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Utilization of zinc solubilizing bacteria for better growth and development of summer groundnut (*Arachis hypogaea* L.)

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Abstract
With an aim to find most efficient zinc solubilizing bacteria (ZSB), isolation was attempted from rhizospheric soil samples of Anand and Main Rice Research Station, Nawagam, wherein total 8 isolates were obtained on basal agar plates supplemented with 1% Zinc oxide and were screened qualitatively for zinc solubilization by observing halo on basal agar plates supplemented with ZnO, $ZnCO_3$ and $Zn_3(PO_4)$. Further, six most efficient isolates were selected on the basis of lowering pH from 7 to 5 in basal broth ability. They were culturally and morphologically characterized. On the basis of qualitative and quantitative assay for zinc solubilization as well as other PGP traits The 3 strains were identified by molecular characterization; isolate ZSB A2, ZSB AF2 and ZSBA6 as *Pseudomonas aeruginosa*, AAUZSB A2 (ACCN ON080844), *Pseudomonas taiwanensis* AAUZSB AF2 (ACCN ON080840) and *Beijerinckia fluminensis* AAUZSB A6 (ACCN ON080839), respectively. All the three cultures were mutually compatible with each other in cross streak assay and a consortium was prepared combining each culture in equal proportion. Inoculation of ZSB alone and/or their consortium @ 5 mL/kg seed, in presence of either $ZnSo_4$ @ 25 kg/ha or Zn EDTA @ 1 kg/ha, increased plant growth and biomass yield of groundnut as compared to their respective controls.

Keywords: Isolation, molecular characterization, zinc solubilizing bacteria, groundnut

1. Introduction
Zinc (Zn) is an essential micronutrient required for plants, animals and human beings for their normal healthy growth and reproduction. In plants, zinc plays a key role as a structural constituent or regulatory co-factor of a wide range of different enzymes and proteins in many important biochemical pathways and these are mainly concerned with carbohydrate metabolism, both in photosynthesis and in the conversion of sugars to starch, protein metabolism, auxin (growth regulator) metabolism, pollen formation, maintenance of the integrity of biological membranes and the resistance to infection by certain pathogens. In soil, it undergoes a complex dynamic equilibrium of solubilization and precipitation that is greatly influenced by pH and microflora (Di *et al*., 1998; Martino *et al*., 2003) [9, 16] and that ultimately affects its accessibility to roots for absorption. Zinc is needed by plant in micro quantity but in critical concentration and if the amount available is not adequate, plants will suffer from physiological stress brought about by the dysfunction of several enzyme systems and other metabolic functions (Alloway, 2008) [2].

Zn deficiency has become a serious problem, affecting almost half of the world's population (Cakmak, 2009) [4]. In fact, this is due to the low Zn content of plants grown in Zn-deficient soils. Many Indian soils are Zn deficient, with levels below the critical 1.5 ppm level. To overcome this limitation, farmers apply Zn in the form of fertilizers such as $ZnSO_4$, which in turn transforms into various insoluble forms depending on soil type and chemical reactions and become completely unavailable in the environment within 7 days of application (Rattan and Shukla, 1991) [17]. High zinc density groundnuts may be a solution to ensure an adequate level of Zn intake which necessitates increasing of Zn concentration of seed through fortification and selection of high Zn density genotypes (Lal and Singh, 2007; Singh *et al*., 2011a, 2011b) [15, 21, 22]. Extensive research demonstrated the role of Zn fertilizers in increasing the Zn density of grain in cereals, suggesting that wherever fertilizers are available, making full use of Zn fertilizers can provide an immediate and effective option to increase grain Zn concentration, and productivity under soil conditions showing Zn deficiency (Cakmak *et al*., 2010) [10].

Zinc solubilizing microorganisms (ZSM) solubilize zinc through several mechanisms, one of which is acidification. These microbes produce organic acids in the soil that bind the zinc cations and lower the pH of the nearby soil (Alexander, 1978) [1]. In addition, the anions can chelate zinc and improve zinc solubility (Jones and Darrah, 1994) [14].

2. Materials and Methods

2.1 Soil sampling

Representative soil samples were collected from various fields of B.A. College of Agriculture, Anand Agricultural University.

2.2 Isolation and purification of zinc solubilizers

Using zinc solubilizing medium supplemented with the insoluble zinc compounds zinc oxide (ZnO), zinc phosphate $Zn_3(PO_4)_2$, and zinc carbonate ($ZnCO_3$) at 100 mg per litre separately as per conventional procedure, zinc solubilizing bacteria from the rhizosphere soil samples were isolated. The spread plate technique was used to transfer each 0.1ml of soil solution from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions to petri plates in zinc solubilizing agar. The growing individual colonies showed evident halo development around the colony from the petri plates which were picked up and transferred to test tubes containing nutrient agar medium after incubation of inoculated petri plates at 28±2 °C for 2 days.

2.3 Screening and selection of efficient zinc solubilizing bacterial isolates

2.3.1 Quantitative assay for zinc solubilization

The serial dilution approach was used to individually dilute a loop of chosen bacterial isolates in sterile distilled water before spot inoculating them on petri plates with zinc solubilizing agar medium with insoluble sources of ZnO, $Zn_3(PO_4)_2$ and $ZnCO_3$ separately. Following incubation at 30 °C, the halo zone surrounding bacterial colony was measured in mm successively after 24 h, up to 7 days. The ZSB isolates, designated as ZSB A1, ZSB A2, ZSB A2, ZSB A4, ZSB A5 and ZSB A6 which had demonstrated the highest solubilization index and were utilized for further research.

2.3.2 Qualitative assay for Zinc solubilization

ZnO, $ZnCO_3$, and $Zn_3(PO_4)_2$ were added to the prepared zinc solubilizing liquid medium, which was then transferred in 50 ml aliquots into 100 ml Erlenmeyer flasks. The mixture was then autoclaved at 121 °C for 20 min. After that, 1 ml suspension of the test isolates with a cell mass of 10^6 cells/ml was added to the flasks as an inoculant. For each treatment, three flasks were kept with an uninoculated control. The samples were incubated in an orbital shaker at 120 rpm for 0, 5, 10, to 15 days at 30 °C. To remove the detritus and cells, the cultured broth was centrifuged at 1000 RPM for 10 min after being filtered using Whatman No. 42 filter paper. For the purpose of determining the amount of soluble zinc, the culture supernatant was immediately put into an atomic absorption spectrometer (AAS). The quantity of zinc that was soluble was calculated by subtracting the inoculation sample's soluble zinc from the uninoculated control, and it was represented as (mg Zn/10Fl) culture. The pH of the filtrates from each flask was checked using a pH meter, at 0, 5, 10, to 15 days following inoculation.

2.4 Morphological characterization

After 48 h of incubation, zinc solubilizing bacterial isolates were cultured in zinc solubilizing agar medium to investigate colony morphological aspects. Gram staining and cell morphology were detected.

2.5 Biochemical characterization

Various biochemical tests *viz.* utilization of carbohydrates, glucose, lactose, citric acid. Production of enzymes like urease, nitrate reductase, protease, lipase, etc., methyl red, Voges Proskauer's, Indole test, etc. were carried out

2.6 Plant Growth Promoting (PGP) traits

2.6.1 IAA production

A spectrophotometric analysis of IAA was performed using Husen's approach (2016) [12]. Growth hormone estimation IAA isolates were grown for 24 h in suitable media containing 0.1 percent tryptophan before being centrifuged at 5000 rpm for 25 min on a rotary shaker (120 rpm). After 30 min at room temperature, the supernatant liquid was blended with Salkowski reagent (1:2), and the intensity of the colour produced was measured using a spectrophotometer at 530 nm.

2.6.2 Phosphate solubilization

On Sperber's medium, all the isolates were evaluated for phosphate solubilization efficiency. (Jha *et al*., 2009). Bacterial isolates were spotted on agar plates and cultured for three days at 30 °C. The effectiveness was measured by the formation of a halo zone of P solubilization surrounding the bacterial colony.

2.6.3 Potash mobilization

On Glucose Yeast Calcium agar medium, all the isolates were spot inoculated. Plates were incubated for 5-6 days at 28±2ºC, and colonies with apparent zones of calcium release were investigated. Colonies having a clear zone were inoculated on Aleksandrov's medium containing mica and feldspar as a raw insoluble potash substrate to test their potash mobilisation activity (Hu *et al*., 2006) [11].

2.6.4 Siderophore production

The ability of the zinc solubilizing bacterial isolates to produce siderophore was assessed as per Schwyn and Neilands (1987) [20]. All the glass wares were first soaked in 2N HCl solution for 24 h to avoid contamination of iron from the glassware. Dehydrated Chrome Azurol S (CAS) solution was prepared by dissolving 60.5 mg dehydrated Chrome Azurol S in 50 ml double distilled water and further mixing with 10 ml of iron solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). This was slowly added to 40 ml aqueous solution containing 72.9 mg Hexa Decyl Trimethyl Ammonium Bromide (HDTMA) by continuous stirring and the final solution was autoclaved. Dehydrated nutrient agar for 200 ml was weighed and dissolved in 180 ml of distilled water and autoclaved. After cooling, CAS solution (20 ml) was added along the wall of flask with gentle agitation to mix without formation of foam. The CAS agar thus prepared was poured into the plates. After solidification, the plates were kept in the refrigeration (40 °C) for 24 h. The overnight grown cultures (10µl each) were spotted on these CAS agar plates and incubated at 28±2ºC for siderophore production. The diameter of orange coloured zone was recorded.

2.7 Molecular characterization

All ZSB bacterial isolates had their genomic DNA extracted

using the protocol described by Sambrook *et al*. (1989) [18].

2.9 Efficacy of zinc solubilizing bacterial isolates on Groundnut (*Arachis hypogaea* L.) in pot trial

To evaluate the efficient zinc solubilizing bacterial isolates for growth, nutrient uptake and yield of groundnut under net house condition, a pot culture experiment was conducted using efficient zinc solubilizing bacteria as single inoculum and their consortium was studied for their performance on growth, yield and nutrient content of groundnut plant as well as pod. The experiment was laid down as per following treatment details.

The experiment was laid down as per following treatment details

T. No	Treatments
T_1	Control
T_2	$ZnSO_4$ @ 25 kg/ha
T_3	Zinc EDTA @ 1 kg/ha
T_4	T_2 + *P.aeruginosa*
T_5	T_2 + *P.taiwanensis*
T_6	T_2 + *B.fluminensis*
T_7	T_2 + consortium
T_8	T_3 + *P.aeruginosa*
T_9	T_3 + *P. taiwanensis*
T_{10}	T_3 + *B. fluminensis*
T_{11}	T_3 + consortium

3. Results and Discussion

3.1. Isolation and Characterization of zinc solubilizing bacteria

3.1.1 Sampling

The usual approach (zig-zag method) was used to collect representative soil samples (500 g) from research farms of the Anand Agricultural University's departments of agronomy, agricultural entomology, and plant pathology. Samples were taken from the top layer of soil (0–15 cm) and kept in polythene bags in the refrigerator at 4 °C until further use. There was total 8 ZSB isolates obtained using zinc solubilizing agar medium supplemented with insoluble zinc sources such as ZnO, ZnCO3 and $Zn_3(PO_4)_2$ (Fig. 1). Isolates were obtained from different farms of Anand Agricultural University, Anand.

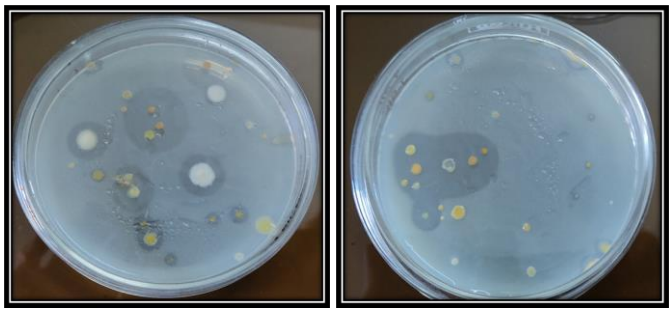

Fig 1: Isolation of ZSB from rhizospheric soil on ZSB agar containing 0.1% ZnO

3.1.2 Morphological characterization of zinc solubilizing bacteria

The cultural features of bacterial isolates are shown in Table 4.2. Bacterial isolates were cultivated on nutrient agar plates for 48 h at 28 ± 2 °C. Different cultural properties of bacterial isolates were investigated in accordance with Bergey's Manual of Determinative Bacteriology, 9th edition (Holt *et al*., 1994) [10].

Table 1: Cultural characteristics of zinc solubilizing bacterial isolates on nutrient agar

Sr. No.	Name of isolate	Size	Margin	Texture	Opacity	Pigment
1.	ZSB A1	Small	Entire	Smooth	Transparent	White
2.	ZSB A2	Medium	Entire	Smooth	Opaque	Off White
3.	ZSB AF2	Small	Entire	Smooth	Opaque	Off white
4.	ZSB A4	Small	Entire	Smooth	Transparent	White
5.	ZSB A5	Small	Entire	Smooth	Transparent	White
6.	ZSB1 A6	Medium	Entire	Smooth	Transparent	White

The colony size of all ZSB was medium to small, margin was entire the texture was smooth, and pigment was white to off white when inoculated in nutrient agar petri plates shown in table 1

Table 2: Morphological characteristics of zinc solubilizing bacterial isolates

Sr. No.	Name of isolate	Shape and arrangement	Gram's Reaction
1.	ZSB A1	Small Rods, single	Gram –ve
2.	ZSB A2	Small Rods, single	Gram –ve
3.	ZSB AF2	Small Rods, single	Gram –ve
4.	ZSB A4	Small rods, single	Gram –ve
5.	ZSB A5	Small Rods, single	Gram –ve
6.	ZSB A6	Small Rods, single	Gram –ve

All 6 isolates were having small rods in shape and arrangement was single and Gram staining reaction (Fig. 2) was negative of all ZSB isolates shown in table 2

Table 3: Biochemical characterization of zinc solubilizing bacterial isolates

Sr. No.	Biochemical Tests	ZSB A1	ZSB A2	ZSB AF2	ZSB A4	ZSB A5	ZSB A6
1	Urease	-	+	+	-	-	-
2	Nitrate reduction	-	+	+	-	-	+
3	Indole production	+	+	+	-	-	+
4	Citrate utilization test	+	+	+	+	+	+
5	Methyl red	-	-	-	-	-	-
6	Voges proskauer's test	-	-	-	-	-	-
7	Glucose	+	+	+	-	-	+
8	Lactose	-	+	+	-	-	+
9	Protease	-	+	+	-	-	+
10	Lipase	-	+	+	-	-	+

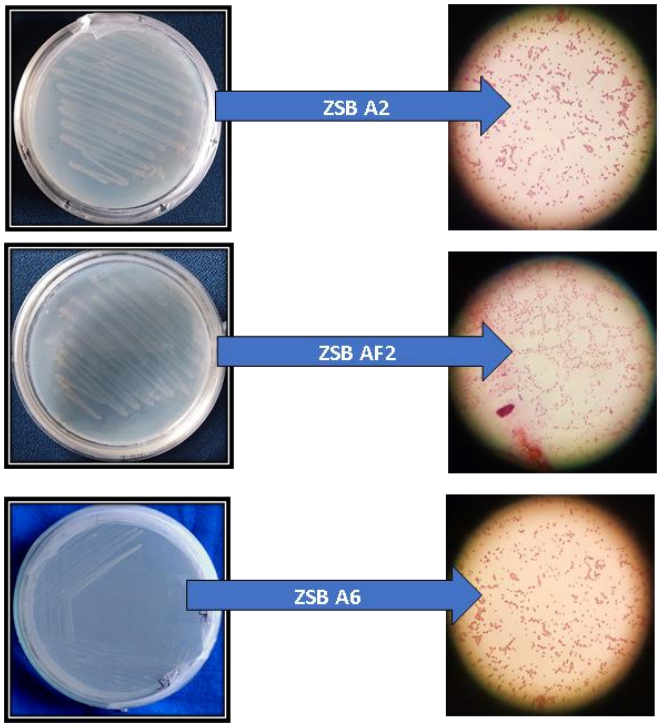

Fig 2: Cultural and morphological characters of ZSB isolates on Nutrient agar

3.2 Screening and selection of efficient zinc solubilizing bacteria

3.2.1 Qualitative and quantitative assay of Zn solubilization

In plate and broth assays, all ZSB were found efficient in solubilizing insoluble zinc (ZnO, $Zn_3(PO_4)_2$, $ZnCO_3$) shown in table 4. The isolates ZSB A2 and AF2 exhibited substantial levels of clear halo zone of zinc solubilization (Fig. 3). ZSB A2 had the highest solubilization on zinc oxide with a diameter of 16.25 mm, zinc carbonate with a diameter of 15.18 mm and $Zn_3(PO_4)_2$ with a diameter of 8.32 mm followed by ZSB AF2 with 14.07 mm in ZnO, 12.38 mm in $ZnCO_3$ and 6.44 mm in $Zn_3(PO_4)_2$ Comparison to other isolates. Whereas, in zinc solubilizing broth containing ZnO, table 4 ZSB A2 demonstrated a wide spectrum of solubilization at 5, 10 and 15 DAI having values of 7.21 mg Zn/100 ml, 8.23 mg Zn/100 ml and 11.63 mg Zn/100 ml, whereas, in $ZnCO_3$ 6.11 mg Zn/100 ml 7.76 mg Zn/100 ml and 10.76 mg Zn/100 ml and in $Zn_3(PO_4)_2$ 5.01 mg Zn/100 ml, 6.30 mg Zn/100 ml and 8.36 mg Zn/100 ml. The pH of the medium was much lower in the inoculated broth with ZSB isolates than in the uninoculated control and was reduced from 7.1 to 4.1 by ZSB A2 at 15 DAI (Table 4).

Table 4: Quantitative assay of zinc solubilizing bacterial isolates on zinc solubilizing agar supplemented with ZnO, $Zn_3(PO_4)_2$ and $ZnCO_3$

Sr. No.	Name	Halo zone diameter* (mm)		
		Zno	$ZnCO_3$	$Zn_3(PO_4)_2$
1	ZSB A1	6.36	5.36	2.23
2	ZSB A2	16.25	15.18	8.32
3	ZSB AF2	14.07	12.38	6.44
4	ZSB A3	4.54	2.06	2.05
5	ZSB A4	7.53	5.54	4.57
6	ZSB A5	9.62	7.55	3.05
7	ASB AF5	4.72	4.07	1.96
8	ZSB A6	10.61	6.74	5.68

Saravanan and Raj (2004) [19] reported ZSB culture from soil and ore sources, both by direct plating and enrichment technique in medium with 0.1% ZnO. Three cultures were isolated by direct plating and one by enrichment technique. Among those, two strains were characterized as *Bacillus* spp. and *Pseudomonas* spp. Further, potential to correct Zn deficiency was assessed using soybean plants. Result revealed that *Pseudomonas* spp. was able to correct Zn deficiency when used along with 1% ZnO.

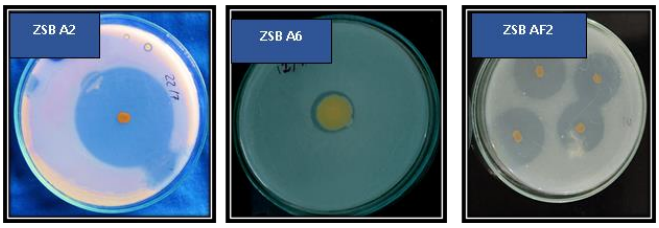

Fig 3: Halo zone on ZSB agar plates containing 0.1% ZnO

Table 5: Qualitative assay of zinc solubilizing bacterial in zinc solubilizing broth supplemented with ZnO, $ZnCO_3$, $Zn_3(PO_4)_2$

Tr. No.	Treatments	Concentration of soluble Zn mg/100 ml in ZnO (DAI)			Concentration of soluble Zn mg/100 ml in $ZnCO_3$ (DAI)			Concentration of soluble Zn mg/100 ml in $Zn_3(PO_4)_2$ (DAI)		
	DAI	5	10	15	5	10	15	5	10	15
T1	ZSB A1	2.1	3.23	6.46	1.80	2.89	5.79	1.01	2.03	4.41
T2	ZSB A2	7.21	8.23	11.63	6.11	7.76	10.36	5.01	6.30	8.36
T3	ZSB AF2	5.11	6.12	10.09	4.61	5.86	10.01	3.01	5.21	7.90
T4	ZSB A4	0.40	0.94	1.12	0.38	0.79	1.01	0.23	0.64	0.90
T5	ZSB A5	1.11	4.62	8.80	0.90	4.40	7.99	0.80	3.54	6.70
T6	ZSB1 A6	3.65	5.66	9.73	3.79	4.67	8.66	2.51	5.61	7.81

Vidyashree (2016) [23] isolated two ZSB from quarry dust powder, after purification and characterization these isolates were classified as *Bacillus aerophilus* and *Enterobacter sp.*

Identified by 16S *r*RNA gene sequencing. They were evaluated for their ability to solubilize zinc in both solid and liquid zinc solubilizing media, along with twenty-two isolates including the reference strain of *Bacillus aryabhattai*, obtained from the microbiological laboratory, ICAR-IIHR, Bengaluru. Among all the isolates, *B. aryabhattai* showed a significant increase in the solubilization and produced a larger clear halo zone on solid agar medium supplemented with 0.1% sources of zinc, namely zinc oxide (42.1 mm), zinc carbonate (46.3 mm) and zinc phosphate (26.7 mm). Similarly, in a liquid zinc solubilizing medium containing 0.1% zinc sources, *B. aryabhattai* significantly improved solubilization and released a greater amount of zinc with zinc oxide (554.8 ppm), zinc carbonate (368.6 ppm), and zinc phosphate (576.5 ppm) after 15 days compared to other isolates. It was found that the pH of the cultured broth was lowered in the range of 3.33 to 3.35. Among all the isolates, *B. aryabhattai* was found to be the most promising ZSB.

Table 6: Change in pH at different days interval

Tr. No.	Treatments	pH values			
		0 DAI	5 DAI	10 DAI	15 DAI
T0	Control	7.1	7.1	7.1	7.1
T1	ZSB A1	7.1	6.9	6.1	4.9
T2	ZSB A2	7.1	6.5	5.3	4.1
T3	ZSB AF2	7.1	6.6	5.6	4.7
T4	ZSB A4	7.1	6.9	6.2	5.1
T5	ZSB A5	7.1	6.9	6.1	5.0
T6	ZSB1 A6	7.1	6.8	5.9	4.8

Bapiri *et al.* (2012) [3] evaluated the zinc solubilizing ability of *Pseudomonas fluorescence* using zinc oxide, zinc carbonate and zinc sulphide in both plate and broth media assays. Forty bacterial strains and 0.1% of each chemical source in six replications were used. Colony and halo diameters were measured after incubating plates for 48 h in an incubator. Zn solubilizing ability of 40 mentioned strains in three repetitions was studied with ZnO and $ZnCO_3$ solutions in broth assay. The soluble zinc and pH were measured after five days. Results showed, that only 8 of 40 strains could form a clearing zone in a plate assay. Halo diameter, ratio of halo diameter to the colony diameter and area respectively for zinc oxide and zinc carbonate were as following, respectively: 0.60- 1.32 cm, 1.20-2.64 and 0.95-2.60 cm^2, 0.13-1.70 cm, 0.27-2.99 and 0.31-4.10 cm^2. There were no halos observed in zinc sulphide. The concentration of soluble Zn for ZnO was 28-625 mg/l and pH shifted from 7.0-7.2 to 3.90-6.50 and for ZnCO3 was 247-753 mg/l and pH shifted from 7.0-7.2 to 3.5-6.3 after 5 days of inoculation in 28ºC.

3.2.2 PGP traits by zinc solubilizing bacteria

3.2.2.1 IAA production

Production of growth hormones is seen to be the most promising aspect of PGPR, and the production of IAA may be seen as one way for promoting plant development. ZSB isolates were inoculated in zinc solubilizing medium supplemented with 1 µg/mL of tryptophan and cultured for 24 h on shaker to evaluate their IAA production capability. For all the six isolates, the IAA production ranged from 0.67 to 7.81 µg/mL. ZSB A2 isolate produced the highest IAA (7.81 µg/mL), followed by ZSB AF2 (6.51 µg/mL).

Dahaji *et al.* (2012) [6] studied symbiotic efficiency of 47 *Rhizobium* strains with 6 common bean cultivars under greenhouse conditions. Fourteen strains showed the best symbiotic efficiency, whereas some isolates could not induce nodules on host plants. The ability of fourteen superior strains to solubilize phosphorus and zinc and to produce auxin, HCN and siderophores was evaluated in laboratory assays. *Rhizobium* strain Rb102 produced the highest amount of auxin (14.2 µg/mL) in the medium containing L-tryptophan. none of the isolates were able to solubilize ZnO and $ZnCO_3$ in solid medium and in liquid medium some of them had negligible solubilization. The highest P and Zn solubility in liquid and solid medium were observed in strains Rb113 and Rb130, respectively.

3.2.2.2 Phosphate solubilization

Tri-calcium phosphate (TCP) was added to Sperber's medium as an insoluble source of phosphate in order to estimate the solubilization of phosphate by native zinc-solubilizing bacterium isolates. The insoluble phosphorous was seen to be dissolved by isolates ZSB A2 and ZSB AF2 (Fig. 4), which also produced a halo zone surrounding the colony, according to the information shown in table 7. The remaining isolates were discovered to be phosphate solubilization-negative,

Desai *et al.* (2012) [7] obtained *Azotobacter*, *Azospirullum*, *Bacillus* and *Pseudomonas* strains from the diverse crop production system and were evaluated for solubilization of zinc and Phosphorous *in vitro* from insoluble Zn and TCP, respectively. After 15 DAI, 15 strains solubilized Zn and produced more than 50 cm^2 solubilization zone on solid media

3.2.2.3 Potash mobilization

The potash solubilization efficiency of six chosen ZSB isolates was tested using a plate assay on GYCa (Glucose Yeast Extract Calcium Carbonate) agar plates, displaying a halo zone of $CaCO_3$ solubilization and Ca^{+3} release. The isolate ZSB A2 and ZSB AF2 displayed the greater zone (Fig. 5) according to the data in table 7, and the findings demonstrated that these isolates could solubilize the immobile potash and make it accessible to the plant. Other isolates were proved to be potash mobilization-negative

Dhaked *et al.* (2017) [8] isolated and screened KMB and ZSB from different rhizosphere soil and found a solubilization zone ranging from 6 to 16 mm for zinc oxide (ZnO). Isolate ZnSB-3 showed a maximum solubilization zone of 16 mm. Isolate ZnSF-1 showed a maximum solubilization zone of 85 mm followed by ZnSF-2 with 34 mm for ZnO. Solubilization zone ranged from 6 to 25 mm for $Zn_3(PO_4)_2$. The isolate ZnSB-8 showed a maximum solubilization zone of 25 mm for zinc phosphate. The solubilization zone for potassium ranged from 160 to 85 mm. Isolate KSB-2 showed a maximum solubilization zone of 160 mm.

3.2.2.4 Siderophore Production

All the native ZSB isolates were screened for siderophore production on CAS agar plates and found negative for it.

Table 7: PGP traits for ZSB isolates

Isolate	IAA concentration at 24 h (µg/mL)	P solubilization	K mobilization
ZSB A1	1.81	-	-
ZSB A2	7.81	+++	+++
ZSB AF2	6.51	++	++
ZSB A4	0.67	-	-
ZSB A5	3.43	-	-
ZSB1 A6	2.71	-	-

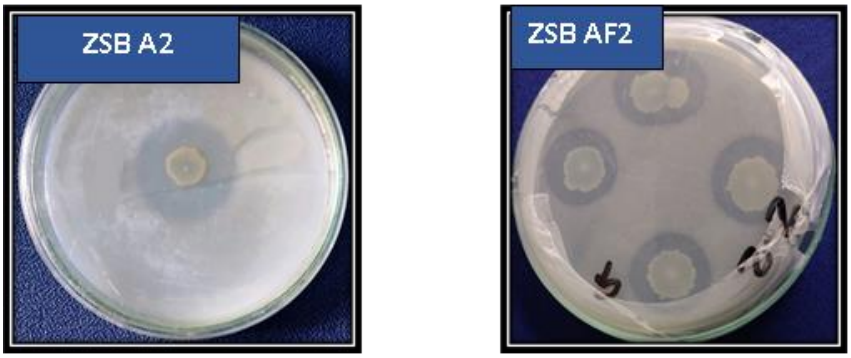

Fig 4: Phosphate solubilization on Sperber agar

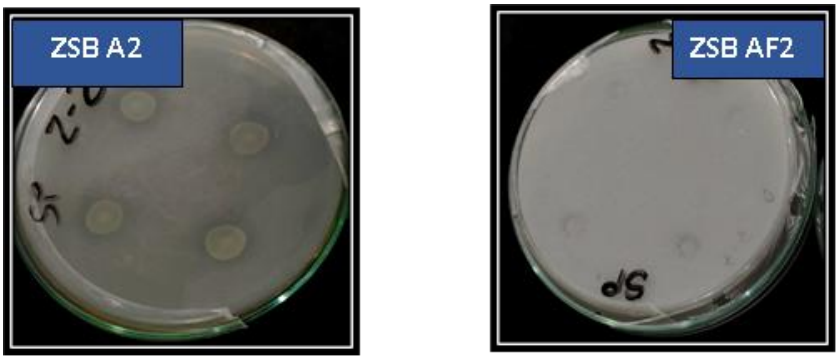

Fig 5: Potash mobilization on GYC agar

3.2.3 Molecular characterization of ZSB isolates

To confirm identity of isolates ZSB A2, ZSB F2 and ZSB A6, the bacterial cultures were subjected to 16S *r*RNA sequencing which is considered as an effective tool for identification of microorganisms up to species level.

Table 8: Molecular Characterization of zinc solubilizing bacterial isolates by 16S *r*RNA gene sequencing

Isolates	Gene bank accession number	Length of 16S *r*RNA gene sequence	Most closely related organism		
			Species	Accession description	% gene identity
ZSB A2	ON080844	1489	*Pseudomonas aeruginosa* strain ATCC 10145	NR_114471.1	99.10
ZSB AF2	ON080840	1469	*Pseudomonas taiwanensis* strain DSM 21245	NR_116172.1	99.56
ZSB A6	ON080839	1448	*Beijerinckia fluminensis* strain UQM 1685	NR_116306.1	99.85

From the 16S *r*RNA molecular characterization, it was revealed that isolate ZSB A2 was found nearest to *Pseudomonas aeruginosa* with gene identity of 99.10%, isolate ZSB AF2 was found nearest to *Pseudomonas taiwanensis* with gene identity of 99.56%, isolate ZSB A6 was found nearest to *Beijerinckia fluminensis* with gene identity 99.86%. (Table 8).

Sequences of all the isolates were submitted in NCBI for acquiring the accession number. The accession numbers obtained from NCBI were ON080844, ON080840, and ON080839 for ZSB isolates AAU ZSB A2, AAU ZSB AF2 and AAU ZSB A6, respectively.

3.3 Evaluation of efficient zinc solubilizing bacterial isolates on growth and yield of groundnut (*Arachis hypogaea* L.) under pot condition

3.3.1 Compatibility testing of selected ZSB

After identification, the selected prominent 3 ZSB isolates were cross streaked on nutrient agar plate to check out their compatibility and observed that all the 3 isolates did not inhibit each other.

3.3.2 Pot study

An experiment was conducted in pot condition after completion of molecular characterization during summer to test the efficacy of ZSB on groundnut growth and biomass yield. A total of three outstanding ZSB (*P. aeruginosa*, *P.*

taiwanensis and B. fluminensis) and their consortium were tested.

3.3.3 Effect of ZSB on plant growth parameters of groundnut

The observations of plant growth parameter measured in different time like 40, 80 and 120 (harvest) DAS are narrated in Table 9. Results revealed that plant growth parameter (plant height, root length, Number of branches per plant, chlorophyll content, days to flowering, plant fresh weight, plant dry weight, fresh root weight, dry root weight) at different time showed significant differences. Results revealed that plant height at 40 DAS showed significant differences wherein, T_{11} receiving soil application of Zn-EDTA @ 1 kg/ha, as well as seed inoculation followed by foliar applications at 30 and 40 DAS of ZSB consortium comprising of *P. aeruginosa* + *P. taiwanensis* and *B. fluminensis* showed significantly higher plant growth parameter as compared to control.

3.3.4 Effect of ZSB on plant yield parameters and available Zn

The observations of yeild parameters and available Zn are narrated in Table 10. Results revealed that yield parameters like No. of seeds, no. of pods pod weight and kernel weight and available Zn present in soil, plant and seed at harvest showed significant differences. Results showed significant differences wherein, T_{11} receiving soil application of Zn-EDTA @ 1 kg/ha, as well as seed inoculation followed by foliar applications at 30 and 40 DAS of ZSB consortium comprising of *P. aeruginosa* + *P. taiwanensis* and *B. fluminensis* showed significantly higher yeild and available Zn as compared to control.

Table 9: Effect of ZSB on Groundnut growth

T. No	Treatments	Plant height			Root length (cm)	Number of branches per plant	Chlorophyll content SPAD value		Days to flowering Fresh Weight (g)	Shoot		Root	
							40 DAS	At harvest		Fresh Weight (g)	Dry Weight (g)	Fresh Weight (g)	Dry Weight (g)
T_1	Control	28.75f	33.00e	36.00h	8.75d	41.75d	36.75c	39.75d	41.75d	21.75f	13.75d	8.00^{e}	5.25e
T_2	$ZnSO_4$ @ 25 kg/ha	31.25e	37.50d	44.50g	9.25d	40.00d	44.00b	49.50c	40.00d	22.25^{f}	14.50d	8.25^{e}	5.75e
T_3	Zinc EDTA @ 1 kg/ha	31.75e	38.00d	45.75g	9.50d	41.00d	45.00b	49.75c	41.00d	23.00^{f}	15.25d	9.25^{e}	5.75e
T_4	T_2 + *P. aeruginosa*	40.50abc	65.75b	68.75c	16.00ab	37.75bc	48.50ab	52.50abc	37.75bc	37.50^{bc}	30.50a	16.75^{cd}	12.50bc
T_5	T_2 + *P. taiwanensis*	37.75cd	53.50c	55.75f	14.00c	39.25bc	45.00b	50.50c	39.25bc	36.00^{cd}	27.00b	16.75^{cd}	11.75cd
T_6	T_2 + *B. fluminensis*	36.00d	56.25c	59.75e	14.75bc	39.00c	46.75ab	52.25abc	39.00c	33.25^{e}	23.75c	15.25^{d}	10.00d
T_7	T_2 + consortium	42.00ab	71.25a	73.25b	17.25a	38.00ab	51.00a	55.75ab	38.00ab	39.50^{ab}	31.75a	19.25^{b}	14.25ab
T_8	T_3 + *P. aeruginosa*	41.50ab	66.50b	69.25c	16.25ab	39.00b	49.75ab	52.75abc	39.00b	38.00^{bc}	30.75a	17.25^{c}	14.00b
T_9	T_3 + *P. taiwanensis*	39.75bc	54.50c	58.5^{e}	14.25c	39.50bc	47.75ab	51.75bc	39.50bc	37.25^{c}	27.25b	17.00^{cd}	11.50cd
T_{10}	T_3 + *B. fluminensis*	36.75d	54.50c	61.75e	15.00bc	38.75bc	47.25ab	52.50abc	38.75bc	34.5^{de}	25.75bc	16.75^{cd}	12.75bc
T_{11}	T_3 + consortium	42.75a	72.75a	76.00a	17.50a	37.00^{a}	52.00^{a}	56.25^{a}	37.00^{a}	40.75^{a}	32.50a	23.00^{a}	16.25a
	S.Em. ±	0.84	1.05	0.78	0.48	0.90	1.77	1.27	0.90	0.67	0.80	0.59	0.62
	C.D. at 5%	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
	C.V. %	4.52	3.83	2.63	6.93	4.62	7.57	4.96	4.62	4.10	6.52	7.75	11.45

Table 10: Effect of ZSB on yield parameters and available Zn

T. No	Treatments	No. of seeds/pod	No. of pods/ plant	Pod weight/ plant (g)	Kernel Weight/ plant (g)	Available Zn in soil (ppm)	Zn content in plant (ppm)	Zn content in seed (ppm)
T_1	Control	1.50	2.00^{d}	1.50h	1.00g	1.00g	3.40f	30.75h
T_2	$ZnSO_4$ @ 25 kg/ha	1.50	3.00^{c}	2.53g	1.93f	2.35f	7.32e	42.52g
T_3	Zinc EDTA @ 1 kg/ha	1.75	3.00^{c}	2.93fg	2.30f	2.80ef	8.20e	46.50f
T_4	T_2 + *P. aeruginosa*	2.00	4.00^{b}	4.75cd	3.68d	4.70b	16.91c	52.75cd
T_5	T_2 + *P. taiwanensis*	2.00	3.00^{c}	3.80ef	3.00e	3.27de	13.37d	47.75ef
T_6	T_2 + *B. fluminensis*	2.00	3.00^{c}	4.50de	3.40de	2.90ef	12.73d	46.25f
T_7	T_2 + consortium	2.00	4.00^{b}	7.53a	6.28b	5.05ab	19.35ab	57.25ab
T_8	T_3 + *P. aeruginosa*	2.00	4.00^{b}	6.38b	5.18c	5.00ab	18.57abc	55.25bc
T_9	T_3 + *P. taiwanensis*	2.00	3.00^{c}	4.35de	3.58d	4.00c	11.45d	51.62cd
T_{10}	T_3 + *B. fluminensis*	2.00	4.00^{b}	5.65bc	4.83c	3.82cd	17.13bc	50.8de
T_{11}	T_3 + consortium	2.00	4.50^{a}	8.48^{a}	7.00^{a}	5.35a	20.57a	59.25a
S.Em. ±		0.14	5.10	0.28	0.17	0.19	0.72	1.16
Test		NS	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
CV%		15.30	0.08	11.86	9.29	10.49	10.63	4.70

3.3.5 Effect of ZSB on soil total bacterial and ZSB population at harvest

Effect of ZSB on soil total bacterial and ZSB population at harvest are presented in fig. 6 and 7. The observations on initial total microbial count are 5.51 log cfu/g and 2.1 log cfu/g for total bacterial count and ZSB count, respectively. Results revealed that T_{11} receiving soil application of Zn-EDTA @ 1 kg/ha as well as seed inoculation followed by foliar applications at 30 and 40 DAS of ZSB consortium comprising of *P. aeruginosa* + *P. taiwanensis* and *B. fluminensis* showed significantly higher soil total bacterial count and ZSB count as compared to control

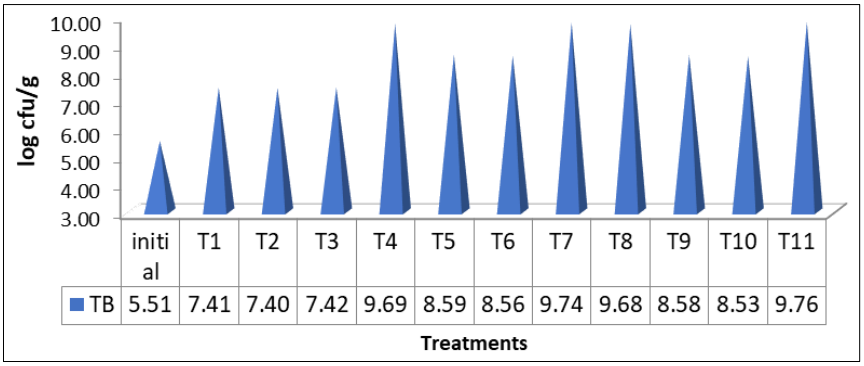

Fig 6: Effect of ZSB on total soil bacterial population on soil extract

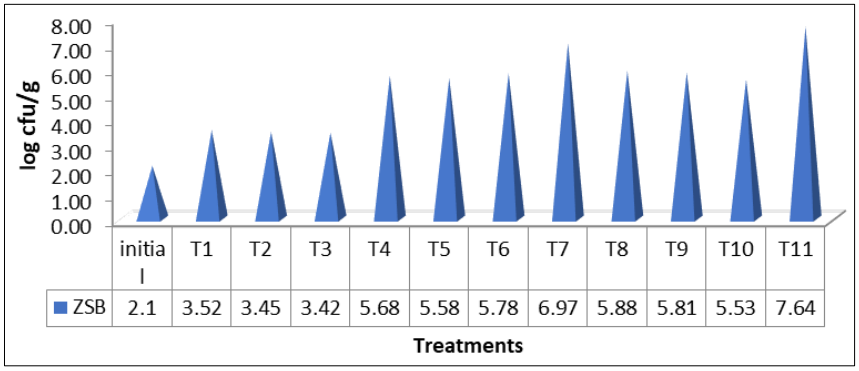

Fig 7: Effect of ZSB on total soil ZSB population on zinc solubilizing agar containing 0.1% Zn

3.8 Conclusion

Overall, results suggested that in presence of zinc fertilizer, seed inoculation of ZSB consortium comprising of *P. aeruginosa*, + *P. taiwanensis* and *B. fluminensis* or individual ZSB culture followed by foliar applications twice has wide scope as agriculturally beneficial bio-input in increasing growth and biomass yield of an important oil seed crop groundnut.

4. Author's Contributions

All authors read and approved the final version of the manuscript and contributed equally

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