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Solubilization of Insoluble Zinc Compounds by Different Microbial Isolates in Vitro Condition

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ABSTRACT: *Lab experiment was carried out to evaluate the zinc solubilizing ability of different microorganisms using zinc oxide, zinc carbonate and zinc phosphate in both plate and broth media assays. Seven microbial strains and 0.1% of each chemical source in three replications were used. Colony and halo diameters were measured after incubating the plates for 72h in incubator. Zinc solubilizing ability of microbial strains in three replications was studied with ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$ solutions in broth assay. Solubilization potential was assessed both qualitatively and quantitatively under in vitro conditions. The laboratory stock cultures (Burkholderia cepacia, Burkholderia cenocepacia, Pseudomonas fluorescence, Pseudomonas striata, Trichoderma viridae, Trichoderma harzianum and Bacillus megaterium) were selected based on the zinc solubilizing zone formation. Results indicated that the, by plate assay Trichoderma viridae formed significantly highest colony diameter (2.33 cm) and halozone diameter (4.10 cm) with zinc carbonate amended media. Pseudomonas striata form highest clearing zone (2.03 cm) by zinc carbonate amended media. Solubilization efficiency (237.77%, 216.07%) and solubilization index (3.36, 3.16) of Burkholderia cenocepacia and Pseudomonas striata were indicated maximum solubility in zinc oxide amended media. In Broth culture assay, Maximum zinc solubilization (458 mg lit^{-1}) was observed with the Trichoderma viridae in zinc carbonate amended media compared to control (140.0 mg lit^{-1}). Maximum reduction in pH was recorded in Pseudomonas fluorescence (3.95) with zinc carbonate amended media compared to control (5.78).*

Key words: *Clearing zone, Solubilization, Zinc, Zinc solubilizing microorganisms.*

INTRODUCTION

Zinc is an essential micronutrient required for growth and metabolism of microorganisms and plants. Zinc is present in the enzyme system as co-factor and metal activator of many enzymes. 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. Many bacterial enzymes contain zinc in the active centre or in a structurally important site. Bacteria can contribute to metal immobilization by several processes such as precipitation and adsorption as reported by Bapiri *et al.* (2012). Zinc occurs in soil as sphalerite, olivine, hornblende, augite and biotite. However, availability of Zn from these sources is guided by many factors among which biochemical actions of rhizomicro-organisms play an important role in converting such unavailable sources into available ones as discussed by Desai *et al.* (2012) Numerous microorganisms especially those associated with roots, have the ability to increase plant growth and productivity. In soil, both macro and micronutrients undergo a complex dynamic equilibrium of solubilization and insolubilization that is greatly influenced by the soil pH and microflora and that ultimately affects their accessibility to plant roots for absorption as noted by Altomare *et al.* (1999).

The organic based zinc nutrition is best since its Zn use efficiency is more. A bacterial based approach was devised to solve the micronutrient deficiency problem. The basic principle behind this approach is decreasing the pH to 5 or below and making zinc soluble and as a consequence the available zinc will get increased in the soil system. The term called zinc solubilizing bacteria was coined for those bacteria that are capable of solubilizing the insoluble zinc compounds or minerals in agar plate as well as in soil as reported by Sarathamble *et al.* (2010). Exogenous application of soluble zinc sources similar to fertilizer application has been advocated to various crops. This

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causes transformation of about 96-99 per cent of applied available zinc to various unavailable forms. The zinc thus made unavailable can be reverted back to available form by inoculating a bacterial strain capable of solubilizing it. Since, zinc is a limiting factor in a crop production as Saravanan *et al.* (2003) concluded. Thus, keeping this in the view present study was undertaken for "Assessing solubilization potential of different insoluble zinc sources by using zinc solubilizing microorganisms in plate as well as broth assay.

MATERIAL AND METHODS

Microbial strains and culture conditions

The laboratory stock cultures (*Burkholderia cepacia, Burkholderia cenocepacia, Pseudomonas fluorescen, Pseudomonas striata, Trichoderma viridae, Trichoderma harzianum, Bacillus megaterium,*) were selected based on the zinc solubilizing zone formation and were procured from Central Research Institute for Dry land Agriculture (CRIDA), Hyderabad, Department of Plant Pathology, Vasantrao Naik Marathwada Krishi Vidhyapeeth, Parbhani and All India Network Project on Soil Biodiversity-Biofertilizers. The solubilization potential was evaluated both qualititively and quantitavely under in-vitro condition.

***Plate assay*:** The isolates were inoculated into modified PKV medium (ingradients g l^{-1}), (glucose-10.0 g; glucose-10.0 g; ammonium sulphate-1.0 g; potassium chloride-0.2 g; dipotassium hydrogen phosphate-0.2 g; magnesium sulphate-0.1g; Yeast-0.2 g; distilled water -1000 ml, pH 7.0) as given by Bapiri *et al.* (2012) containing 0.1% insoluble zinc compounds (ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$). The test organisms were inoculated on these media and incubated at 28°C for 72 hours in dark and were examined for pH changes and Zn solubilization visualized by the formation of halos around colonies.. All glassware was soaked for 1 hr in 0.1 M HNO_3 and rinsed three times in distilled deionised water prior to use. Zinc solubilization efficiency (SE) was calculated. Solubilization Efficiency (SE) is the ratio of total diameter i.e. clearance zone including bacterial growth and the colony diameter as described by Poonam Sharma *et al.* (2014).

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

The solubilization index (SI) was calculated using the following formula : {Sadiq [9]}

$$\text{Solubilization Index} = \frac{\text{Colony diameter} - \text{Halozone diameter}}{\text{Colony diameter}}$$

Broth assay

The microbial isolates were inoculated separately to basal medium supplemented with 0.1% insoluble zinc compounds. The solubilization of zinc from laboratory grade ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$ by isolates was assessed. Modified PKV medium was prepared, splitted in 100 ml aliquots in 250 ml Erlenmeyer flasks and 0.1% of these chemicals were added and steam sterilized for 30 minutes in autoclave. Then the flasks were inoculated with 0.1 ml suspension of the test culture. Three flasks were maintained with an uninoculated control for each treatment. Experiments were done in triplicate. The samples were withdrawn after 3 days, centrifuged to remove the debris and cells. One ml of this solution was directly fed to Atomic Absorption Spectrophotometer (AAS) to determine the available Zn content.

Determination of pH

The pH of the isolates culture filtrates and the uninoculated samples were determined after 3 days of inoculation. The culture was filtered using Whatman No.42 filter paper. The pH was measured using pH meter.

Statistical analysis: All statistical tests were done by FCRD design and significant differences was calculated at $P \leq 0.05$.

RESULTS AND DISCUSSION

Zinc solubilization by microbial inoculants in solid medium

In plate assay, all the eight strains of microbial isolates produced a clear solubilization halo on PKV medium supplemented with three insoluble Zn compounds used (ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$) at 0.1% zinc concentration. The diameter of colony and solubilization halo produced by different microbial inoculants at 0.1% insoluble Zn is presented in Table 1 Solubilization of zinc compounds was higher in *Trichoderma viride* than in other strains and the diameter of colony and halo zone was high when $ZnCO_3$ was supplemented. *Trichoderma viride* obtained the highest potential in $ZnCO_3$ containing medium, producing a colony diameter 2.33 cm and halozone diameter 4.10 cm. Its performance in zinc oxide was 1.93 cm and 3.80 cm. The mechanisms of acquisition of zinc by these strains from insoluble zinc compounds might be a consequence of proton extrusion and

Table 1
Solubilization of insoluble zinc compounds (per cent) in solid medium by different microbial inoculants

	Zinc sources at 0.1%					
	Colony diameter(cm)			*Halo zone diameter (cm)*		
Microbial Inoculants	*ZnO*	$ZnCO_3$	$Zn_3(PO_4)_2$	*ZnO*	$ZnCO_3$	$Zn_3(PO_4)_2$
T_1 : *Burkholderia cepacia*	1.56	1.10	1.40	2.46	1.66	2.20
T_2 : *Burkholderia cenocepacia*	1.50	1.16	1.70	3.53	1.50	2.20
T_3 : *Pseudomonas fluorescence*	1.60	1.50	1.80	3.25	2.13	2.73
T_4 : *Pseudomonas striata*	1.66	2.06	1.30	3.60	4.10	2.03
T_5 : *Trichoderma viridae*	1.93	2.33	1.40	3.80	4.10	2.30
T_6 : *Trichoderma harzianum*	1.76	1.96	1.30	3.00	3.96	2.10
T_7 : *Bacillus megaterium*	1.93	2.20	1.93	2.93	4.06	2.86
	Z	**T**	**Z x T**	**Z**	**T**	**Z x T**
SE±	0.01	0.01	0.028	0.02	0.03	0.05
CD at 5%	0.02	0.04	0.079	0.05	0.08	0.15

Table 2
Solubilization of insoluble zinc compounds (per cent) in solid medium by different microbial inoculants

	Zinc sources at 0.1%					
	Solubilization Efficiency			*Solubilization Index*		
Microbial Inoculants	*ZnO*	$ZnCO_3$	$Zn_3(PO_4)_2$	*ZnO*	$ZnCO_3$	$Zn_3(PO_4)_2$
T1 : *Burkholderia cepacia*	157.53	151.50	157.20	2.57	2.51	2.57
T2 : *Burkholderia cenocepacia*	235.77	128.47	129.40	3.36	2.28	2.29
T3 : *Pseudomonas fluorescens*	203.33	142.27	151.83	3.03	2.42	2.52
T4 : *Pseudomonas striata*	216.07	193.80	156.63	3.16	2.98	2.56
T5 : *Trichoderma viride*	196.57	175.73	164.57	2.96	2.75	2.64
T6: *Trichoderma harzianum*	169.87	201.73	161.80	2.69	3.02	2.61
T7: *Bacillus megaterium*	151.70	185.07	148.37	2.52	2.84	2.48
	Z	**T**	**Z x T**	**Z**	**T**	**Z x T**
SE±	1.04	1.59	2.76	0.013	0.019	0.034
CD at 5%	2.89	4.41	7.65	0.036	0.055	0.029

production of organic acids of microbial origin possibly in a non-specific way leading to solubilization of zinc and thereby influencing the bioavailability of zinc. The solubilization efficiency and solubilization index of each isolate based on colony diameter and halozone is presented in Table 2.

Zinc solubilizing organisms formed clear halozones on medium plates which revealed that they can solubilize ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$, the extent of solubilization varies in different isolates which is measurable. The solubilization index ranged from 2.28-3.36. Results showed that *Burkholderia cenocepacia* in ZnO amended media showed maximum solubilization efficiency and solubilization index (S.E. = 235.77 and S.I. = 3.36) followed by *Pseudomonas striata* which showed solubilization efficiency and solubilization index (S.E. = 216.07 and S.I. = 3.16). In *Pseudomonas sp.* it was 2-ketogluconic acid that mediated solubilization process (Di Simine *et al.* (1998) and Fasim *et al.* (2002).

Zinc solubilization by microbial inoculants in liquid medium: The findings were also confirmed in broth assay using the same chemicals. On evaluation under in vitro, inoculation of microbial strains produced substantially higher soluble zinc content in liquid broth as compared to uninoculated control (Fig. 1). In zinc carbonate supplemented medium, *Bacillus megaterium* and *Trichoderma viride* solubilize zinc up to 294.33 mg l^{-1} and 293.33 mg l^{-1} as compared to uninoculated control (88.33 mg l^{-1}).

The results indicate that inoculation increased soluble zinc in medium and can be ascribed to production of organic acids by microbial isolates. This might be related to different genomics and plasmid

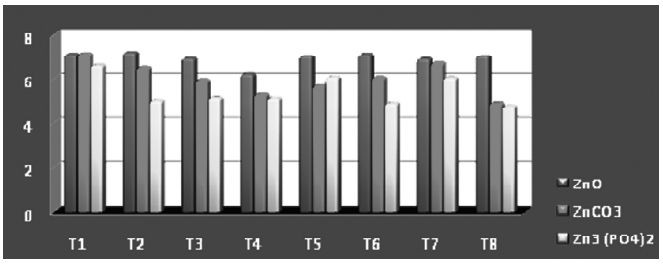

Figure 1: Solubilization of insolubal zinc compounds in broth

properties of strain that affected by the location from which they were isolated. The isolates showed higher solubilizing ability in $ZnCO_3$ containing medium which may be attributed to the fact that these strains isolated from calcareous soil, presenting a higher potential than other Zn containing chemical substrates, making their adherence with the carbonate particles capable of solubilizing zinc carbonate and also it is depend the chemical properties of $ZnCO_3$ that easier than others affected by acidic exudates of microorganisms. The results are in close agreement the findings of Bapiri *et al.* (2012).

A significant pH change was observed as compared to uninoculated control incubated for a period of 72 hours as recorded in Fig. 2. The pH ranged from 4.79-7.21. Among all isolates, *Bacillus megaterium* showed drop in pH to 4.91 from 7.12 (control) followed by *Trichoderma viridae* which showed pH 4.79 in $Zn_3(PO_4)_2$ supplemented cultures. Among the zinc compounds, solubilization of zinc was found to be higher in zinc phosphate supplemented medium as compared to zinc oxide or zinc carbonate with inoculation of *Bacillus megaterium*. Similar findings were also reported by Ramesh *et al.* (2014) and Fasim *et al.* (2002). The reduction in pH of inoculated broth medium was also correlated positively with production of organic acids.

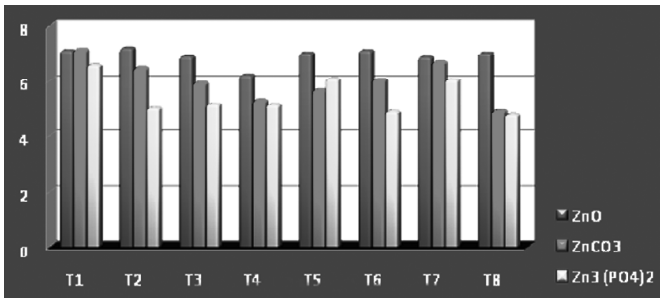

Figure 2: Effect of change in pH in broth on different microorgansms

The pH decrease observed beneath the fungal strains is unlikely to derive uniquely from the dissociation of organic acids, as reported by Martino *et al.* (2003). The results showed a varied solubilization potential that were found among the microbial isolates in ZnO, $ZnCO_3$ and $Zn_3(PO)_4$ containing media. In acidic environments, such as all the cultures showed a shift in pH towards acidic range, it gives a clue that organic acid might be involved. Thus the obtainment of an elite culture or a consortium of strains capable of utilizing different unavailable insoluble forms of zinc and tolerant to higher zinc levels may be useful to make zinc available in the soil system [Saravanan *et al.* (2003)]. Soils are naturally rich in total zinc but poor in available forms. Application of soluble zinc component to alleviate zinc deficiency in certain soils is a costly practice. Zinc in soil system is constantly changing forms and being converted to unavailable forms. This particular study gains momentum for zinc nutrition using microbes.

CONCLUSION

We isolate 7 strains that formed clearing zone in plate assay and available zinc in broth assay. Selection and inoculation of zinc solubilizing bacteria either alone in soils inherently rich in native zinc or along with cheaper insoluble zinc compounds, like ZnO, $ZnCO_3$ or $Zn_3(PO_4)_2$ will lead to lot of saving in crop husbandry, besides curtailing the expenditure on agro input.

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