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RESEARCH ARTICLE

SCREENING FOR ELITE ZINC SOLUBILIZING BACTERIAL ISOLATE FROM RICE RHIZOSPHERE ENVIRONMENT

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ABSTRACT

Zinc deficiency is one of the major constraints to rice cultivation all over the world. Hence, zinc fertilizer is applied in the soil as zinc sulphate. However the major portions of applied zinc sulphate become unavailable to plant in the soil. In the recent days, the inoculation of plant growth promoting microorganisms for improving the crop growth, yield and quality without affecting the environment is being adopted in the agriculture. There was 240 zinc solubilizing bacterial strains were isolated from rhizosphere of rice. Based on the performance of bacterial strains, there were 15 isolates found to be potential zinc solubilizer. The ZOB-6 bacterial isolate showed the maximum zone of solubilization zone (16.5mm) with ZO supplemented medium when compared to other isolates. Similarly, the ZCB-13 produced maximum clearing zone of (12.5) mm in zinc carbonate supplemented medium. The ZOB-6 and ZCB-13 zinc solubilizing bacterial isolates were confirmed as species of Acinetobacter species and Acinetobacter calcoaceticus.

INTRODUCTION

Among different nutrients, zinc and phosphorus are the major nutrient in realizing sustainable productivity of the plant system (Subba Rao *et al*; Ganeshamurthy *et al*; 1994 and Singh *et al*, 2005). Zinc is an essential micronutrient for the growth in plants and it is vital to the crop nutrition as required in various enzymatic reactions, metabolic process and redox reaction. Zinc supplied to the plants as fertilizers like zinc sulphate which inturn transformed into different insoluble complexes depending on the soil types and soil chemical reaction. This makes it unavailable to the plant within the seven days of application (Rattan and Shukle, 1991). Zinc deficiency is a serious constraint to rice production in many parts of the world (Anon, 1993) and this could only be compensated by the application of costly chemical fertilizers, either as foliar or soil applications (Reyes and Brinkman, 1989). In India, the lives of more than one billion people rely on diets predominately containing cereals (rice and wheat), pulses and oil seeds that are lacking sufficient Zn for adequate nutrition (Prasad, 2010). It has been estimated that more than 50% of the Indian soils are zinc deficient (Singh *et al*, 2005) but zinc is predominant in the soils of semiarid tropical regions of India. The crop and soil management practice mine large amounts of zinc from native pool of soil. In India, the major cause for increased zinc deficiency is the adoption of intensive cultivation, imbalanced nutrient application generally without zinc and dispensing with organic manures and the very nature of soils predominated with high pH, calcareous and low in organic matter content (Behera *et al*, 2011). Alternately, numerous microorganisms especially those associated with roots can increase plant growth and productivity (Okan *et al*, 1985; kloepper *et al*, 1998; Yanni *et al*, 2001; Rodriguiz *et al*, 2004) by increasing the supply of nutrients of low mobility in the soil like P, Zn, and Cu (Cunningham and Kuiack *et al*, 1992; Tarafdar and Marschner *et al*, 1994; Goldstein *et al*, 1995; Thompson *et al*, 1996; Bashan *et al*, 2004). Several studies have also documented solublization of insoluble Zn compounds by bacteria (Disimine *et al*, 1998 and Fasim *et al*, 2002). This zinc thus made unavailable can be reverted back to available by inoculating a bacterial strain capable of solubilizing it (Saravanan *et al*, 2003). In this context, the uses of beneficial rhizosphere microorganisms as bio-inoculants to increase availability of native zinc to crop assimilation and achieve the objective of low-input and sustainable agriculture (He *et al*, 2010).

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from rice rhizosphere from 5 locations (Kurinchippadi, Bhuvanagiri, Sethiyathoppu, Mettuppalayam, & Aalappakkam) of Cuddalore district of Tamilnadu, India. The samples were selected randomly during the stage of standing crop and it was transported to laboratory, shady dried, powdered and stored at 4^{o}C for further study.

Processing of soil samples

Serial dilution technique was used for isolating the zinc solubilizing bacterial strain. 1.0 gm of rice rhizosphere

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Table 1 Diameter of colony and diameter of holozone formed by Zinc solubizing bacterial isolates from rhizosphere soil of rice.

Isolates	Diameter (mm) of colony and holo zone at 6th day of inoculation			
	Zinc oxide		Zinc carbonate	
	Colony diameter	Holo zone Diameter	Colony diameter	Holo zone Diameter
ZOB-1	4.4	7.5	3.8	4.8
ZOB-2	4.6	6.2	3.6	5.4
ZOB-3	5.3	7.4	4.2	6.7
ZOB-4	4.2	6.3	5.5	9.2
ZOB-5	6.2	12.08	5.3	8.1
ZOB-6	9.7	16.5	6.6	12.4
ZOB-7	7.3	15.1	4.2	7.4
ZOB-8	6.4	14.3	4.9	8.2
ZOB-9	8.5	12.3	3.5	5.0
ZOB-10	4.6	6.8	4.1	6.3
ZCB-11	2.8	3.6	3.9	5.8
ZCB-12	4.5	5.3	5.7	8.3
ZCB-13	5.6	6.5	7.8	12.5
ZCB-14	4.4	5.25	5.3	11.9
ZCB-15	5.4	6.1	6.1	10.3

Data represents mean value of three determinations

Samples was suspended in 90 ml of sterile distilled water in test tube. From the first dilution 1ml was transferred to 9ml of sterile distilled water in test tube to get 10^{-3} dilution. The same method was followed for preparing up to 10^{-6} dilution. The Tris Mineral salts medium containing Dextrose-10.0g, (NH4)2 SO4- 1.0gm, KCl- 0.2gm, K_2HPO_4- 0.1gm, $MgSO_4$-0.2gm Distilled water-1000ml and pH-7.0 was prepared (Saravanan *et al*, 2007). The source of insoluble zinc compounds ZO and ZC (0.1%) and agar was added into the medium & autoclaved at 121°C for 30mins. The sterile agar medium was poured in sterile petri plates under aseptic condition. After solidification 0.1ml from 10^{-4}, 10^{-5}, and 10^{-6} dilutions were taken by sterile pipette, transferred and spread onto petri plates. The inoculated plates were incubated at 27-30°c for 48 hrs.

Table 2 Soluble zinc Content in Tris –minimal liquid broth supplemented with zinc oxide and zinc carbonate on 4th, 8th and 16th day after inoculation of selected zinc solubilizing bacterial isolates.

Isolates	Soluble Zinc content (mg/l-1 broth)					
	Zinc oxide			Zinc carbonate		
	4th day	8th day	16th day	4th day	8th day	16th day
ZOB-6	8.4	13.5	38.3	5.4	12.3	27.6
ZOB-7	6.7	10.0	32.5	3.9	8.6	22.0
ZCB-13	4.2	9.6	24.6	5.2	13.4	26.3
ZCB-15	4.5	8.3	18.0	4.4	7.8	16.8

Data represents mean value of three determinations

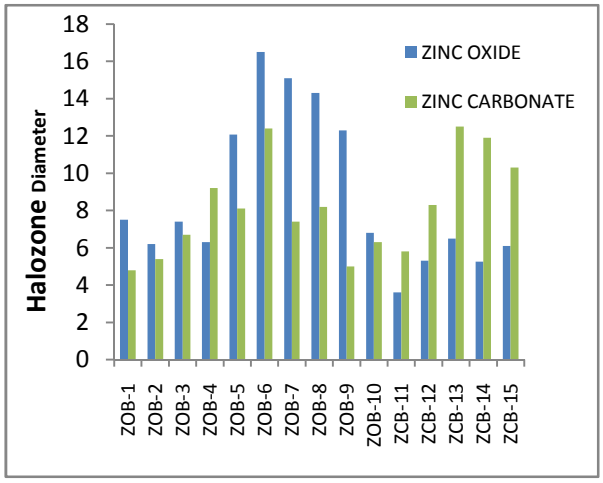


Fig 1 Diameter of colony (CD) and diameter of holozone (HD) formed by Zinc solubizing bacterial isolates from rhizosphere soil of rice.

Screening for Zinc solubilizer

After the incubation period the grown individual colonies exhibited halozone around the colony from 10^{-4}, 10^{-5}, and 10^{-6} dilution were transferred into nutrient slants. The pure cultures were used for zinc solublization test, and biochemical characterization.

Identification of Zinc Isolates

Characterization of bacterial strains

Colony morphological futures of bacterial isolates were observed by culturing zinc solubilizing bacterial isolates after 48 hours of spreading on liquid mineral salts agar medium in petri plates. The capsule staining, cell motility, spore formations were observed. A loop full of bacterial culture was transferred on glass slide with a drop of sterile water and observed under light microscope for recording cell morphology.

Bacterial identification

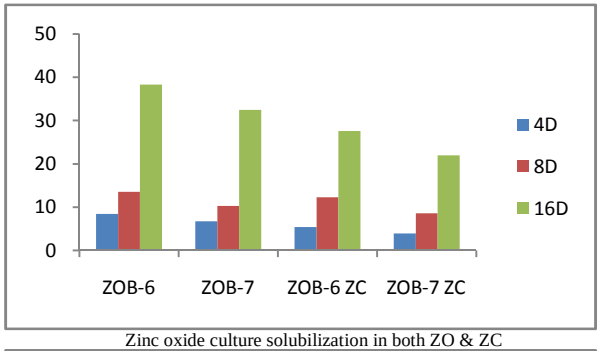
Genomic DNA was extracted from selected isolates previously characterized by conventional methods of biochemical test by modified phenol: chloroform procedure of Sambrook and Russel (2001). The primers specific for 27 region of 16S rRNA as Forward primer and 1492 sequence as Reverse primer were used for amplification. Each 50 µl of polymerase chain reaction (PCR) reaction mixture contained: 10µg of total DNA, 10mM mM of each dNTP, 5 unit of Taq DNA polymerase (Invitro- gen, USA) in 5 µl of 10x Taq buffer, 1 mM MgCl2, 5mM of each primer, and 38-µl sterile deionized water. The thermal cycling program for amplification consisted of 1 cycle of 95°C for 4 min (Denaturation), 56°C for 1 min (Annealing), and 72°C for 1 min (Extension), 34 cycles of 95°C for 1 min, 56°C for 30 sec, and 72°C for 1min; and a Final elongation cycle of 72°C for 7 min. The sequence data of the 16S rDNA was compared with sequences in the National Center for Biotechnology Information data bank using the BLAST program.

Solubilization Index of bacterial isolates

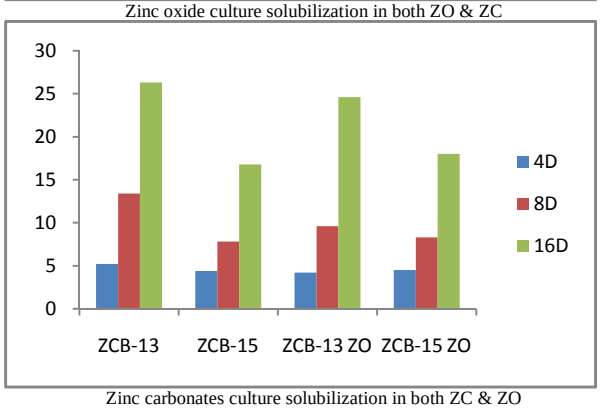
Sterilized mineral salts agar medium was poured into sterile petri plates. After the solidification of medium a loop full of screened bacterial isolates were inoculated onto the plates individually under aseptic condition. The inoculated plates

were incubated at 28°c for 72hrs. The diameter of bacterial colony and halozone around colony was measured and the values were calculated using solubilizing index formula:

SI= Colony diameter + Halozone diameter / colony diameter.
SI = solubilizing index



Zinc oxide culture solubilization in both ZO & ZC



Zinc carbonates culture solubilization in both ZC & ZO

Fig 2 Soluble zinc Content in Tris –minimal liquid broth supplemented with zinc oxide and zinc carbonate on 4th, 8th and 16th day after inoculation of selected zinc solubilizing bacterial isolates.

Invitro Zinc Solubilization Assay

The quantum of zinc solubilized by the bacterial culture was determined using Atomic adsorption Spectrophotometer (AAS) at 4th, 8th and 16th day of incubation. In the broth assay, the selected bacterial isolates were grown in 100ml of mineral salts medium broth supplemented with insoluble source of zinc oxide and zinc carbonate at 0.1% concentration at 28°c in an orbital shaker at 120 rpm. There were five replicates maintained for zinc oxide and zinc carbonate solubilizing bacterial isolates. The cultured broth was filtered through 0.2 µm filter paper then fed into the AAS and the available zinc concentration was recorded at 4th, 8th and 16th day of incubation.

RESULT AND DISCUSSION

There were 240 bacterial colonies showed holozone in Tris mineral salt medium supplemented with zinc oxide (ZO) and zinc carbonate (ZC) picked from medium and maintained in nutrient agar slants. Among the total bacterial isolates, 185 and 55 were obtained from ZO and ZC supplemented medium respectively. From the 240 bacterial isolates, 15 were selected based on their zinc solubilizing halozone and the number of isolated was further reduced to 4 which exhibited high zinc solubilizing halozone in both ZO and ZC supplemented medium for zinc solubiliztion assay using AAS. The selected isolates were named as ZOB and ZOC which isolated using ZO and ZC supplemented tris mineral salt medium respectively with serial number from 1 to 15.

Based on the diameter of colony and halozone of selected 15 bacterial isolates (Table.1), the potential isolates ZOB-6, ZOB-7, ZCB13 and ZCB15 were selected for determining their zinc solubilizing capacity in broth culture (Table.2). All these isolates were able to form a clear halo zone with varying size on both the two zinc compounds included in this study. In the present investigation, the ZOB-6 bacterial isolate showed the maximum colony diameter (9.7mm) and highest Zinc solubilizing zone (16.5mm) in ZO supplemented medium when compared to other isolates. But among the 15 bacterial isolates, the ZCB-13 produced maximum colony diameter (7.8mm) and highest clearing zone of (12.5 mm) in zinc carbonate supplemented medium. The bacterial isolates ZOSB-6 and ZCSB-13 exhibited better value both in colony diameter and halozone diameter in ZO as well as ZC supplemented medium.

Among the effective isolates ZOB-6, ZOB-7, ZCB-13 and ZCB15, the ZOB-6 and ZCB-13 significantly increased the availability of zinc in tris mineral salt liquid medium supplemented with zinc oxide and zinc carbonate at 16th day of incubation respectively as compared with the other isolates (Table 2). So far, only bacterial species belong with species of Bacillus and Pseudomonas were reported to be zinc solubilizer as they form a clear halo zone (Di simine *et al*, 1998; Sachdev *et al*, 2010 and Saravanan *et al*, 2003). The molecular characterization for ZOB-6 and ZOB-13 confirms as species of *Acinetobacter sp* and *Acinetobacter calcoaceticus* respectively. The zinc solubilizing bacterium ZOB-6 showed the maximum value of available zinc in broth at 4th, 8th and 16th day of incubation both in ZO and ZC supplemented growth medium followed by ZCB-13, ZOB7 and ZCB-15.M

Summary

The artificial application of zinc as fertilizer in crop production increases cost of cultivation. The available Zn in soil in different form may be unavailable to plants, but it can be made available to them by inoculating zinc solubilizing bacterial species as an inoculant. From the present investigation, based on growth efficiency and zinc solubilizing potentiality, among the 240 zinc solubilizing isolates ZOSB-6 and ZCSB-13 were selected as elite PGPR for making availability zinc to rice crop from unavailable zinc sources such as zinc oxide and zinc carbonate. Even though the two bacterial stains showed better results in laboratory level, further it should be tested by conducting field experiments on rice to confirm their performance and also for recommendation to farmers. Selection and inoculation of zinc solubilizing bacteria either alone in soil inherently rich in native zinc or along with cheaper insoluble zinc compounds, like zinc oxide and zinc carbonate, will lead to lot of saving in crop cultivation. In this present situation, isolation and identification of effective PGPR for improving crop growth, yield and quality as well as saving soil fertility is being important one in sustainable crop production.

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