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Enhancing Uptake of K and Zn and Improving Yield of Groundnut by Application of K- and Zn- Solubilizing Bacteria

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ABSTRACT

Background: Potassium and zinc are important with respect to mineral nutrition of groundnut and play a significant role in physiological and biochemical processes and thus affect productivity. To make these important minerals available to the growing plants in calcareous soil having fixation problem, the current investigation was undertaken to identify both K- and Zn-solubilizing bacteria for improving growth, nutrient uptake and yield of groundnut.

Methods: Soil samples were collected from groundnut rhizosphere during 2018 for the isolation of Zn- and K- solubilizing bacteria. Estimation for Zn- and K- solubilization was done using zinc phosphate and potassium aluminosilicate as insoluble form of Zn and K, respectively. Promising Zn and K solubilizing bacterial isolates were selected and evaluated in pots.

Result: Inoculation of different isolates of KSB and ZSB improved K and Zn contents of shoot and seed during summer and *kharif* 2019. Evaluation of KSB and ZSB isolates, in potted condition, resulted in improved pod yield upto 39.6% (KSB) and upto 37.1% (ZSB) during summer and upto 24.1% and 25.1% during *kharif* 2019, respectively. Therefore, uptake of Zn- and K- and yield of groundnut can be enhanced significantly by application of both potential ZSB and KSB.

Key words: *Acinetobacter*, *Enterobacter,* K-solubilizing bacteria, *Pseudomonas*, Zn-solubilizing bacteria.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is the third most important oilseed crop of India. However, poor and imbalanced plant nutrition is one of the major factors affecting productivity of groundnut, more so in calcareous shallow depth medium black soils. Availability of major macronutrients like potassium (K), phosphorus (P) and micronutrients like zinc (Zn), iron (Fe) and manganese (Mn) is limited in calcareous soils due to fixation and competing calcium ions (Wahba *et al.* 2019). Potassium is a key nutritional element which is required for the development of root nodules and nitrogen fixation; maintenance of turgor pressure for stomatal regulation; drought tolerance; protein synthesis and in other pathways.

Although earth's crust has 2.6% K (Schroeder, 2019), it's availability to plants is limited due to its presence as structural components of soil minerals (structural K, 90-98%), which is available after the breakdown of soil minerals. Rest 2-10% K is available in three pools, namely soil solution-, exchangeable- and fixed- K. As available pool is limited, it is important to use naturally available potash minerals in conjunction with potassium solubilizing bacteria (KSB) to restore the delicate balance of soil-microbe-plant-atmosphere continuum.

Zinc, an important micronutrient, is a regulatory co-factor of enzymes and structural constituent of many proteins involved in plant metabolism. Groundnut plants having zinc deficiency show stunted growth, with chlorotic uneven young leaves. Zinc deficiency reduces carbohydrate and phytohormone synthesis, decreases nitrogen metabolism and flower and pod development. In India, 70 mha of cultivable area is Zn deficient (Singh, 2008). It was reported that Zn deficiency in Indian soils is likely to increase from 49 to 63% by the year 2025 (Arunachalam *et al.* 2013). Availability of Zn in soil is controlled by several factors which include pH, $CaCO_3$ content, soil CEC (cation exchange capacity), organic matter content, soil solution equilibration dynamics of Na^+, Mg^{2+}, HCO_3^-, Ca^{+2}, PO_4^{2-} ions and soil moisture (Alloway, 2009). Thus, agronomic bio-fortification of groundnut with zinc solubilizing microorganism (ZSM) will help in improving dietary intake of Zn by increasing kernel Zn concentration.

Considering the importance of both K- and Zn- nutrition in groundnut, present investigation was undertaken to identify potential K- and Zn- solubilizing bacteria for improving uptake of these nutrients and to enhance growth and yield of groundnut.

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MATERIALS AND METHODS

Isolation and assay (qualitative and quantitative) of groundnut rhizobacteria for Zn and K solubilization ability

The rhizospheric soil samples from 60 days old groundnut plants (cv. TG37A) were collected, during 2018 from the

farm of ICAR-Directorate of Groundnut Research, Junagadh, Gujarat following standard protocol and soil dilutions were plated onto Tris minimal salt medium (Tris-HCl-6 g, NaCl-4.7 g, KCl-1.5 g, NH_4Cl-1.0 g, Na_2SO_4-0.5 g, Agar Agar -18.0 g/l) amended with insoluble 0.1% $Zn_3(PO_4)_2$ and 2% $AlKO_6Si_2$, respectively, for isolation of Zn- and K- solubilizing rhizobacteria. Morphologically distinct colonies, showing clear zones of solubilization were picked and spotted on the same medium and incubated at 28± 2°C for seven days. The halozone and colony diameter were measured to get the solubilization index (SI) and was calculated using the formula of Sadiq *et al.* (2014). Quantitative assay was done in broth amended with insoluble $Zn_3(PO_4)_2$ and $AlKO_6Si_2$ and quantified after seven days of incubation with atomic absorption spectrophotometer and flame photometer, respectively, following the procedure of Fuwa (1964) and Hald (1947). The formulae used for calculating solubilization index was:

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter (mm)} + \text{Halozone diameter (mm)}}{\text{Colony diameter (mm)}}$$

Evaluation of Zn and K solubilizing bacteria for growth, yield and nutrient uptake of groundnut in potted condition

Twenty-seven Zn-solubilizing and fourteen K solubilizing bacteria were evaluated in a pot trial during summer and *kharif* season of 2019 to study their effects on growth, yield, Zn and K uptake of groundnut (cv. TG3A) in potted condition following the procedure of Dey *et al.* (2004). There were a total of 29 and 16 treatments for Zn- and K- solubilizing bacteria, each having 3 replications. Nitrogen @ 20 kg/ha (0.01 g/kg) as ammonium sulphate and P_2O_5 @ 40 kg/ha (0.02 g/kg) as single super phosphate were applied just before sowing. Zinc phosphate @ 1 g/pot and $AlKO_6Si_2$ @1.0 g/pot were applied as insoluble source of zinc and potassium. Each isolate was grown overnight in Nutrient Broth. Each broth was centrifuged at 12,000 rpm, washed with phosphate buffer three times and then pellets were dissolved in 0.1M phosphate buffer (pH 7.0) and OD was adjusted to 1.2 before being used for pot experiments. An OD of 1.2 was equivalent to 2.0×10^8 cfu/ml. The seeds of each treatment were soaked in phosphate buffered saline (PBS) containing the suspension of the ZSB and KSB for one hour to maintain a population of 10^8 CFU/seed. In each pot, eight seeds (95% germination) were sown at a depth of 5 cm. After germination, five seedlings were maintained in each pot. The shoot length, plant biomass and pod yield were recorded after harvest. Total K and Zn in plant samples was analyzed using the method of Chapman and Pratt (1961) in Flame Photometer (Elico® Model: CL378) and Atomic Absorption Spectrophotometer (Perkin Elmer® Model:AAnalyst400), respectively.

Identification and phylogenetic analysis of selected isolates

Genomic DNA were isolated from all the isolates having capacity to solubilize insoluble Zn and K. The 16S rRNAs were amplified and purified following protocols of the manufacturer (Qiagen Inc.) using universal 8F and 1492R primers and custom sequenced on an ABI 3,730×l Genetic Analyzer (Applied Biosystems, Foster City) at Sequencher Tech. Pvt. Ltd., Ahmedabad, Gujarat, India. The 16S rRNA gene sequences were processed and deposited in the National Center for Biotechnology Information (NCBI) GenBank database and accession numbers were obtained. The identities of the isolates were obtained by comparing sequences of the isolated 16S rRNA gene with available sequences in the GenBank (http://www.ncbi.nlm.nih.gov/) using the BLASTn program. Phylogenetic analyses of 16S rRNA sequences were performed using MEGA (Molecular Evolutionary Genetics Analysis software) version 6.0 (Tamura *et al*., 2013). Phylogenetic trees were constructed by maximum likelihood method using bootstrap (Felsenstein, 1985; 1000 replications).

Statistical analysis

All the statistical analyses were performed following Gomez and Gomez (1984) and SPSS package. The data were analysed by analysis of variance (ANOVA) applicable to design of experiment. Mean were separated by Tukey's multiple range test as per experimental need. Differences at $P \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

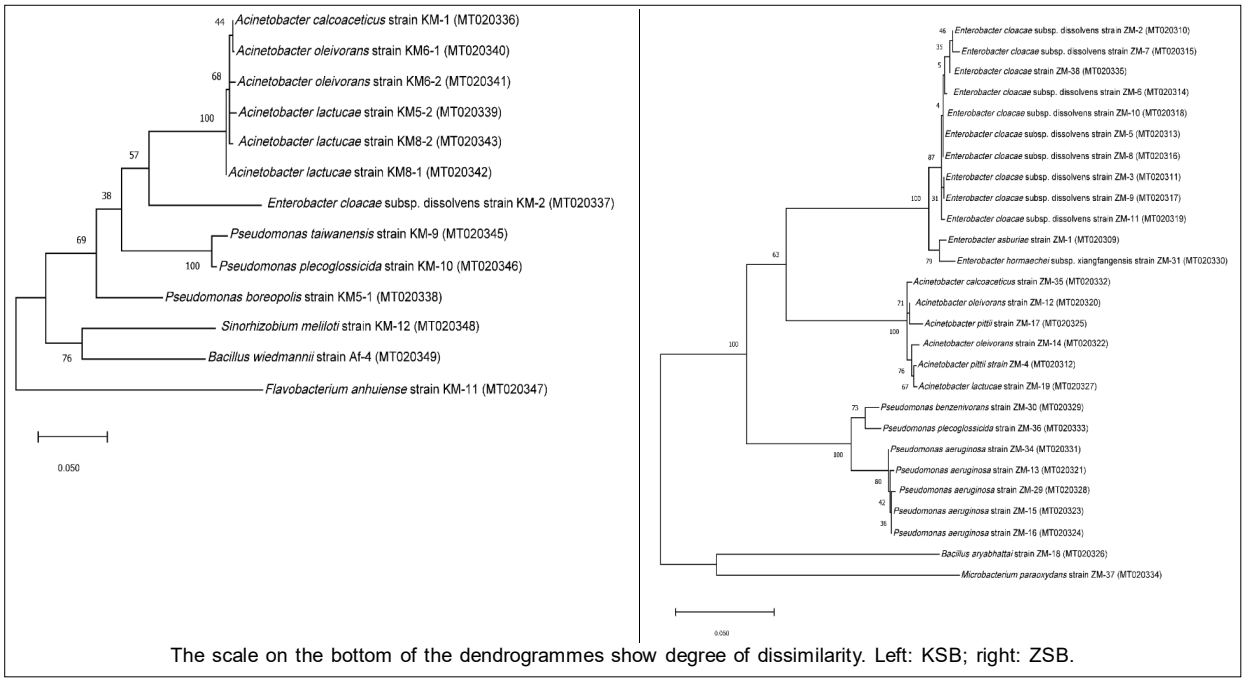
Identification, qualitative and quantitative assay of KSB and ZSB

Identification of the potash- and zinc-solubilizing bacteria was made by BLASTn of 16S rRNA sequences at NCBI. The sequences of the isolates were subsequently submitted to NCBI and accession numbers were obtained (Table 1). The phylogenetic tree revealed one major and two minor clusters (Fig 1) for KSB and three major and one minor cluster (Fig 1) for ZSB. The solubilization index (SI) of KSB and ZSB ranged from 2.02±0.03 to 3.99±0.24 and 2.07±0.01 to 4.24±0.57 for KSB and ZSB, respectively (Fig 2). Among different isolates of ZSB, *Enterobacter* was the dominant genera (12), followed by *Pseudomonas* (7), *Acinetobacter* (6) and one each of *Bacillus* and *Microbacterium*. Maximum SI was found with *Pseudomonas aeruginosa* ZM-16 (4.24), followed by *Pseudomonas aeruginosa* ZM-13 (3.89) (Fig 3). Predominant bacteria reported to solubilize zinc include *Thiobacillus* sp., *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter* sp., *etc*. (Saravanan *et al.,* 2007). Out of the 14 KSB isolates, six isolates belonged to *Acinetobacter* sp., three belonged to *Pseudomonas* sp. and one each to *Enterobacter* sp., *Streptomyces* sp., *Flavobacterium* sp., *Sinorhizobium* sp. and *Bacillus* sp.

Quantitative assay showed that pH of the broth drastically reduced from 6.80 in control to 4.22-5.19 for ZSB isolates and that for KSB isolates from 7.18 in control to 4.56-6.81 in different treatments. The maximum drop in pH was observed with *Enterobacter cloacae* subsp.

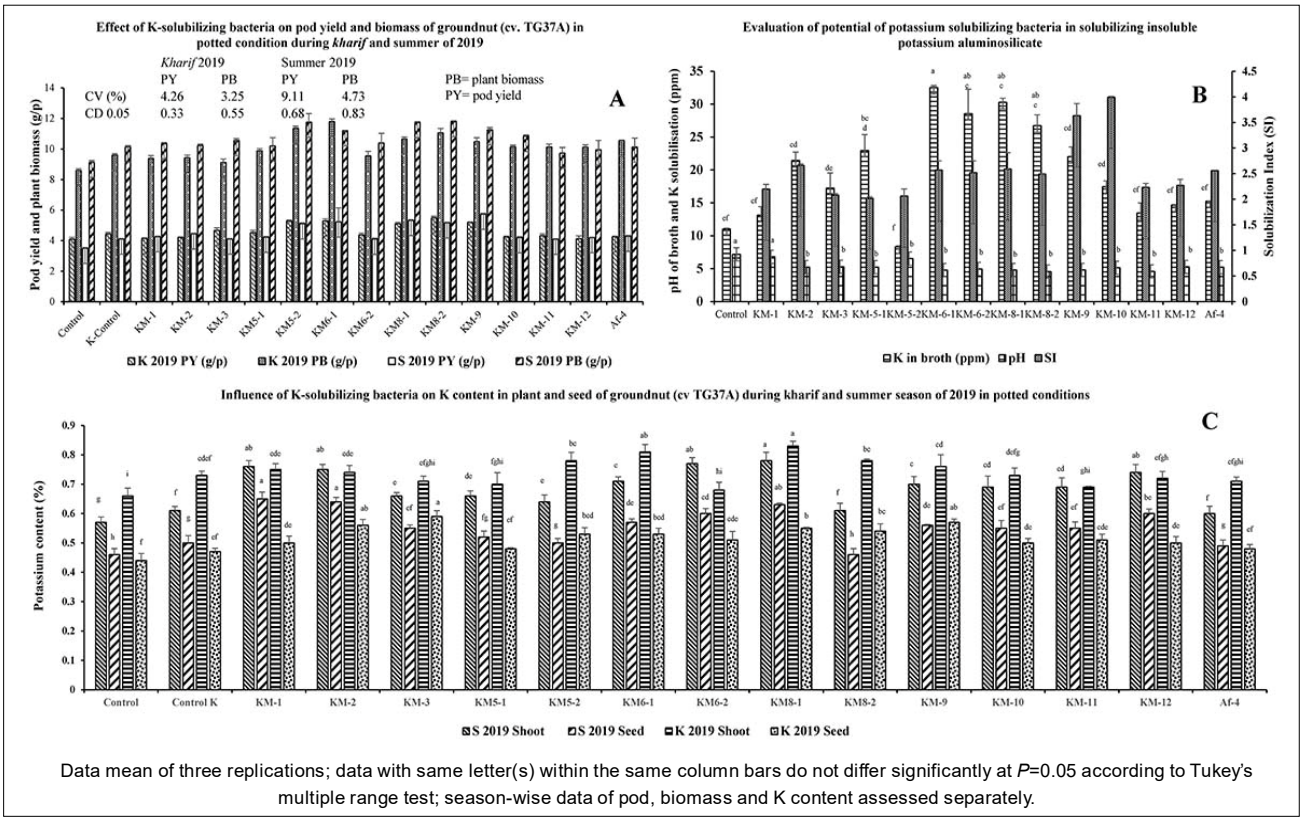
Table 1: Identity of Zn- and K- solubilizing bacteria based on 16S rRNA sequence.

Zn-solubilizing bacteria (ZSB)	Accession no.	K-solubilizing bacteria (KSB)	Accession no.
Enterobacter asburiae ZM-1	MT020309	*Acinetobacter calcoaceticus* KM-1	MT020336
Enterobacter cloacae subsp. *dissolvens* ZM-2	MT020310	*Enterobacter cloacae* subsp. *dissolvens* KM-2	MT020337
Enterobacter cloacae subsp. *dissolvens* ZM-3	MT020311	*Streptomyces roseofulvus* KM-3	-
Acinetobacter pittii ZM-4	MT020312	*Pseudomonas boreopolis* KM5-1	MT020338
Enterobacter cloacae subsp. *dissolvens* ZM-5	MT020313	*Acinetobacter lactucae* KM5-2	MT020339
Enterobacter cloacae subsp. *dissolvens* ZM-6	MT020314	*Acinetobacter oleivorans* KM6-1	MT020340
Enterobacter cloacae subsp. *dissolvens* ZM-7	MT020315	*Acinetobacter oleivorans* KM6-2	MT020341
Enterobacter cloacae subsp. *dissolvens* ZM-8	MT020316	*Acinetobacter lactucae* KM8-1	MT020342
Enterobacter cloacae subsp. *dissolvens* ZM-9	MT020317	*Acinetobacter lactucae* KM8-2	MT020343
Enterobacter cloacae subsp. *dissolvens* ZM-10	MT020318	*Pseudomonas taiwanensis* KM-9	MT020345
Enterobacter cloacae subsp. *dissolvens* ZM-11	MT020319	*Pseudomonas plecoglossicida* KM-10	MT020346
Acinetobacter oleivorans ZM-12	MT020320	*Flavobacterium anhuiense* KM-11	MT020347
Pseudomonas aeruginosa ZM-13	MT020321	*Sinorhizobium meliloti* KM-12	MT020348
Acinetobacter oleivorans ZM-14	MT020322	*Bacillus wiedmannii* Af-4	MT020349
Pseudomonas aeruginosa ZM-15	MT020323		
Pseudomonas aeruginosa ZM-16	MT020324		
Acinetobacter pittii ZM-17	MT020325		
Bacillus aryabhattai ZM-18	MT020326		
Acinetobacter lactucae ZM-19	MT020327		
Pseudomonas aeruginosa ZM-29	MT020328		
Pseudomonas benzenivorans ZM-30	MT020329		
Enterobacter hormaechei subsp. *xiangfangensis* ZM-31	MT020330		
Pseudomonas aeruginosa ZM-34	MT020331		
Acinetobacter calcoaceticus ZM-35	MT020332		
Pseudomonas plecoglossicida ZM-36	MT020333		
Microbacterium paraoxydans ZM-37	MT020334		
Enterobacter cloacae ZM-38	MT020335		



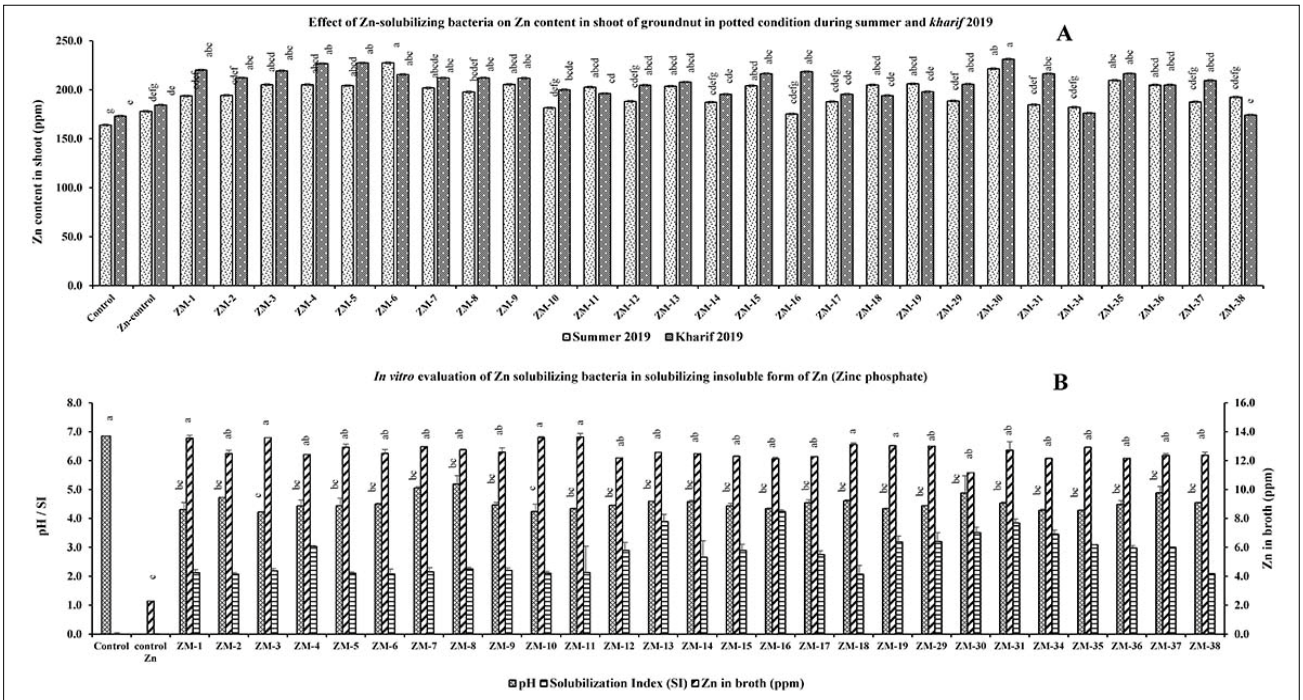
The scale on the bottom of the dendrogrammes show degree of dissimilarity. Left: KSB; right: ZSB.

Fig 1: Phylogenetic trees of 13 KSB and 27 ZSB constructed on the basis of 16S rDNA sequences in MEGA 6.0.



Data mean of three replications; data with same letter(s) within the same column bars do not differ significantly at *P*=0.05 according to Tukey's multiple range test; season-wise data of pod, biomass and K content assessed separately.

Fig 2: *In vitro* evaluation of KSB for solubilization index (SI) and solubilization potential (B) and assess their role on growth and yield (A) and nutrient content (C) in groundnut during summer and *kharif* season of 2019.



Data mean of three replications; data with same letter(s) within the same column bars do not differ significantly at *P*=0.05 according to Tukey's multiple range test; season-wise data of pod, biomass and K content assessed separately.

Fig 3: *In vitro* evaluation of ZSB for solubilization index (SI) and solubilization potential (B) and assess their role on Zn content (A) in shoot of groundnut during summer and *kharif* season of 2019.

dissolvens strain ZM-3 (pH 4.22). Soluble Zn in broth ranged from 11.17 to 13.63 ppm (Fig 2 and 3). Maximum solubilization of Zn was exhibited by *Enterobacter cloacae* subsp. *dissolvens* strain ZM-11 (13.63 ppm), which was at par with rest of ZSB isolates. While significant negative correlation (-0.885) was noted between Zn content in liquid broth and pH, it was -0.663 for K content.

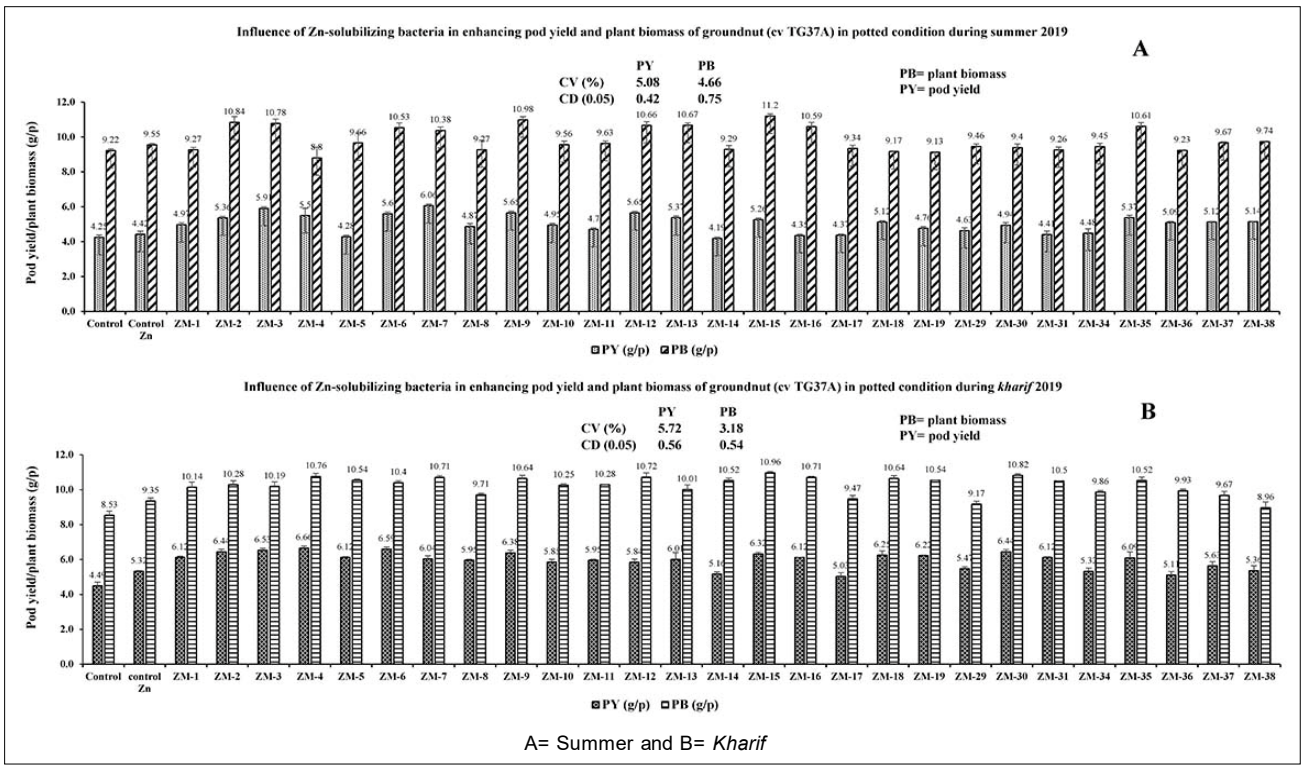
Soluble K in broth, due to solubilization of insoluble potassium aluminosilicate, of different isolates ranged from 8.33 to 32.53 ppm (Fig 2), maximum being found in *Acinetobacter oleivorans* KM6-1 (32.53 ppm), which was at par with isolates KM6-2, KM8-1 and KM8-2. The steepest drop in pH was observed with the isolate *Acinetobacter lactucae* KM8-2 (pH 4.56).

The drop in pH is attributed to production of organic acids with solubilization of ZnO (Ramesh *et al.,* 2014). Similarly, Meena *et al.* (2014) have reported that organic acids and exo-polysaccharide secretion play a key role in releasing K from K-containing minerals. A strong inverse correlation was observed between pH and amount of K released/solubilized from insoluble K sources (Zarjani *et al.,* 2013). Similar results were also obtained in the present study. The potassium solubilizing bacteria (KSB) release K from inorganic and insoluble pools of soil K through solubilization and their inoculation has beneficial effect on plant growth (Bakhshandeh *et al.,* 2017). Badr (2006) noted that potassium silicate solubilizing bacteria release upto 35.23 mg/l of K when pH of media decreases from pH 8.0 to 6.5 in seven days.

Evaluation of KSB and ZSB for growth promotion, K and Zn uptake and yield

Experiments were conducted in pots during summer and *kharif* seasons of 2019 with groundnut variety TG37A, in combination with insoluble potassium aluminosilicate and KSB (14) and with Zinc phosphate and ZSB (27). In summer 2019, the pod yield and plant biomass (g/plant) were significantly higher in treatments inoculated with strains KM-9 (5.74 g/plant) and KM8-2 (11.81 g/plant) respectively, which were at par with strains KM8-1, KM5-2 and KM6-1 (Fig 2). In *kharif* 2019, significantly higher pod yield (5.50 g/plant) and plant biomass (11.80 g/plant) were obtained with KM8-2 and KM6-1, respectively. Similarly, pod yield was significantly high in treatment inoculated with strain ZM-7 (6.06 g/plant), which was at par with strains ZM-3, ZM-9 and ZM-12 during summer 2019. However, plant biomass and shoot Zn content was significantly higher with strains ZM-15 (11.2 g/plant) and ZM-6 (227.5 ppm) respectively (Fig 4). In *kharif* 2019, pod yield was significantly higher in treatment inoculated with strain ZM-4 (6.66 g/plant) which was at par with ZM-1, ZM-2, ZM-3, ZM-5, ZM-6, ZM-7, ZM-9, ZM-15, ZM-16, ZM-18, ZM-19, ZM-30 and ZM-31. Moreover, plant biomass and shoot Zn content was significantly high with strains ZM-15 (10.96 g/plant) and ZM-30 (231.3 ppm; Fig 3), respectively.

The shoot and seed K content was significantly higher in treatments inoculated with KM8-1 (0.78%) and KM-1 (0.65%) in summer and with KM8-1 (0.83%) and KM-3 (0.59%) in shoot and seed, respectively during *kharif* (Fig 2)



A= Summer and B= *Kharif*

Fig 4: Effect of ZSB on growth and yield of groundnut in potted conditions.

over control. Similarly, inoculation of KSB improved K content of shoot (4.9-27.8%, 1.4-13.7%) and seed (4.0-30.0%, 2.1-25.5%) during summer 2019 and *kharif* 2019, respectively, over control. Similarly, inoculation of ZSB improved Zn content of shoot (2.0-27.8%, 5.2-25.4%) during summer 2019 and *kharif* 2019, respectively, over control.

While inoculation with KSB improved pod yield by 2.2-39.6% and 2.2-24.1% during summer 2019 and *kharif* 2019, respectively, over control, it was 1.3-37.1% and 0.7-25.1% during summer 2019 and *kharif* 2019, respectively, over control for ZSB.

Different KSB isolates inoculated on groundnut have been reported to improve germination, number of pods/plant and plant biomass (Verma *et al.* 2016). Similar results were obtained in the present study. Sunflower inoculated with KSB (KSBR-41 isolate) significantly increased test weight, seed yield and potassium content (Kammar *et al.* 2016). Soil rhizospheric bacteria can transform soil unavailable K to the plant available forms, which is brought by different bacterial species like *Bacillus mucilaginosus*, *Burkholderia, Acinetobacter* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Enterobacter hormaechei, Burkholderia*, *etc.* (Meena *et al.* 2016).

Increased mobilization of zinc by Zn-solubilizing *Bacillus aryabhattai* in wheat and soybean was reported (Ramesh *et al.* 2014). About 7-12% enhanced zinc translocation in wheat grains by strains of *Serratia* sp., *Bacillus* sp., *Pseudomonas* sp., as compared to chemical zinc supplementation, along with yield enhancement was reportedearlier (Lefèvre *et al.* 2014). A study conducted by Raut *et al.* (2019) found that application of 100% recommended dose of Zn through Zinc sulphate @ 20 kg ha^{-1} along with 5% ZSB as seed treatment at sowing and through drenching at 30 DAS along with 100% recommended dose of nutrients to summer groundnut improved zinc use efficiency and yield parameters. Therefore, the groundnut growth improvement due to inoculation of Zn- and K-solubilizing bacteria, in the present study, can be attributed to significant quantity of Zn-and K- solubilization.

CONCLUSION

To minimize the external application of chemical fertilizers and to improve productivity in a sustainable manner, inoculation of microorganisms would be useful in the current and future farming scenarios. To lessen the burden on national exchequer consequent upon import of potassic fertilizer, application of K-solubilizing bacteria will play a pivotal role by improving the availability of K in soil utilizing unavailable forms. It is also imperative to bank on application of Zn-solubilizing bacteria to improve Zn availability in soil and improve the uptake by the plants. In this context, identification of potential KSB and ZSB from groundnut and their application will improve yield parameters and K- and Zn-uptake and content in both summer and *kharif* groundnut in India.

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