>	7.0 <sup>C</sup>	9.5 <sup>B</sup>
<i>S. liquefaciens</i> FA-2	104 <sup>A</sup>	59 <sup>A</sup>	13 <sup>B</sup>	8.0 <sup>B</sup>	10.9 <sup>A</sup>
<i>B. thuringiensis</i> FA-3	105 <sup>A</sup>	56 <sup>B</sup>	13 <sup>B</sup>	7.8 <sup>B</sup>	10.7 <sup>A</sup>
<i>S. marcescens</i> FA-4	104 <sup>A</sup>	58 <sup>A</sup>	12 <sup>C</sup>	8.0 <sup>B</sup>	10.2 <sup>AB</sup>
Consortium <sup>1</sup>	105 <sup>A</sup>	59 <sup>A</sup>	14 <sup>A</sup>	8.5 <sup>A</sup>	10.9 <sup>A</sup>
Zinc fertilizer	104 <sup>A</sup>	55 <sup>B</sup>	12 <sup>C</sup>	7.8 <sup>B</sup>	10.7 <sup>A</sup>
Non ZSB FA-1	106 <sup>B</sup>	52 <sup>C</sup>	11 <sup>D</sup>	7.2 <sup>C</sup>	10.5 <sup>B</sup>
<b>Varieties (average over treatments)</b>					
Chakwal-50	104 <sup>B</sup>	56.1 <sup>AB</sup>	13.3 <sup>A</sup>	8.1 <sup>A</sup>	10.7 <sup>A</sup>
Inqlab 91	105 <sup>A</sup>	55.2 <sup>BC</sup>	11.9 <sup>B</sup>	6.9 <sup>C</sup>	10.1 <sup>B</sup>
Lasani-08	104 <sup>B</sup>	53.9 <sup>C</sup>	12.6 <sup>A</sup>	8.3 <sup>A</sup>	10.7 <sup>A</sup>
SH-2002	104 <sup>B</sup>	57.1 <sup>A</sup>	11.5 <sup>B</sup>	7.7 <sup>B</sup>	10.5 <sup>AB</sup>
Treatments (P)	<0.001	<0.001	<0.001	<0.001	0.001
Varieties (P)	0.005	0.002	<0.001	<0.001	0.054
Treatments x Varieties (P)	0.841	<0.001	0.170	0.007	0.086

\*Mean values (n=3); Mean values with different letters in the same column differ significantly at P<0.05 based on LSD

1. *S. liquefaciens* FA-2 + *B. thuringiensis* FA-3 + *S. marcescens* FA-4

Grains spike<sup>-1</sup> were significantly higher (consortium = 59, FA-2 = 59, FA-4 = 58, FA-3 = 56) as compare to other treatments (fertilizer = 55, negative Zn mobilizer strain = 52). The lowest number of grains per spike (50) was recorded from control, where no fertilizer/inoculant was applied.

Data on number of tillers plant<sup>-1</sup> is presented in Table 6 indicated that different treatments of Zn mobilizing PGPR were found to be highly significant. Highest number of tillers plant<sup>-1</sup> (14) was obtained where consortia of three strains (FA-2 + FA-3 + FA-4) was applied while treatments FA-2 and FA-3 was observed with similar number (13) of tillers plant<sup>-1</sup>. Lower number of tillers was recorded for Zn fertilizer treatment (12) followed by non Zn mobilizer strain (11) while minimum number of tillers plant<sup>-1</sup> (11) was

attained from control where neither fertilizer nor inoculum was applied.

Relative study of the means confirmed that, maximum grain yield (8.5 t ha<sup>-1</sup>) was recorded in plots where consortia (FA-2 + FA-3 + FA-4) was applied. This treatment showed 9% increase in yield as compared to Zn fertilizer treatment (Fig. 2). It was statistically at par with FA-2 (8.0 t ha<sup>-1</sup>), FA-3 (8.0 t ha<sup>-1</sup>), FA-4 (8.0 t ha<sup>-1</sup>) and Zn fertilizer treatment (7.8 t ha<sup>-1</sup>). The lowest value was noticed with non Zn mobilizer strain treatment (7.2 t ha<sup>-1</sup>) followed by control having yield 7.0 t ha<sup>-1</sup>. The results further showed that Zn mobilizing rhizobacterial application significantly increased the grain yield of wheat as illustrated (Table 6).

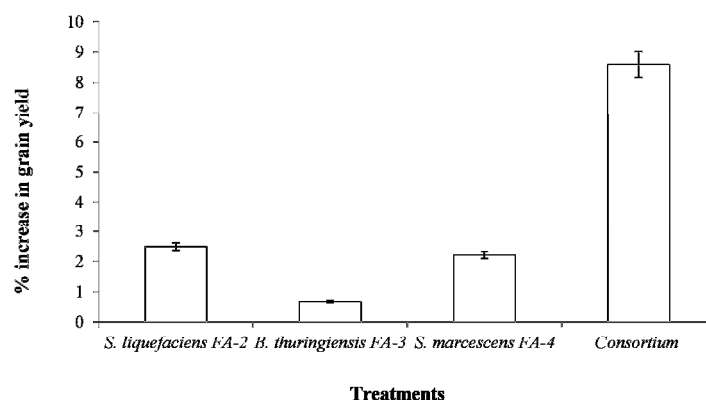
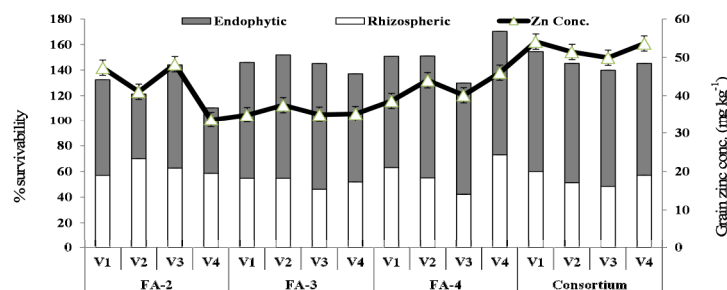
Among wheat cultivars, variations were observed for

**Table 7:** Grain Zn concentration of four wheat genotype inoculated with Zn solubilizing rhizobacteria at Ayub Agriculture Research Institute (AARI), Faisalabad

Treatments	Zn contents (mg kg <sup>-1</sup> )			
	Chakwal-50	Inqlab 91	Lasani-08	SH-2002
Control	33.0 <sup>*D</sup>	28.6 <sup>D</sup>	27.6 <sup>D</sup>	32.6 <sup>E</sup>
<i>S. liquefaciens</i> FA-2	47.3 <sup>B</sup>	33.6 <sup>BC</sup>	35.0 <sup>BC</sup>	44.0 <sup>BC</sup>
<i>B. thuringiensis</i> FA-3	41.0 <sup>C</sup>	34.9 <sup>BC</sup>	35.2 <sup>BC</sup>	40.1 <sup>CD</sup>
<i>S. marcescens</i> FA-4	48.2 <sup>B</sup>	37.5 <sup>B</sup>	38.6 <sup>B</sup>	46.0 <sup>B</sup>
Consortium <sup>1</sup>	54.1 <sup>A</sup>	51.4 <sup>A</sup>	49.9 <sup>A</sup>	53.6 <sup>A</sup>
Zinc fertilizer	35.1 <sup>D</sup>	30.6 <sup>CD</sup>	31.8 <sup>BCD</sup>	36.7 <sup>DE</sup>
Non Zn mobilizing strain FA-1	34.1 <sup>D</sup>	31.6 <sup>CD</sup>	31.3 <sup>CD</sup>	34.0 <sup>E</sup>
LSD	4.9	4.68	7.24	5.37

\*Mean values (n=3); Mean values with different letters in the same column differ significantly at  $P < 0.05$  based on LSD

1. *S. liquefaciens* FA-2 + *B. thuringiensis* FA-3 + *S. marcescens* FA-4

**Fig. 2:** Percent (%) increase in grain yield of wheat crop inoculated with Zn solubilizing rhizobacteria over chemical Zn fertilizer; Data are mean values of three replicates; error bars indicate the standard error; Consortia = *S. liquefaciens* FA-2 + *B. thuringiensis* FA-3 + *S. marcescens* FA-4**Fig 3:** Bacterial percent (%) survivability (rhizospheric and endophytic colonization) and grain zinc concentration (mg kg<sup>-1</sup>) of wheat after inoculation with Zn solubilizing rhizobacteria; Data are mean values of three replicates; error bars indicate the standard error; *S. liquefaciens* FA-2, *B. thuringiensis* FA-3, *S. marcescens* FA-4; Consortia = *S. liquefaciens* FA-2 + *B. thuringiensis* FA-3 + *S. marcescens* FA-4

growth and yield parameters. The cultivars Chakwal-50 and Lasani-08 showed similar number of tillers plant<sup>-1</sup>, grain yield and biomass followed by the cultivars SH-2002 and Inqlab-91 (Table 6).

Grain Zn concentration affected by different treatment is given in Table 7. A significant increase in the Zn concentration 64, 79.2, 80.5 and 64.1% was found over control in wheat genotypes Chakwal-50, Inqlab-91, Lasani-

08 and SH-2002, respectively treated with consortium (FA-2, FA-3 and FA-4). Variable effect was observed by the individual bacterial inocula applied to all four wheat varieties. Data revealed that the consortium contributed more to grain Zn concentration compared with individual bacterial strains (Fig. 3). Less increase in grain Zn concentration 6.5, 7.0, 15.2 and 12.5% was noticed over control by Zn fertilizer treatment with genotypes Chakwal-50, Inqlab-91, Lasani-08 and SH-2002 in that order (Table 7).

## Discussion

In this study, we selected potential Zn solubilizing rhizobacteria after intensive qualitative and quantitative screening of wheat associated rhizobacteria. Low population of rhizobacteria (4.6%) as Zn mobilizers was observed in wheat rhizosphere in this study. These strains significantly increased wheat yield as well as Zn content of grains under field conditions.

PGPR solubilized the insoluble Zn ores efficiently by plate assay namely zinc carbonate, zinc oxide and zinc phosphate while zinc sulphide was not solubilized. As the plate assay has some limitations, it is not taken as authentic procedure to assess the solubilization or mineralization ability of bacteria. Therefore, the rhizobacteria showing good potential of Zn solubilization on agar plate were further tested in a liquid broth supplemented with the insoluble Zn compounds. The strain *S. marcescens* FA-4 solubilized the zinc carbonate compound distinctly over strain *B. thuringiensis* FA-3 and *S. liquefaciens* FA-2 respectively. The strain *B. thuringiensis* FA-3 solubilized the zinc oxide appreciably over *S. marcescens* FA-4 and *S. liquefaciens* FA-2, respectively.

The mechanisms of Zn solubilization by the PGPR from insoluble Zn compounds might be a result of proton extrusion and production of organic acids by microbes. The solubilization of insoluble Zn is due to production of organic acids such as gluconic acid or derivatives of gluconic acids and consequent release of Zn in external surroundings had been reported previously by (Costa and Duta, 2001). Solubilization of Zn was found higher with zinc carbonate as compared to zinc oxide and zinc phosphate. This is in contrast to the finding of Ramesh *et al.* (2014), who reported that MDSR7 and MDSR14 efficiently solubilized zinc phosphate than zinc oxide and zinc carbonate. Our results are however, in accordance with (Saravanan *et al.*, 2007), who indicated that *Gluconacetobacter diazotrophicus* PAL5 more effectively solubilized zinc oxide than zinc carbonate and zinc phosphate. However, Zn solubilization was not observed with zinc sulphide ore as described earlier (Amalraj *et al.*, 2012). The solubilization of zinc sulphide by the rhizobacteria is dependent on the potential to oxidize sulphide ions (Fowler and Crundwell, 1998). Less or lack of the ability to oxidize sulphide ions might be the conceivable

explanation for its slight or no solubilization in broth and plate assays.

Zn mobilizing rhizobacteria FA-2, FA-4 and FA-3 were identified as *S. liquefaciens*, *S. marcescens* and *B. thuringiensis* on the basis of 16S rRNA and *gyrB* gene sequencing. It has been recognized that Zn mobilizing abilities appear to be recurrent among different bacterial taxa (Di Simine *et al.*, 1998; Rajkumar and Freitas, 2008; He *et al.*, 2010). In addition to Zn mobilizing ability of *S. liquefaciens* FA-2 and *S. marcescens* FA-4, their human pathogenicity can not be ignored and needs further toxicological studies to allow field application. The genus *Bacillus* is the center of attention for researchers as they are omnipresent in nature and have numerous traits concerning plant growth promotion (Kohler *et al.*, 2007; Ramírez and Kloepper, 2010; Zhao *et al.*, 2011). However, it is difficult to differentiate the *B. cereus* group (*B. anthracis*, *B. thuringiensis* and *B. cereus*) from one another via 16S rDNA sequence analysis due to their indistinguishable features (La Duc *et al.*, 2004). The *Bacillus* sp. strain FA-3 was further characterized by *gyrA* and *gyrB* gene analysis and based on that is 99% similar to *B. thuringiensis*.

All three Zn solubilizing PGPR produced the exopolysaccharide (EPS) *in vitro* which significantly enhanced the attachment of bacteria to roots of plants for colonization. Soil environment is quite complex consisting of numerous biotic and abiotic factors, which greatly influence the traits of particular strains in the rhizosphere environment. It is well documented that the stress factors prevailing in plant rhizosphere have great impact on plant and change the metabolites of rhizosphere leading to alteration in soil microbial communities and their interaction with the host plant (Compant *et al.*, 2010). Root colonization by bacteria is a basic step to initiate the positive plant microbe interaction, which indirectly depends on the characteristics of bacteria as well as host plant. Root exudates secreted by plants are rich in nutrition for PGPR and facilitate their colonization (Bhattacharjee *et al.*, 2012). An efficient rhizobacteria must be competent to colonize roots to establish itself in the rhizosphere at population density enough to exert the beneficial effects. Inoculation of the combination of three strains showed enhanced effect as compared to individual rhizobacterial inoculation in our study. Similar results have been reported earlier by (Rosas *et al.*, 2009; Yadegari *et al.*, 2010; Mäder *et al.*, 2011; Rana *et al.*, 2012; Ramesh *et al.*, 2014). Zn solubilizing rhizobacteria significantly influenced the growth, yield and Zn concentration of wheat grain over un-inoculated control and Zn fertilizer. These PGP activities are associated with their ability to ensure high availability of free zinc to the plants either through mineralization or solubilization of bound zinc with other compounds in the form of carbonates, bicarbonates and hydroxyl carbonates. This is confirmed with an increase in soil enzyme activities representing rhizobacterial climax and a turn down in the rhizosphere pH



upon bacterial inoculation as reported (Neumann and Romheld, 2002; Oburger *et al.*, 2009). It was noticed that an increase in percent survivability appreciably enhanced Zn concentration in wheat grain. Moreover, percent survivability for endophytic PGPR was higher as compared to rhizospheric zone in this study. This is also in agreement with previous studies in which *B. thuringiensis* was reported efficient for endophytic colonization (Praca *et al.*, 2012). PGPR that colonizes root is best as bio-control agent application against soil-borne diseases, thus improving plant growth (El-Mehalawy *et al.*, 2004). Evaluation of root colonization by Zn mobilizer strains showed that rhizospheric bacterial population declines significantly at harvesting stage as compared to germination.

All the four cultivars showed varied rate of increase in grain Zn concentration over Zn fertilizer after inoculation with PGPR. The crop cultivar is an additional factor as exemplified during a study, where inoculation of wheat with *Pseudomonas* strains promoted plant growth differently depending upon the wheat genotypes in saline soil (Egamberdieva, 2010). Our data suggested that cultivars should be selected based on their capacity to interact with PGPR.

## Conclusion

We report the potential for using Zn solubilizers as a strategy to enhance yield and Zn contents of wheat grain. The consortium of Zn solubilizers could be optimised to introduce and maintain a suitable density of bacteria in the soil for maximum benefit to wheat crop. Co-inoculation of PGPR in wheat crop for Zn biofortification can be an economical source to overcome Zn deficiency in the diet to save poor nations in the world. Commercial field application of these bacterial strains is recommended.

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