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SCREENING OF ZINC SOLUBILIZING, POTASSIUM RELEASING BACTERIAL AND FUNGAL ISOLATES FROM DIFFERENT RHIZOSPHERE SOILS

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ABSTRACT

Zinc is key micronutrient plays vital roles in plant metabolic processes. Indian conditions are tend to be deficient in zinc due to adverse climatic conditions, high pH and several other factors, whereas potassium is most important for plants in maintaining plant heath, drought resistance, involves in many plant metabolic processes. In present study attempts were made to isolate and quantify the zinc solubilizing and potassium releasing microbes from the rhizospheric soils. The isolates were screened for their solubilization efficiency and quantitative estimation of available zinc after 7^{th}, 14^{th} and 21^{st} days after inoculation. Results showed that ZnSF-4 and ZnSF-1 has shown maximum solubilization of 54 mm on zinc phosphate and 65 mm on zinc oxide supplemented plate assay respectively whereas the highest amount of potassium solubilized by KSF-2 (58 mm). Total available zinc and in broth assay was maximum in case of ZnSF-4 (2.84 μg ml^{-1}), whereas potassium is maximum in KSB-5 (2.50 μg ml^{-1}). Total available zinc and potassium was found more in the 21^{st} day of inoculation (0.84 to 2.84 μg ml^{-1}).

INTRODUCTION

Zinc is an essential micronutrient for microorganisms and plants. This element is present in the enzyme system as co-factor and metal activator of many enzymes (Parisi *et al.*, 1969). The role of zinc in the nutrition and physiology of both eukaryotic and prokaryotic organisms, especially its importance for activity of many enzymes is widely studied (Hughes and Poole, 1989). Many bacterial enzymes contain zinc in the active center or in a structurally important site (Clarke and Berg, 1998). Bacteria can contribute to metal immobilization by several processes such as precipitation and adsorption.

Since zinc is a limiting factor in crop production in alkaline and calcareous soils of the world. This study on zinc solubilization by bacteria has an immense importance in zinc nutrition to plants. The rhizospheric microorganisms play a pivotal role in the enhancement of crop production by the solubilization of unavailable form of metal into available form.

Hutchins *et al.*, (1986), reported that *Thiobacillus thioxidans*, *Thiobacillus ferroxidans* and facultative thermophilic iron oxidizers solubilize zinc from sulphide ore (sphalerite). This metal solubilization was due to the production of organic acids and pH drop by organisms. Plants take up Zn as (Zn^{2+}) divalent cation. The release of organic acids that sequester cations and acidify the micro environment near root is thought to be a major mechanism of Zn solubilization. A number of organic acids such as acetic, citric, lactic, propionic, glycolic, oxalic, gluconic acid etc., have been considered due to its effect in pH lowering by microorganisms (Cunnigham and Kuiack, 1992).

Potassium is essential macronutrient for plant growth and plays significant roles in activation of several metabolic processes including protein synthesis, photosynthesis, enzyme activation, as well as in resistance to diseases and insects etc (Rehm and Schmitt, 2002). Very little of potassium source is available for plant use. Potassium though present in as abundant element in soil or is applied to fields as natural or synthetic fertilizers, only one to two percent of this is available to plants, the rest being bound with other minerals and therefore unavailable to plants. For optimal nutrition of a crop, the replenishment of a K depleted soil solution is affected predominantly by the release of exchangeable K from clay minerals.

Consequently, for maximal crop growth, soil solution and exchangeable K need to be replenished continually with K through the release of non-exchangeable K by the weathering of K reserves (i.e. micas and feldspars) (Sparks and Huang, 1985) or the addition of K fertilizers. Many microorganisms in the soil are able to solubilize 'unavailable' forms of K-bearing minerals, such as micas, illite and orthoclases, by excreting organic acids which either directly dissolve rock K or chelate silicon ions to bring the K into solution (Groudev, 1987; Friedrich *et al*., 1991; Ullman *et al*., 1996; Bennett *et al*., 1998). Silicate solubilizing bacteria were found to resolve potassium, silicon and aluminum from insoluble minerals (Aleksandrov *et al*., 1967). Therefore, the application of K-Solubilizing Microorganisms (KSM) (Zahra *et al*., 1984; Vandevivere *et al*., 1994; Barker *et al*., 1998) is a promising approach for increasing K availability in KSM amended soils.

In India most of the crops were showing the symptoms of zinc and potassium dearth in the soil in turn which makes them susceptible to pest and diseases resulted in the yield reduction considerably. Presently there is a dearth of chemical fertilizers in India and that to for potassic fertilizers we are fully/partly dependent on other countries in order to meet the domestic farmer's needs. Though our country has less reserve sources of potassium and zinc but there is a recoverable amounts were present in the soil to meet the crop requirements. To avail this to available for the plants the only solution is using the biological methods which can recover the unavailable zinc and potash with minimum cost and they are proved to be excel. However, this research paper evaluates the best zinc solubilizing and potassium releasing isolates from the rhizospheric soils of Rajendranagar, Hyderabad, by using both plate assay and broth assay.

MATERIALS AND METHODS

Isolation of zinc solubilizing and potassium releasing isolates from rhizospheric soils

Rhizospheric soils (Black cotton and red sandy soils) were collected within the top 20 cm from the root surfaces of Rice, Maize, Sunflower, Castor, Redgram, Cotton, Safflower and Soybean in the Rajendranagar, Hyderabad, India. A total of 8 (from each crop four samples collected and after mixing as a composite soil it has taken as 1 sample) soil samples were collected stored at 4 ^{0}C for further use. Isolation of Potassium Releasing Bacteria (KSB) and fungi were carried out on Aleksandrov medium (Aleksandrov *et al.,* 1967) supplemented with 0.2 % potassium alumino silicate by following dilution plate technique. KSB colonies were identified by the formation of clear/ transparent zone around the bacterial/fungal colonies due to the release of K source (Rekha *et al.,* 2015). For zinc solubilizing, study was done on TRIS (Tris amino methane) minimal medium supplemented with 0.1 % of either zinc phosphate, zinc oxide and zinc sulphate in triplicate plates. A clear zone around the bacterial colony indicated solubilization of Zn sources and quantitative study in respective liquid medium.

Plate assay

Screening of isolates for zinc solubilizing capacity on TRIS minimal medium

The isolates were inoculated into modified TRIS medium (ingredients g l^{-1}), (Glucose-10.0 g; Zinc phosphate- 1 g; Ammonium sulphate- 0.5 g; Potassium chloride- 0.2 g; Yeast extract- 0.5 g; Ferrous sulphate- 0.01 g; Manganese sulphate- 0.01 g; Di-potassium hydrogen phosphate- 0.25 g; Agar- 20 g and Double distilled water- 1000 mL) containing 0.1 % insoluble zinc compounds (ZnO, $ZnPO_4$ and ZnS) (Fasim *et al.,* 2002). The test organisms were inoculated on these media and incubated at 28 ± 2ºC for 48-72 hr. The diameters of the colony and clearing zones around the colonies were measured. Zinc Solubilization Efficiency (SE) was calculated as described by Ramesh *et al*. (2014)

$$SE = \frac{\text{Diameter of solubilization halo zone}}{\text{Diameter of colony}} \times 100$$

Screening of potassium releasers on Alexondrov's medium

Potassium releasing isolates were inoculated on the modified Alexondrov's medium (Glucose- 5 g; Magnesium sulphate- 0.5 g; Ferric chloride- 0.005 g; Calcium carbonate- 0.1 g; Tri-calcium phosphate- 2g; Potassium alumino silicate- 2 g; agar 15-20 g; Double distilled water- 1000 mL) containing 0.2 % potassium alumino silicate as a potassium source (Prajapati, 2012). The test organisms were inoculated on the media and incubated at 28 ± 2ºC for 48-72 hr. The diameter of the colony and clearing zones around the colonies were measured.

Broth assay

Broth assay for zinc solubilizing isolates

The isolates were inoculated separately to TRIS minimal medium supplemented with 0.1 % insoluble zinc compounds (ZnO and $ZnPO_4$). The solubilization of zinc from laboratory grade ZnO and $ZnPO_4$ by the isolates were assessed. Modified TRIS minimal medium was prepared, distributed in 25 ml aliquots in 50 mL Erlenmeyer flasks and 0.1 % of $ZnPO_4$ and ZnO were added in different flasks steam sterilized for 30 min in autoclave. Then the flasks were inoculated with 0.1 ml suspension of the test culture containing 10^5 CFU ml^{-1}. Three flasks were maintained with an uninoculated control for each treatment. Experiments were done in triplicate. The samples were collected after 7th, 14th and 21st days after inoculation. Samples were centrifuged at 10000 rpm for 10 min to remove cell debris. The pH of the supernatant was measured. One ml of this supernatant was directly fed to Atomic Absorption Spectrophotometry (AAS) to determine the available zinc content (Suseelendra *et al.,* 2012).

Broth assay for potassium releasing isolates

The total available Potassium content in broth was estimated by using flame photometer method. Aleksandrov's broth supplemented with 0.2 % of Potassium alumino silicate is prepared autoclaved and inoculated with the culture containing 10^5 CFU ml^{-1} in each flask. The culture was sampled on 7th, 14th and 21st days after incubation and centrifuged to collect supernatant. This cell free culture filtrate solution (supernatant) was used to determine the available K content was measured using flame photometer (Bagyalakshmi *et al.,* 2014).

RESULTS AND DISCUSSION

Screening of isolates for potassium releasing on the Alexondrov's medium

A total of five bacterial and two fungal isolates were screened for their potassium releasing capacity (Aleksandrov, 1967). All the seven isolates were positive for potassium releasing ability. The isolates has shown the solubilization zone ranging from a maximum of 58 to a minimum of 8 mm was found after

72 hr of incubation at 28 ± 2 ^{0}C. Potassium releasing ability of the isolates varied with the isolate due differential acid production. The isolate KSF-2 had shown the maximum solubilization zone of 58 mm followed by KSF-1 (43 mm), KSB-1 (12 mm), KSB-4 (12 mm), KSB-3 (11 mm), KSB-5 (10 mm) and with least solubilization zone in KSB-2 (8 mm). The maximum solubilization efficiency was showed by KSB-1 with the efficiency of 150 % followed by KSB-4 (140 %), KSB-3 (120 %), KSB-1 (100 %), KSB-2 (60 %), KSF-1 (34 %) and with minimum solubilization efficiency in KSF-2 (28 %). K dissolution rate along with its corresponding change in pH was recorded, these results were supported by Brindavathy and Gopalaswamy, (2014). The results of the present study were concordance with the findings of Hu *et al.,* (2006) (Graph 1).

Muentz, (1890) showed the first evidence of microbial involvement in solubilization of rock potassium. The solubilization process of minerals may be due to the production of various organic acids such as acetic, formic, gluconic, oxalic and succinic acids Lal, (2002). Adeleke *et al.* (2010) have reported the ability of ectomycorhizal fungi in mobilization of P and K sources from insoluble ore. *B. muciloginosus* is reported to have a greater capacity to release K from muscovite (Sugumaran and Janarthanam, 2007). Similar results were reported by Kalavati *et al.* (2012) who isolated and screened for potassium solubilizing fungi from ceramic industry soils on Aleksandrov's agar medium supplemented with 0.5 % potassium aluminium silicate. Microorganisms like *Aspergillus niger*, *Bacillus extroquens* and *Clostridium pasteurianum* were found to grow on muscovite, biotite, orthoclase microclase and mica *in vitro* (Archana, 2013).

Screening of potassium releasing isolates for zinc solubilization

Potassium releasing isolates were screened for the zinc solubilization ability on TRIS minimal medium supplemented with zinc phosphate. The solubilization zone is ranging from 43 mm to 9 mm. The isolate KSF-2 has showed the maximum solubilization zone of 43 mm followed by KSB-1 (12 mm), KSB-4 (9 mm) and KSB-5 (9 mm). The maximum solubilization efficiency was recorded by KSB-1 with the efficiency of 300 % followed by KSF-2 (138.8 %), KSB-4 (125 %) and with minimum solubilization efficiency was found in KSB-5 (28 %). The solubilization zone was maximum for KSF-2 (43 mm) and least for the KSB-5 (9 mm), no solubilization was observed with the KSB-2, KSB-3 and KSF-1 isolates. The potassium releasing isolates were efficient in solubilization of zinc the possible mechanism is due to organic acids production by isolates (Table 1).

Solubilisation of zinc can be accomplished by a range of mechanisms, which include excretion of metabolites such as organic acids, proton extrusion, or production of chelating agents (Nahas, 1996; Sayer and Gadd, 1997). It is apparent from the zinc solubilization data that the solubilization potential varied with each isolate. Organic acid production by microbial isolates has been reported to be a major mechanism of solubilization (Nguyen *et al.,* 1992)

Broth assay to estimate total available potassium by using flame photometer

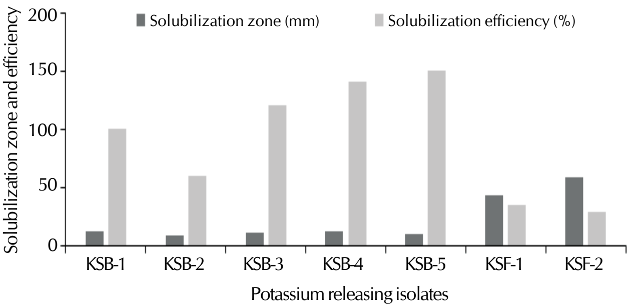
Quantitative estimation of total available potassium by flame photometer revealed that the total available potassium found to be more on 21st day of inoculation (1.5 to 2.50 μg ml^{-1}) followed by 15th (1.0 to 2.10 μg ml^{-1}) and 7th day (0.20 to 1.0 μg ml^{-1}). Among the isolates KSB-5 released maximum amount of K from potassium alumino silicate (2.50 μg ml^{-1}) and minimum potassium was found in KSF-1 (1.50 μg ml^{-1}) on 21st day after inoculation (Graph:2). After 7 days, the pH of the broth was acidic in some of cultures. The pH shifted from 7.0-7.2 to 4.5-6.5. The dissolution potential was worked as dissolution rate and expressed as per cent. KSB-1, KSF-2and KSB-3 produced significantly higher rate of K dissolution with 92 per cent with the pH reduction of 4.4, 4.2 and 4.1 respectively, followed by KSB-2 having 72 % (pH of 4.8) of K in the culture medium. The results of the present study were concordance with the findings of Chengsheng *et al.* (2014).

Screening of isolates for zinc solubilization on TRIS minimal medium

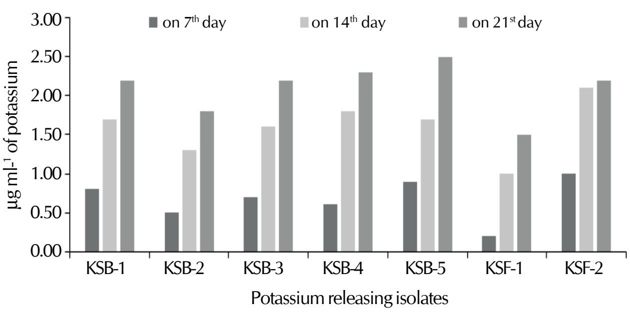
The zinc solubilizing isolates evaluated for their efficiency on TRIS minimal medium supplemented with three zinc sources namely $ZnPO_4$, ZnO and ZnS. Results revealed that the isolate

Table 1: Screening of zinc solubilizing and potassium releasing isolates on TRIS minimal medium and Alexondrov's medium respectively

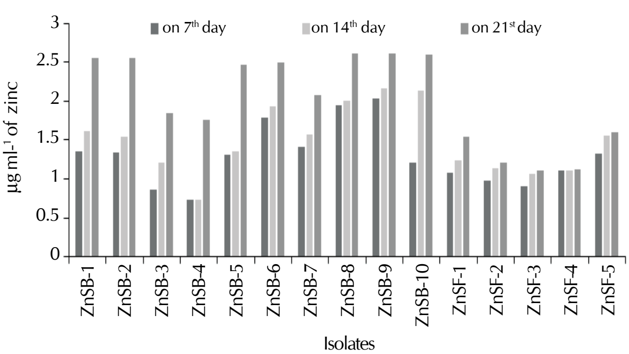
S. No	Isolates	Solubilization of Zinc and Potassium Sources (mm)							
		$ZnPO_4$	SE %	ZnO	SE %	ZnS	SE %	K	SE %
1	ZnSB-1	10	42	14	100	0	0	0	0
2	ZnSB-2	9	50	14	27.2	0	0	0	0
3	ZnSB-3	9	28	12	20	0	0	0	0
4	ZnSB-4	12	33	8	33.3	0	0	0	0
5	ZnSB-5	15	25	0	0	0	0	0	0
6	ZnSB-6	11	37.5	0	0	0	0	0.6	50
7	ZnSB-7	8	33	0	0	0	0	0.5	66
8	ZnSB-8	7	16	19	137.5	0	0	0.6	100
9	ZnSB-9	15	50	0	0	0	0	0	0
10	ZnSB-10	13	62.5	0	0	0	0	0	0
11	ZnSF-1	36	63	65	140.7	0	0	0	0
12	ZnSF-2	30	66.6	63	142.3	0	0	0	0
13	ZnSF-3	21	10.5	0	0	0	0	0	0
14	ZnSF-4	54	315	59	247	0	0	0	0
15	ZnSF-5	42	162	58	152	0	0	0	0
	C.D	1.479		1.179				0.075	



Graph 1: Screening of potassium releasing isolates on Alexondrov's medium containing potassium alumino silicate as a potassium source



Graph 2: Quantitative estimation of available potassium by using flame photometer



Graph 3: Quantitative estimation of available zinc by using AAS on 7th, 14th and 21st day after inoculation

ZnSF-4 has showed the maximum solubilization zone of 54 mm and least solubilization zone observed in ZnSB-8 (7 mm) on $ZnPO_4$ supplemented medium. The maximum solubilization efficiency was observed with ZnSF-4 having 315 % and minimum solubilization efficiency was found in ZnSF-3 (10 %). On zinc oxide (ZnO) supplemented medium zinc solubilizing isolate ZnSF-1 was recorded with the maximum solubilization zone of 65 mm and lowest solubilization was observed in ZnSB-4 (8 mm), and no solubilization zone was found with the isolates ZnSB-5, ZnSB-6, ZnSB-7, ZnSB-9, ZnSB-10 and ZnSF-3. Maximum solubilization efficiency was found in ZnSF-4 with 247 % and minimum solubilization efficiency was observed in ZnSF-3 (20 %). On zinc sulphate supplemented medium no isolates was found to be positive for soulubilization.

So far, only bacterial species belong with species of *Bacillus* spp and *Pseudomonas* spp were reported to be zinc solubilizer as they form a clear halo zone (Simine *et al*, 1998 and Saravanan *et al*., 2003). It was found in Supernatant analysis by GC MS, identified large amounts of 2-ketogluconic acid which is the main cause of solubilization (Fehmida *et al.*, 2002). The presence of the insoluble metal compounds was not necessary to stimulate acid production. Other important factors a!ecting solubilization in the aqueous phase include pH, inorganic anions which can form salts of low solubility with metal ions and organic ligands that form complexes with metal ions (Kanopa and Zakharova, 1999). Solubilization of zinc phosphate by *Pseudomonas fluorescens* has also been reported (Simine *et al.*, 1998). Similarly, several fungi including *Penicillium simplicissimum* have been shown to solubilize zinc oxide (Franz *et al.,* 1991; Franz *et al.,* 1993).

Screening of zinc solubilizing isolates for potassium releasing ability

The zinc solubilzsers were screened for potassium releasing ability on alexondrov's medium, results revealed that there was only 3 isolates given positive for potassium releasing ability among 15 isolates. The isolates ZnSB-6 and ZnSB-8 has shown the maximum solubilization zone of 6 mm followed by ZnSB-7 (5 mm). Maximum solubilization efficiency was recorded by ZnSB-8 with the efficiency of 100 % followed by ZnSB-7 (66 %), and with minimum solubilization efficiency was found in ZnSB-6 (50%). The zinc solubilizers were very poor solubilizers of potassium releasers when compared to zinc solubilizers on potassium releasing capacity (Table 1).

The results of the present study were concordance with the findings of Bagyalakshmi *et al.* (2014). Studies conducted by Han *et al.* (2006) shown that increased availability of P and K in soil, the uptake of N, P and K by shoot and root, and the growth of pepper and cucumber when the application of integrated rock P with inoculation of PSB.

Broth assay for estimation of total available zinc by using AAS

The total available zinc in broth assay was found more during the 21st day after inoculation (1.12 to 2.62 $\mu g\ ml^{-1}$) followed by 15th (0.74 to 2.17 $\mu g\ ml^{-1}$) and 7th day (0.74 to 2.04 $\mu g\ ml^{-1}$). Among the isolates ZnSB-9 and ZnSB-8 released maximum amount of Zn from zinc phosphate (2.62 $\mu g\ ml^{-1}$) and minimum solubilization found in ZnSB-3 (0.86 $\mu g\ ml^{-1}$), whereas in zinc oxide supplemented broth the total available zinc was found more in the 21st day (0.84 to 2.84 $\mu g\ ml^{-1}$) followed by 15th (0.25 to 2.53 $\mu g\ ml^{-1}$) and 7th day (0.21 to 1.28 $\mu g\ ml^{-1}$). Among the isolates ZnSF-4 released maximum amount of Zn from zinc oxide (2.84 $\mu g\ ml^{-1}$) and minimum was found in ZnSF-5 (0.84 $\mu g\ ml^{-1}$) (Graph:3).

The results showed a varied solubilization potential that were found among the isolates in ZnO and $ZnCO_3$ containing media. This might be related to differences in genomics and plasmid properties of strain that affected by the location from which they were isolated. All strains showed higher solubilizing ability in the ZnO containing medium. From the results, it is clear that available zinc levels increased with the decrease pH after 7 days of inoculation.

Dissolution of the zinc carbonate and zinc oxide may be due to production of organic acids, like gluconic acids. Gluconic acid, and its 2 and 2,5-keto-derivatives, are produced by fungi, such as *Penicillium luteum* and *Aspergillus niger,* and bacteria belonging to *Pseudomonas* or related genera as a result of an external oxidative pathway effective on glucose and other aldose sugars (Whiting *et al.,*1976; Babu-Khan *et al.,* 1995; Williams *et al.,* 1996). The gluconic acid is subsequently taken up by transport systems of the cell and utilized by cellular metabolic pathways therefore the external oxidation of glucose usually produces only transient increases in the concentration of gluconic acid. The zinc phosphate solubilization by *Pseudomonas fluorescens* was investigated by Simine *et al.,* (1998). They found that gluconic acid and 2 ketogluconic acids produced in the culture broth helped in the solubilization of the zinc salts. Acidic environments, such as those of the present investigation, revealed that the expected mechanism by which gluconic acid is able to dissolve insoluble metal compounds is mainly by acidification.

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