Isolation screening and molecular characterization of zinc solubilizing bacteria and their effect on the growth of wheat (*Triticum aestivum*)

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Abstract. Wheat (*Triticum aestivum*) is a major cereal crop grown worldwide. Most of the world population depends on wheat for their nutrient requirement. Zinc (Zn) is one of the most crucial elements required for the development of wheat plant. It is one of the micronutrients required in many biochemical cycles. It has been found that the concentration of Zn is below the required level in the soil and hence it remains deficient in the crops. To ameliorate the deficit, chemical fertilizers are added in the soil, where as biofertilizers are preferred over chemicals in sustainable agriculture. The paper describes the isolation, screening and molecular characterization of the zinc solubilizing bacteria (ZSB) to improve plant growth. A total of 100 soil samples were collected from the rhizospheric soil of wheat plants. ZSB were isolated by dilution plating on Bunt and Rovira media. The 50 isolates were selected and screened for their Zn solubilization. The zinc tolerance of all the isolates varied from 0.5% to 2% of insoluble Zn. Based on the Zn tolerance ability, 15 bacterial isolates were screened for Phosphate solubilization and further analyzed for the synthesis of IAA, NH₃, siderophore production and chitinase activity. The three isolates were selected on the basis of the plant growth promoting characteristics for molecular characterization and were found to be homologous to *Bacillus cereus*, *Pseudomonas aeruginosa* and *Bacillus tropicus*. This study documented the establishment and survival of ZSB in the wheat rhizosphere and enhanced plant productivity, thus indicating the potential of isolates as commercial biofertilizers.

Keywords: Rhizosphere, wheat, Zn, zinc solubilising bacteria (ZSB)

INTRODUCTION

Worldwide nutrient deficiency is a major concern affecting plants as well as humans. Nutrients are classified into two groups as micro and macronutrients. Sodium, potassium, phosphorus, calcium, magnesium and sodium are characterized as macronutrients as they are present in abundance, while iron, manganese, copper, zinc, boron and molybdenum are required by plants in minute quantities, therefore, they are called trace elements or micronutrients (Sharma *et al.*, 2013).

Malnutrition due to trace elements is an issue that needs to be addressed globally (Roohani *et al.*, 2013). Zn deficiency is an emerging challenge seen in humans which directly or indirectly is dependent upon the Zn status of the crops (Noulas *et al.*, 2018).

Zn is the most ubiquitous nutrient requisite of most plant enzymes. There are approximately 3000 protein Zn prosthetic groups in humans and animals (Alloway, 2009). Zn is a catalytic

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component of several enzymes and is crucial for their activity. In plants Zn deficiency leads to impeded photosynthesis, metabolism of nitrogen, carbohydrate synthesis, delayed growth of flowers and fruits and additionally delays maturity in crops thus eventually affecting the yield and nutrient quality (Cele and Maboeta, 2016).

Like several other developing nations, India also faces a challenge, and it is estimated that around 50% of Indian semi-arid soil is Zn deficient (Malik *et al.*, 2017). Soils mainly contain a large concentration of total Zn that occur in the inactive forms in minerals like franklinite (ZnFe_2O_4), hopeite $\text{Zn}_3(\text{PO}_4)_2$, sphalerite (ZnS), smithsonite (ZnCO_3), Znite (ZnO), willemite (Zn_2SiO_4) (Broadley *et al.*, 2007). The critical value of Zn in the soil varies from 0.6 to 1.2 ppm or mg/kg of soil; less than 0.6 ppm means the soil is Zn deficient, and above 1.2 ppm indicates Zn toxicity. Whereas in the plants the concentration of Zn could reach 20-300 ppm, only a minute level is in readily available form as the solubility of Zn is very low in the soil as compared to other nutrients. The solubility of Zn is affected by the pH and moisture content of soil (Athokpam *et al.*, 2018).

There are various strategies to counteract the deficiency of Zn which includes biofortification and agronomic practices (Vejan *et al.*, 2016). The application of Zn fertilizers is also one of the practices used to overcome Zn deficiency in Zn-deficient soil. Even though they seem fast and easy to process, the cost of fertilizers are exorbitant (Hafeez *et al.*, 2013). Moreover, the applied Zn gets transmuted into various inactive forms based on the types of soil and physicochemical composition of soils (Vidyashree *et al.*, 2016). The use of microorganisms is a prospective alternate strategy used in this respect (Abaid Ullah *et al.*, 2015). Plant growth-promoting rhizobacteria (PGPR) are registered for their potential to solubilise inactive forms of Zn and thus air crop yield (Goteti *et al.*, 2013). Zn solubilising bacteria belongs to the category of PGPR consisting of numerous beneficial bacteria that reside in the rhizosphere of plants (Prasad *et al.*, 2015). ZSB assists in the growth and development of plants via stimulating biochemical processes like atmospheric nitrogen fixation and synthesizing siderophores (El-Sayed *et al.*, 2014). Siderophores can link with Zn to

form a complex and make Zn available for plant uptake. These bacteria convert insoluble Zn into a soluble form in various ways like the production of organic acids, siderophores, ammonia and mineral acids resulting in acidification of surrounding soil which also solubilises insoluble Zn chelates (Kamran *et al.*, 2017). Another mechanism through which PGPR enhances the growth of plants is due to the production of Exopolysaccharide (EPS). EPS is high molecular weight and biodegradable polymer which comprises a vital part of the organic matter of the soil. EPS aids in sustaining soil aggregation, water potential and keeping stress condition under control (Gouda *et al.*, 2018). EPS producing bacteria are capable of balancing the drought-like situation and promote growth (Upadhyay *et al.*, 2011). Considering the necessity of the desired amount of Zn in the plants and its available status in the soil, this study was carried out to isolate, screen and study the establishment of potential Zn solubilizing bacteria in the rhizosphere of the wheat.

MATERIALS AND METHODS

Collection of rhizospheric soil samples

Samples of soil were collected from rhizosphere of wheat from the different regions of district Faridabad, Haryana. In the Faridabad region wheat is one of the major crops. The uprooting of plants is done in a sterilized manner and the narrow region of soil surrounding the roots of the plant called rhizospheric soil is collected by vigorous shaking in sterilized Ziplock bags. Post collection, soil samples were transferred to the lab where they were dried to remove excess moisture and stored at 4°C for further analysis.

Isolation of ZSB

ZSB were isolated from the stored soil samples using serial dilution plating on Bunt and Rovira (B&R) medium. The composition was (g/l): glucose (20g), peptone (1g), NH_4SO_4 (0.5g), K_2HPO_4 (0.4g), Yeast extract (1g), FeCl_4 (0.01g), MgCl_2 (0.1g), agar (2%), ZnO (0.1%) (Abaid-Ullah *et al.*, 2015). The rhizospheric soil samples were diluted up to the 10^{-7} dilution in the sterile water blanks, and the last three dilutions were

spreaded on Bunt & Rovira media. The incubation was done for 2 days at $30 \pm 2^\circ\text{C}$. Colonies around which a halo zone appeared were considered as Zn solubilising bacteria and these zone forming isolates were again streaked for purification before transferring them on Bunt & Rovira slants for further experimentation.

Zn solubilising assay

The selected bacterial isolates were inoculated on Bunt & Rovira media containing 0.1% ZnO to determine Zn solubilisation and incubated at 30°C for 48 hours (Abaid-Ullah *et al.*, 2015). The halo zone diameter and colony zone diameter were estimated using a measuring scale. Zinc solubilization efficiency (ZSE) was estimated as:
$$\text{ZSE} = (\text{Halozone diameter} - \text{colony diameter}) / \text{diameter of colony} \times 100$$
 (Khanghahi *et al.*, 2018).

Screening for Zn tolerance

Zn becomes toxic to plants as well as to microbes when it is present in higher concentration in the soil. As the concentration of Zn increases, it retards bacterial growth. If the bacteria cannot tolerate higher concentration of Zn, they are unable to solubilize it. Hence Zn tolerance of isolates was determined on Bunt & Rovira media at different concentrations of zinc oxide (0.5%, 1%, 1.5% and 2%). If the bacteria continue to grow at higher concentrations it is considered tolerant to Zn. At the same time if the halo zone is observed around the colonies it indicates that the bacteria are capable of Zn solubilization as well. The concentration at which the bacteria were unable to form colony was considered as its Zn tolerance value or minimal inhibitory concentration (MIC) of Zn (Sarvana *et al.*, 2004).

Phosphate solubilization efficiency

Phosphate solubilisation of bacterial isolates was determined by inoculating the isolates on Pikovskaya's Medium (PVK) having the composition: glucose (10 g), KCl (0.2 g), MgCl_2 (0.1 g), NH_4SO_4 (0.2 g), NaCl (0.2 g), FeSO_4 (0.02 g), MgSO_4 (0.2 g) Yeast extract (0.5 g), Tricalcium phosphate (0.02 g), distilled water 1000 ml. The pH was adjusted to 7. The plates were then incubated for 48 h at $30 \pm 2^\circ\text{C}$. The colonies that formed clear halo zone were noted as phosphate

solubilizers (Pande *et al.*, 2017). Phosphate solubilization efficiency is estimated as:

$$\text{PSE} = (\text{Halozone diameter} - \text{colony diameter}) / \text{diameter of colony} \times 100.$$

Siderophores production

The siderophore production assay was performed by Chrome azurol S (CAS) reagent (121 mg of chromeazurol-S added to 100 ml distilled water and 20 ml of 1 mM ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) ferric chloride solution mixed with 10mM HCl). To this mixture 20 ml of 1.8 g/l Hexadecyl trimethyl ammonium bromide (HDTMA) solution was added. CAS plates were made using CAS reagent and LB agar. An aliquot of the selected bacterial isolates containing approximately 10^8 cfu/ml was inoculated and incubated for 7 days at 28°C to check for production of orange halo zone (Arora and Verma, 2017).

Indole acetic acid production

Production of auxin was evaluated using lab assay (Agbodjato *et al.*, 2018). Around 3 ml of bacterial culture is mixed with 2 ml of Salkowski reagent consisting of 2 ml of 0.5 M FeCl_3 and 98 ml of 35% HClO_4 . The mixture was kept at room temp for 30 minutes to develop a pink to dark red colour. The intensity of the colour developed is quantified at 500 nm after the slight colour change was noticed by using a spectrophotometer (Mohite, 2013).

Ammonium production

Isolates were inoculated in 10 ml of peptone broth and incubated at 30°C for a period of 48 h. After the incubation, 0.5ml of Nessler's reagent was added into the tube. The appearance of light yellow to brown colour was considered as positive for ammonia production (Mumtaz *et al.*, 2017).

Exopolysaccharide production

Exopolysaccharide (EPS) production was tested by inoculating the bacterial isolates on RCV sucrose media containing 0.1 g yeast extract, 15 g agar and 30 g sucrose in 100 ml distilled water. The culture was incubated for 4 days at 30°C . The EPS production was characterized as having slimy appearance along with mucoid growth (Yadav *et al.*, 2018).

Chitinase activity

Chitinase activity in bacterial isolates was determined by growing bacterial cultures on colloidal chitin agar consisting of 10 g colloidal chitin, 0.5 g K_2HPO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g Na_2HPO_4 , 3.0 g $NaNO_3$, 1 g Yeast extract. Total volume was made to 1000ml by distilled water and pH adjusted to 7 after adding agar @ 2%. An aliquot of the isolates containing approximately 10^8 cfu/ml was spotted on the plates and incubated at 30°C for 7 days. Chitinase activity was characterized by the halo zones formed around bacterial colonies (Moustaine *et al.*, 2017).

Nitrogen fixation

The screening for nitrogen fixation was done by growing bacterial isolates on solid nitrogen - free malate medium consisting of 6.0 g K_2HPO_4 , 0.02 g $CaCl_2$, 4.0 g KH_2PO_4 , 2.8 g H_3BO_3 , 0.2 g $MgSO_4 \cdot 7H_2O$, 3.0g $NaOH$, 1.0 g NH_4Cl , 0.1 g $NaCl$, 0.01 g $FeCl_3$, 5.0 g DL-malic acid, 0.02 g $NaMoO_4 \cdot 2H_2O$, 0.002 g $MnSO_4$, 0.04 mg $Cu(NO_3)_2 \cdot 3H_2O$, 0.24 mg $ZnSO_4 \cdot 7H_2O$ per 1000 ml of distilled water. The pH was adjusted to 7 and isolates are incubated at 32°C for 24 h. The nitrogen fixation was characterized by the change of colour of media from pale green to blue. Blue colour Zone producing bacteria were characterized as nitrogen fixers (Kuan *et al.*, 2016).

Pot experiment to determine crop response and establishment of ZSB in wheat rhizosphere

The selected ZSB isolates were checked for the crop response and their establishment in the rhizosphere of wheat. Seeds were inoculated with the culture broth of the selected isolates containing approximately 10^6 cells/ml for 30 minutes. The pot experiment was carried out in pots made of plastics which were filled with sterilized soil (4 kg). (Khan and Bano, 2019). The soil that was used for this experiment was sandy loam, pH 7.8, E.C.–0.182 dS/m, nitrogen –0.040, % organic carbon–0.12% and total phosphorus – 8.0 kg/ha. Wheat seeds coated with bacterial isolates (ZA1, ZI4, ZJ3, ZK41, ZL25, ZM9 and ZT19) were sown along with control (uninoculated). Ten seeds were sown/pot which was later thinned to 5. Zn oxide and Zn sulphate were taken as a source of Zn. The experiment was performed with these treatments in the triplicates:

control, 5 ppm Zn as ZnO + culture, 10 ppm Zn as ZnO + culture, 5 ppm Zn as $ZnSO_4$ + culture and 10 ppm Zn as $ZnSO_4$ + culture. Pots were watered manually once every 2-3 days till 90 days. Plants were cautiously uprooted after 30, 60, and 90 days of sowing (DAS) from the plastic pots. The rhizospheric soil was taken from every pot, 5gm of soil is dissolved in 45 ml of sterilized water and dilutions were prepared up to 10^{-6} and the last three dilutions were streaked on Bunt & Rovira media plates. After incubation for 48 h at $30 \pm 2^\circ C$, bacterial colonies showing halo zone around them were taken as Zn solubilizers and were counted. The average of three plates (cfu/g of soil) was considered the final value (Khanghahi *et al.*, 2018).

Determination of shoot biomass

The plants were uprooted from all the treatments at 30, 60, and 90 days after sowing (DAS) and dried in oven at 80°C to a constant shoot weight. The shoot biomass of the plants from all the treatments was calculated. The results are analysed using SPSS software of data analysis (Kamran *et al.*, 2017).

16S rRNA gene sequencing

To determine the phylogenetic relationships, genomic DNA was isolated using the CTAB method (Kumar *et al.*, 2016). Quality of genome was evaluated on 1.0% agarose gel. 27F and 1492R universal primers were used to amplify fragments of 16SrDNA. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forwarding primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A consensus sequence of the 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on the maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and MEGA 7 is used to generate a phylogenetic tree. The Maximum Likelihood method is used to infer evolutionary history. The bootstrap consensus tree was used for representing evolutionary history. The Gene sequence of the bacterial isolates was analysed at

NCBI GenBank using the BLAST programme and a phylogenetic tree was constructed (Kumar *et al.*, 2016).

RESULTS

Screening of bacterial isolates and ZSE determination

The current study was conducted with the aim to isolate and characterize Zn solubilizing bacterial isolates from the rhizosphere of wheat crops in

soils of Haryana, India. Their potential to solubilize inorganic forms of Zn is determined. A total of 50 isolates were purified and named as depicted in Table 1. Eight out of these 50 isolates showed strong solubilisation of inorganic Zn. The isolates showing strong solubilisation were ZA1, ZI4, ZJ3, ZK7, ZM15, ZW22, ZB37, and ZK41. The halo zone diameter of the isolates ranged from 0.8 cm to 2.75 cm. ZSB strains ZA1, ZJ3 and K41 displayed maximum halo zone diameter, while least solubilisation efficiency was observed in ZW49 and ZM32. The most efficient isolates with respect to ZSE were ZA1 and ZK41.

Table 1. Colony diameter, Zn halozone diameter, Zn solubilising efficacy, and minimal inhibitory concentration of rhizospheric bacterial isolates on B & R media.

Isolates	Colony diameter (cm)	Zn halozone diameter (cm)	ZSE (%)	MIC (% of ZnO)	pH
ZA1	0.393±0.012	2.367± 0.058	508.667±15.011	2	3.633±0.379
ZJ2	0.483±0.029	1.717±0.058	255.333±9.238	2	5.067±0.839
ZJ3	0.407±0.012	2.417±0.029	494.333±9.815	2	3.300±0.100
ZI4	0.0597±0.006	2.417±0.029	305.000±8.660	2	4.333±0.321
ZK5	0.390±0.017	2.067±0.058	430.000±8.660	2	3.540±0.461
ZK6	0.967±0.058	2.200±0.100	128.333±23.629	1.5	5.247±0.344
ZK7	0.397 ±0.006	1.767±0.058	345.000±8.660	2	3.967±0.115
ZK8	0.683±0.029	2.500±0.100	265.667±7.572	1.5	5.130±0.256
ZM9	0.683±0.029	2.300±0.000	244.333±7.506	1	5.127±0.329
ZM10	0.683±0.029	1.467±0.029	118.333±9.866	1.5	5.900±0.200
ZM11	0.893±0.012	1.467±0.029	66.667±7.371	2	6.750±0.229
ZM12	0.697±0.006	1.267±0.012	78.667±1.528	2	6.420±0.381
ZM13	0.867±0.058	1.950±0.044	128.000±15.602	1.5	5.790±0.271
ZM14	0.593±0.012	1.183±0.029	116.000±15.588	1	6.600±0.173
ZM15	0.593±0.012	2.400±0.000	304.333±7.506	2	4.300±0.436
ZM16	0.493±0.012	1.000±0.000	104.333±7.506	2	5.733±0.208
ZO17	0.693±0.012	1.400±0.050	101.667±10.599	2	5.540±0.393
ZS18	0.393±0.012	0.877±0.006	125.667±4.933	2	5.833±0.208
ZT19	0.593±0.012	1.793±0.012	201.333±4.163	2	5.267±0.289
ZW20	0.817±0.029	1.363±0.006	70.333±0.577	2	6.420±0.509
ZW21	1.000±0.000	2.653±0.006	165.333±0.577	2	6.100±0.200
ZW22	0.400±0.000	1.900±0.100	375.000±25.000	2	3.867±0.058
ZW23	0.794±0.012	1.707±0.012	115.667±4.041	1.5	5.883±0.225
ZW24	0.693±0.029	2.133±0.058	212.667±21.939	2	5.400±0.361
ZL25	0.493±0.006	2.000±0.000	302.667±4.619	2	4.467±0.451
ZR26	1.167±0.058	2.167±0.058	85.333±4.041	2	6.233±0.115
ZM27	0.967±0.058	2.167±0.058	120.000±0.000	2	5.600±0.173
ZB28	0.967±0.058	1.667±0.029	72.667±9.292	1	6.570±0.517
ZC29	0.667±0.058	1.683±0.029	125.000±43.301	2	6.093±0.040
ZM30	0.967±0.058	1.683±0.058	69.333±16.166	1.5	6.023±0.741
ZC31	0.967±0.058	1.667±0.058	72.333±4.041	2	6.023±0.503

ZM32	0.967±0.058	1.500±0.000	55.333±9.238	2	6.467±0.379
ZC33	0.667±0.058	2.500±0.100	271.333±29.000	2	6.433±0.229
ZS34	1.000±0.000	2.300±0.100	130.000±10.000	2	4.650±0.336
ZC35	0.967±0.058	2.233±0.058	131.333±12.055	1.5	5.737±0.284
ZC36	0.500±0.000	1.967±0.058	292.333±11.547	1.5	5.583±0.451
ZB37	0.500±0.000	2.167±0.058	333.333±11.547	1.5	4.133±0.208
ZK38	0.933±0.058	2.000±0.000	114.667±12.702	1.5	4.333±0.416
ZM39	0.680±0.017	1.533±0.058	123.667±14.012	1.5	5.567±0.252
ZJ40	0.497±0.006	1.600±0.100	222.000±23.065	1.5	5.467±0.231
ZK41	0.497±0.006	2.750±0.000	453.667±6.351	2	3.767±0.152
ZJ42	0.700±0.000	2.483±0.029	254.667±4.041	1.5	4.567±0.404
ZC43	1.167±0.058	2.167±0.058	88.667±9.815	2	6.133±0.551
ZC44	0.967±0.058	1.693±0.012	75.333±9.238	2	6.433±0.510
ZC45	1.000±0.000	1.693±0.012	69.333±1.155	1.5	6.583±0.401
ZF46	1.000±0.000	1.933±0.058	93.333±5.774	1.5	6.033±0.223
ZW47	0.967±0.058	2.967±0.058	207.333±12.702	2	4.667±0.252
ZK48	0.683±0.029	2.493±0.006	265.000±15.620	2	4.667±0.153
ZW49	0.933±0.058	1.497±0.006	55.000±9.539	2	6.640±0.539
ZC50	0.933±0.058	2.033±0.058	118.333±16.803	2	5.653±0.134
CD at 5%	0.3674243	0.7457546	198.30672		0.491429

The data is the mean of 3 experiments. Critical difference (CD) is calculated at 5% significance level.

Figure 1. Zn Solubilisation by ZSB on Bunt & Rovira medium containing 0.1% ZnO (a) isolate ZA1, (b) isolate ZJ3, (c) isolate ZK14.

Phosphate solubilisation efficiency

Table 2 displays that all the 15 bacterial isolates have capability to solubilize phosphate. The phosphate solubilisation efficiency (PSE) of isolated bacterial strains in Pikovskaya media showed that isolates ZA1 (550%), ZJ3 (517%), and ZK41 (450%) were efficient.

Plant growth promoting attributes of the isolates

The plant growth promoting attributes were assessed for the selected bacterial isolates. All the

isolates displayed IAA and auxin production. The results of various assays such as siderophores production, ammonia production, chitinase activity, exo-polysaccharides (EPS) production are summarized in Table 3. Siderophore production was characterized by an orange halo zone which was displayed by all isolates except ZK7, ZK8 and ZB37. Similarly, EPS production characterized by having slimy appearance was present in all except ZK7, ZK8 and ZC36. All the isolates were also checked for ammonia production and the isolates exhibiting a change of

colour to a faint yellow and brown were considered positive for ammonia production. The iodide and mercury present in Nessler's reagent react with ammonia to produce brown coloured substance (Zhao *et al.*, 2019). The isolates ZK7, ZK8, ZB37 and ZC36 did not display ammonia production while the rest were tested positive for the production of ammonia.

Table 2. Phosphate solubilizing efficiency of rhizospheric bacteria.

Isolates	PSE (%)	pH
ZA1	550.667±40.017	3.497±0.300
ZJ3	517.333±46.876	3.553±0.446
ZI4	350.333±16.563	4.347±0.050
ZK5	428.667±15.373	3.327±0.112
ZK7	299.667±7.024	5.273±0.318
ZK8	200.000±11.060	6.202±0.093
ZM9	300.333±18.009	5.293±0.107
ZM15	152.000±15.3950	6.367±0.058
ZW22	300.667±10.066	5.450±0.132
ZL25	302.000±13.856	5.483±0.411
ZC36	230.000±7.053	5.723±0.103
ZB37	330.667±20.033	3.783±0.388
ZK41	450.000±20.207	3.400±0.312
ZJ42	242.667±16.577	4.404±0.356
ZK48	248.648±10.408	4.807±0.121
CD at 5%	190.95254	1.755361

The data is the mean of 3 experiments ± standard deviation. Critical Difference (CD) is calculated at 5% significance.

Establishment of ZSB in rhizosphere of wheat plant

The nutrient broth was inoculated with approximately 10^4 cfu/ml of selected 7 isolates and incubated for 24h. The seeds of wheat are soaked in the culture broth of the selected isolates for 30 min. Bacterial load on the seeds was found in the range of $3.2-5.2 \times 10^6$ cfu/seed. The count of ZSB was calculated after 30, 60, and 90 days of sowing. The rhizospheric soil was taken from every pot, dilutions were prepared up to 10^{-6} and last three dilutions were streaked on Bunt & Rovira media plates to calculate the number of ZSB. The maximum count was found to be after 90 DAS in the rhizosphere of the wheat inoculated with ZK41 i.e. 3.2×10^5 cfu/g (Table 4).

Effect of inoculation of ZSB on shoot biomass under pot conditions

The inoculation of seeds with bacterial isolates for pot culture experiment displayed improvement in the shoot biomass of the wheat plant as compared to the control. The inoculation of ZSB showed significant difference as compared to control (Figure 7). The maximum shoot biomass was observed in case of wheat inoculated with ZJ3 and ZK41 (Table 5).

Table 3. Plant growth promoting attributes of ZSB isolates.

Isolates	IAA production	NH ₃	EPS	Siderophores	Chitinase activity
ZA1	+	+	+	+	+
ZJ3	+	+	+	+	+
ZI4	+	+	+	+	+
ZK5	+	+	+	+	+
ZK7	+	-	-	-	-
ZK8	+	-	-	-	-
ZM9	+	+	+	+	+
ZT19	+	+	+	+	+
ZW22	+	+	+	+	+
ZL25	+	+	+	+	+
ZC36	+	-	-	+	-
ZB37	+	-	+	-	+
ZK41	+	+	+	+	+
ZJ42	+	+	+	+	+
ZK48	+	+	+	+	+

+ Stands for production of biochemical, - stands for absence.

Table 4. ZSB count in wheat rhizosphere under pot house conditions at different days after sowing.

Treatments	ZSB Count in the rhizosphere(X 10 ⁴ cfu/gm)		
Isolates	30 DAS	60 DAS	90 DAS
Control(uninoculated)	3.667±0.0577 ^e	5.833±0.289 ^f	8.667±0.577 ^c
5 ppm Zn	4.667±0.0577 ^e	7.500±0.500 ^f	10.333±0.577 ^c
5 ppm Zn as ZnO+ ZA1	9.333±1.1555 ^{bc}	20.000±1.000 ^b	30.333±0.577 ^a
5 ppm Zn as ZnO+ ZJ3	14.333±1.1555 ^a	23.000±2.646 ^a	31.667±1.528 ^a
5 ppm Zn as ZnO+ ZM9	6.667±0.5777 ^d	12.667±2.082 ^{de}	21.667±1.528 ^b
5 ppm Zn as ZnO+ ZT19	6.333±0.5777 ^d	13.000±1.000 ^{de}	21.667±0.577 ^b
5 ppm Zn as ZnO+ ZL25	6.333±0.5777 ^d	11.333±0.577 ^e	23.667±0.577 ^b
5 ppm Zn as ZnO+ ZI4	8.333±0.5777 ^c	15.000±1.000 ^{cd}	22.667±2.887 ^b
5 ppm Zn as ZnO+ ZK41	10.333±2.517 ^b	17.667±2.517 ^{bc}	32.000±1.000 ^a
CD at 5%	1.5801	2.7745	2.1939

The data represent mean ± SD. Means with the same letter are not significantly different across the column. CD is calculated at 5% significance.

Table 5. Shoot biomass of wheat after different days of sowing (DAS) under pot conditions.

Treatments	Shoot Biomass (mg/plant)		
Isolates	30 DAS	60 DAS	90 DAS
Control (Uninoculated)	83.333±7.024 ^d	312.000±19.079 ^c	1319.000±110.729 ^b
5 ppm Zn	96.000±4.000 ^{cd}	354.000±56.045 ^{bc}	1324.000±305.005 ^{ab}
5 ppm Zn as ZnO+ ZA1	112.000±9.165 ^{cd}	445.000±16.093 ^a	1673.000±228.515 ^a
5 ppm Zn as ZnO+ ZJ3	136.000±15.716 ^{ab}	476.667±89.120 ^a	1690.000±325.447 ^a
5 ppm Zn as ZnO+ ZM9	120.000±14.422 ^{abc}	466.000±71.393 ^a	1675.000±225.000 ^a
5 ppm Zn as ZnO+ ZT19	122.000±12.014 ^{abc}	357.000±51.856 ^{bc}	1665.000±403.129 ^a
5 ppm Zn as ZnO+ ZL25	126.000±4.000 ^{abc}	423.000±61.147 ^{ab}	1651.333±138.818 ^{ab}
5 ppm Zn as ZnO+ ZI4	112.000±22.000 ^{cd}	411.000±73.000 ^a	1664.000±39.000 ^a
5 ppm Zn as ZnO+ ZK41	152.333±43.662 ^a	455.000±18.028 ^{ab}	1694.333±10.693 ^a
CD at 5%	33.853	83.292	385.01

The data represent mean ± SD. Means with the same letter are not significantly different across the column. CD is calculated at 5% significance.

Table 6. Similarity index of isolates with the various bacterial species.

Bacterial isolates	Bacterial strains showing maximum homology	Similarity Index (%)	Gen Bank accession No.
ZA1	<i>Bacillus cereus</i> CCM2010	99.2 %	MW856664
ZJ3	<i>Pseudomonas aeruginosa</i> DSM50071	100%	MW856663
ZK41	<i>Bacillus tropicus</i> MCCC1A01406	99.42 %	MW856667

16S rRNA sequencing and phylogenetic tree

On the basis of the effect on the shoot biomass and morphology, the three isolates were selected for gene sequencing. To identify the bacterial isolates, genomic DNA was isolated (Figure 2) and amplified using 27F and 1492R universal primers (Figure 3). 16S rDNA sequencing was done and phylogenetic tree was constructed. The data obtained from the NCBI GenBank database

revealed a sequence similarity as depicted in Table 6. The isolates were found to be homologues to *Bacillus cereus*, *Pseudomonas aeruginosa*, *Bacillus tropicus* whose phylogenetic trees are shown in Figure 4, Figure 5, and Figure 6 respectively.

Figure 2. Genomic DNA of ZA1, ZJ3, ZK41, Lad-Ladder.

Figure 3. (a) PCR amplification of 16S r-DNA fragment; the size of PCR amplified product is ~1.5kb, (b) Ladder Specification.

DISCUSSION

Zn is a vital micronutrient that is required in minute quantities and it is indispensable for the growth of all forms of life. Humans and animals

depend directly or indirectly upon plants for their growth. In context to this, it is imperative to know the status of the Zn in the soil. Many studies revealed that most of the Indian agricultural soils are deficient in Zn. To accomplish the requirements of the crops, chemical fertilizers have been used extensively and nowadays it has become a very common practice to achieve desired results. But still, the amelioration of Zn deficiency remains the major concern as most of the applied Zn fertilizers (60%) get converted to insoluble forms and hence no longer available to plants (Kamran *et al.*, 2017). The Zn solubilising bacteria promote growth in plants via nutrient uptake, solubilisation of nutrients, nitrogen fixation and mineralization (Gouda *et al.*, 2018). This can be attributed to many factors like the type of the soil, moisture content, presence of other nutrients etc. The main factors affecting Zn solubilisation is acidification through proton production, chemical transformation via redox reaction and chelating of siderophores (Costerousse *et al.*, 2018).

In the present study Zn solubilising bacteria were isolated from the rhizospheric soil samples of wheat grown in Faridabad district by serial dilution and screened on B & R media. Post 3 days incubation period colonies with clear halo zone were picked and solubilisation efficiency of the bacteria was determined. The results divulged that out of 50 isolates 8 displayed high Zn solubilisation efficiency. The halo zone diameter of these ranged from 0.8 cm to 2.8 cm. Maximum solubilisation efficiency was displayed by (ZJ3) *Pseudomonas aeruginosa* (494%), (ZK41) *Bacillus tropicus* (453%) and (ZA1) *Bacillus cereus* (508%). Mumtaz *et al.* (2017) showed that isolates displayed various Zn solubilisation efficiency (ZSE) that ranges from 123-296% which corroborate the present data. In this study, various plant growth-promoting characteristics were studied and it was observed that most of the isolates have these traits. For example, all bacterial strains were tested positive for phosphate solubilisation efficiency (PSE) ranging from 150-550%. The results are similar to the finding of Batool and Iqbal (2019) who reported quite similar findings concerning PSE.

The isolates were also checked for IAA synthesis and all strains have positive IAA synthesis following Mohite (2013) and Goswami

et al. (2013) who reported that IAA a phytohormones present in plants stimulates root elongation and formation. Siderophores production was also tested and results revealed all isolates to be positive for the same. Siderophores assists in solubilisation and chelating of iron from its inactive form (Arora and Verma, 2017).

Siderophores chelates Zn and enhances its uptake (Pahari and Mishra, 2017). Ammonia production was also noted in all isolates except ZK7, ZK8, ZC36 and ZB38 and according to Agbodjato *et al.* (2015) production of ammonia indirectly affects plant growth thus it plays an important role.

Figure 4. Phylogenetic analysis of ZA1 (bacteria 2) based on 16SrDNA sequence analysis. NR133704.2 (*B. tianshenii* YIM M13235); NR159143.1 (*B. solisilvae* NEAU-cbsb5); NR024691.1 (*B. flexus* IFO15715); NR041942.1 (*B. acidicola* 105-2); NR074914.1 (*B. cytotoxicus* NVH 391-98); NR114422.1 (*B. pseudomycoides*); NR115714.1 (*B. cereus* CCM 2010); NR152692.1 (*B. wiedmannii* FSL W8-0169); NR121761.1 (*B. toyonensis* BCT-7112).

Figure 5. Phylogenetic analysis of ZJ3 (bacteria 1) based on 16SrDNA sequence analysis. NR103934.2 (*P. stutzeri* ATCC 17588); NR116489.1 (*P. stutzeri* VKM B-975); NR042435.1 (*P. nitroreducens* IAM 1439); NR114975.1 (*P. nitroreducens* DSM14399); NR043289.1 (*P. otitidis* MCC10330); NR112062.1 (*P. resinovorans* ATCC 14235); NR026078.1 (*P. aeruginosa* DSM 50071); NR114471.1 (*P. aeruginosa* ATCC 10145); NR117678.1 (*P. aeruginosa* DSM 50071); NR113599.1 (*P. aeruginosa* NBRC 12689 16S).

Figure 6. Phylogenetic analysis of ZK41 (bacteria 6) based on 16S rDNA sequence analysis. NR157729.1 (*B. albus* MCCC 1A02146); NR157732.1 (*B. nitratireducens* MCCC 1A00732); NR157730.1 (*B. luti* MCCC 1A00359); NR157736.1 (*B. tropicus* MCCC 1A01406); NR157734.1 (*B. paramycoides* MCCC 1A04098); NR115714.1 (*B. cereus* CCM 2010); NR115526.1 (*B. cereus* IAM 12605); NR157728.1 (*B. paranthracis* MCCC 1A00395); NR157733.1 (*B. pacificus* MCCC 1A06182); NR074540.1 (*B. cereus* ATCC 14579).

Figure 7. Growth of wheat inoculated with (a)ZA1, (b)ZJ3 and (c)ZK41 in comparison with control after 15 days of sowing.

Another important attribute exhibited by plant growth promoting bacteria is production of EPS that plays an important role in the growth of bacteria. EPS is vital for balancing moisture content and growth during drought period. EPS forms a protective sheath around the rhizosphere root region thus shielding the plant root from invasion and desiccation. By improving soil structure and aggregation certain plants treated with EPS producing bacteria also displayed resistance to stress (Khan and Bano, 2019). EPS producing bacteria can even outlast under severe drought conditions as they can enhance the retention of water as well as monitor diffusion of organic carbon (Yadav *et al.*, 2018).

All ZSB isolates survived and established well in wheat rhizosphere even after 90 DAS. The inoculation of isolates remarkably increased the growth of plants as evident by the shoot biomass calculated at various intervals. Plants treated with 5 ppm of ZnO displayed increased shoot biomass. The control had a mass of 1319 mg/kg post 90 days of inoculation and the rest of isolates showed greater mass than the control. The maximum shoot biomass of 1690 mg/kg was noted in ZJ3 and ZK41. ZA1 was found to be 99.2% similar to *Bacillus cereus*. ZJ3 shared 100% similarity to *Pseudomonas aeruginosa* while ZK41 showed 99.4% similarity to *Bacillus tropicus*.

CONCLUSION

The results concluded that the isolates ZA1, ZJ3 and ZK41 possessing maximum homology with *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Bacillus tropicus*, respectively can solubilise insoluble and inactive forms of Zn and thus ameliorate the growth of wheat crops. These isolates possess various PGPR characteristics like ammonia production, EPS production, IAA production, phosphate solubilisation, siderophores production, and chitinase activity that promote plant growth. The isolates can be thus used to boost the plant nutrient quality and yield and therefore help in eliminating Zn deficit. The isolated strains can be used as a potential bioinoculants. The current research provides data for future study on molecular pathways and

mechanisms employed by these zinc solubilising bacteria.

REFERENCES

- Abaid-Ullah, M., Nadeem, M., Hassan, M., Ganter, J., Muhammad, B., Nawaz, K., Shah, A. S., & Hafeez, F. Y. 2015. Plant growth promoting rhizobacteria: an alternate way to improve yield and quality of wheat (*Triticum aestivum*). *International Journal of Agriculture and Biology* 17(1): 51-60.
- Agbodjato, N. A., Amogou, O., Noumavo, P. A., Dagbénonbakin, G., Salami, H. A., Karimou, R., Alladé, A. M., Adedayo, O., Baba-Moussa, F., Adjanohoun, A., & Baba-Moussa, L. S. 2015. Biofertilising, plant-stimulating and biocontrol potentials of maize plant growth promoting rhizobacteria isolated in central and northern Benin. *African Journal of Microbiology Research* 12(28) :664-672.
- Alloway, B. J. 2009. Soil factors associated with Zn deficiency in crops and humans. *Environmental Geochemistry and Health* 31(5): 537-548.
- Arora, N. K. & Verma, M. 2017. Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech* 7(6): 1-9.
- Athokpam, H. S., Ralte, L., Chongtham, N., Singh, N. B., Devi, K. N., Singh, N. G., & Sharma, P. T. 2018. Status and forms of Zn in acidic soils of Imphal West District, Manipur (India). *International Journal of Current Microbiology and Applied Science* 7(4): 2349-2355.
- Aznar, A. & Dellagi, A. 2015. New insights into the role of siderophores as triggers of plant immunity: what can we learn from animals? *Journal of Experimental Botany* 66(11): 3001-3010.
- Batool, S. & Iqbal, A. 2019. Phosphate solubilizing rhizobacteria as alternative of chemical fertilizer for growth and yield of *Triticum aestivum* (Var. Galaxy 2013). *Saudi Journal of Biological Sciences* 26(7): 1400-1410.
- Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., & Lux, A. 2007. Zn in plants. *New Phytologist* 173(4): 677-702.
- Cele, E. N. & Maboeta, M. 2016. Response of soil enzyme activities to synergistic effects of biosolids and plants in iron ore mine soils. *International Journal of Environmental Science and Technology* 13(9): 2117-2126.
- Costerousse, B., Schönholzer-Mauclaire, L., Frossard, E., & Thonar, C. 2018. Identification of heterotrophic Zn mobilization processes among bacterial strains isolated from wheat rhizosphere (*Triticum aestivum* L.). *Applied and Environmental Microbiology* 84(1): e01715-17.
- Crowley, D. E. 2006. Microbial siderophores in the plant rhizosphere. In *Iron nutrition in plants and rhizospheric microorganisms* pp. 169-198. Springer, Dordrecht.
- El-Sayed, W. S., Akhkha, A., El-Naggar, M. Y., & Elbadry, M. 2014. *In vitro* antagonistic activity, plant growth promoting traits and phylogenetic affiliation of rhizobacteria associated with wild plants grown in arid soil. *Frontiers in Microbiology* 5: 651.
- Goswami, D., Vaghela, H., Parmar, S., Dhandhukia, P., & Thakker, J. N. 2013. Plant growth promoting potentials of *Pseudomonas* spp. strain OG isolated from marine water. *Journal of Plant Interactions* 8(4): 281-290.
- Goteti, P. K., Emmanuel, L. D. A., Desai, S., & Shaik, M. H. A. 2013. Prospective Zn solubilising bacteria for enhanced nutrient uptake and growth promotion in maize (*Zea mays* L.). *International Journal of Microbiology* 2013: 1-7.

- Gouda, S., Kerry, R. G., Das, G., Paramithiotis, S., Shin, H. S., & Patra, J. K. 2018. Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. *Microbiological Research* 206: 131-140.
- Hafeez, F. Y., Abaid-Ullah, M., & Hassan, M. N. 2013. Plant growth-promoting rhizobacteria as Zn mobilizers: a promising approach for cereals biofortification. In *Bacteria in Agrobiology: Crop Productivity*. pp. 217-235. Springer, Berlin, Heidelberg.
- Hina, J., Akhtar, M. J., Asghar, H. N., & Amer, J. 2018. Screening of Zn solubilizing bacteria and their potential to increase grain concentration in wheat (*Triticum aestivum*). *International Journal of Agriculture and Biology* 20(3): 547-553.
- Kamran, S., Shahid, I., Baig, D. N., Rizwan, M., Malik, K. A., & Mehnaz, S. 2017. Contribution of Zn solubilizing bacteria in growth promotion and Zn content of wheat. *Frontiers in Microbiology* 8(2017): 2593.
- Kour, R., Jain, D., Bhojiya, A. A., Sukhwai, A., Sanadhya, S., Saheewala, H., Jat, G., Singh, A. & Mohanty, S. R., 2019. Zn biosorption, biochemical and molecular characterization of plant growth-promoting Zn-tolerant bacteria. *3 Biotech* 9(11): 1-17.
- Kaushal, M., Mandyal, P., & Kaushal, R. 2019. Field based assessment of Capsicum annum performance with inoculation of rhizobacterial consortia. *Microorganisms* 7(3): 89.
- Khan, N. & Bano, A. 2019. Exopolysaccharide producing rhizobacteria and their impact on growth and drought tolerance of wheat grown under rainfed conditions. *PLoS One* 14(9): e0222302.
- Khanghahi, M. Y., Ricciuti, P., Allegretta, I., Terzano, R., & Crecchio, C. 2018. Solubilization of insoluble Zn compounds by Zn solubilizing bacteria (ZSB) and optimization of their growth conditions. *Environmental Science and Pollution Research* 25(26): 25862-25868.
- Kuan, K. B., Othman, R., Abdul Rahim, K., & Shamsuddin, Z. H. 2016. Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. *PLoS One* 11(3): e0152478.
- Kumar, S., Stecher, G., & Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33(7): 1870-1874.
- Malik, R. S., Yadav, H. K., Narender, R., & Sangwan, P. 2017. Background levels of Zn, iron, manganese and copper in soil series of Haryana and their relationship with soil properties. *Indian Journal of Agricultural Sciences* 87(8): 1094-1098.
- Mohite, B. 2013. Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *Journal of Soil Science and Plant Nutrition* 13(3): 638-649.
- Moustaine, M., Elkahkahi, R., Benbouazza, A., Benkirane, R., & Achbani, E. H. 2017. Effect of plant growth promoting rhizobacterial (PGPR) inoculation on growth in tomato (*Solanum lycopersicum* L.) and characterization for direct PGP abilities in Morocco. *International Journal of Environment, Agriculture and Biotechnology* 2(2): 590-596.
- Mumtaz, M. Z., Ahmad, M., Jamil, M., & Hussain, T. 2017. Zn solubilizing *Bacillus* spp. potential candidates for biofortification in maize. *Microbiological Research* 202(2017):51-60.
- Noulas, C., Tziouvakas, M., & Karyotis, T. 2018. Zn in soils, water and food crops. *Journal of Trace Elements in Medicine and Biology* 49: 252-260.
- Pahari, A. & Mishra, B. B. 2017. Characterization of siderophore producing Rhizobacteria and Its effect on growth performance of different vegetables. *International Journal of Current Microbiology and Applied Sciences* 6(5): 1398-1405.
- Pahari, A., Pradhan, A., Nayak, S. K., & Mishra, B. B. 2017. Bacterial siderophore as a plant growth promoter. In *Microbial Biotechnology*. pp. 163-180. Springer, Singapore.
- Pande, A., Pandey, P., Mehra, S., Singh, M., & Kaushik, S. 2017. Phenotypic and genotypic characterization of phosphate solubilizing bacteria and their efficiency on the growth of maize. *Journal of Genetic Engineering and Biotechnology* 15(2): 379-391.
- Prasad, R., Kumar, M., & Varma, A. 2015. Role of PGPR in soil fertility and plant health. In *Plant-growth-promoting rhizobacteria (PGPR) and medicinal plants*. pp. 247-260. Springer, Cham.
- Roohani, N., Hurrell, R., Kelishadi, R., & Schulin, R. 2013. Zn and its importance for human health: An integrative review. *Journal of Research in Medical Sciences: The Official Journal of Isfahan University of Medical Sciences* 18(2): 144-157.
- Saravanan, V. S., Subramoniam, S. R., & Raj, S. A. 2004. Assessing in vitro solubilization potential of different Zn solubilizing bacterial (ZSB) isolates. *Brazilian Journal of Microbiology* 35(1-2): 121-125.
- Sharma, A., Patni, B., Shankhdhar, D., & Shankhdhar, S. C. 2013. Zn—an indispensable micronutrient. *Physiology and Molecular Biology of Plants* 19(1): 11-20.
- Upadhyay, S. K., Singh, J. S., & Singh, D. P. 2011. Exopolysaccharide-producing plant growth-promoting rhizobacteria under salinity condition. *Pedosphere* 21(2): 214-222.
- Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., & Nasrulhaq Boyce, A. 2016. Role of plant growth promoting rhizobacteria in agricultural sustainability-a review. *Molecules* 21(5): 573.
- Vidyashree, N. D. 2016. Isolation and characterization of Zn solubilizing bacteria from stone quarry dust powder. *International Journal of Agriculture Sciences*: 0975-3710.
- Yadav, A. N. & Yadav, N., 2018. Stress-adaptive microbes for plant growth promotion and alleviation of drought stress in plants. *Acta Scientifica Agriculture* 2(6): 85-88.
- Zhao, Y., Shi, R., Bian, X., Zhou, C., Zhao, Y., Zhang, S., ... & Zhang, T. 2019. Ammonia detection methods in photocatalytic and electrocatalytic experiments: how to improve the reliability of NH₃ production rates? *Advanced Science* 6(8): 1802109.