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Manasa SG
M. Sc. (Agri), Department of Agricultural Microbiology, AC Raichur, UAS, Raichur, Karnataka, India

Mahadevaswamy
Prof. and Head, Department of Agricultural Microbiology, AC Raichur, UAS, Raichur, Karnataka, India

Nagaraj M Naik
Asst. Prof., Department of Agricultural Microbiology, AC Raichur, UAS, Raichur, Karnataka, India

Ramesh Y
Asst. Prof., Department of Agronomy, AC Raichur, UAS, Raichur, Karnataka, India

Gundappagol RC
Prof. Department of Agricultural Microbiology, AC Kalaburgi, UAS, Raichur, Karnataka, India

Correspondence
Manasa SG
M. Sc. (Agri), Department of Agricultural Microbiology, AC Raichur, UAS, Raichur, Karnataka, India

In vitro screening of the isolates for zinc solubilization and growth promoting attributes

Manasa SG, Mahadevaswamy, Nagaraj M Naik, Ramesh Y and Gundappagol RC

Abstract
In our present study, screening was carried out for the Zinc solubilizing ability of bacteria isolated from the rhizosphere of rice. All the 40 isolates subjected for screening for their efficiency to produce solubilization zone around the colonies on TRIS-minimal agar plates supplemented with 0.1% ZnO. Out of 40 bacterial isolates, two of them produced highest zone of solubilization which were notified as MZSB- 8 and MZSB- 6 with a hallow zone of 21 mm and 19 mm diameter respectively. In the broth assay, total zinc in the media was found to be solubilized by MZSB8 on the 21st day *i.e.* 17.675 ppm. Later, the isolates were examined for their plant growth influencing characters *viz.*, production of IAA, siderophore and phosphate solubilization for which MZSB8 and MZSB6 showed maximum results.

Keywords: Zinc, ZnO, rhizosphere, TRIS-minimal agar medium, solubilization, etc.

Introduction
Zinc (Zn) is said to be an essential element required for the growth and development of plants and animals. It is recognized as one of the eight essential micronutrients required for crop growth and production (Alloway, 2004) [1]. It is present up to 0.008% of earth's crust. Zinc assumes a si in metabolic activities of both eukaryotes and prokaryotes which acts as a cofactor for activation of different enzyme systems (Hughes and Poole, 1989) [8]. Zinc plays a vital role in the human immune system as well. The optimum dietary intake of zinc for human adults is 15 mg per day. Zinc is an essential part of the active catalytic center of the enzyme carbonic anhydrase, which increases the rate at which equilibrium is achieved between CO_2 and bicarbonate ions in solution. (Rains, 1976) [14]. It extends great impact on basic life processes of plants such as metabolism and absorption of N_2 and quality of the protein; photosynthesis and chlorophyll synthesis, resistance to abiotic and biotic stresses and protection against oxidative damage (Potarzycki and Grzebisz, 2009) [13]. The inadequate supplement of Zn to the plant harms the yield quantity and production quality. Thus, for the proper growth and development of plants, a certain amount of Zn has to be supplied.
Although plants require relatively smaller concentrations in tissues for healthy growth, zinc deficiency is found to reduce membrane integrity and carbohydrates synthesis, auxins, nucleotides, cytochromes, chlorophyll and develops a susceptibility to heat stress (Singh *et al.*, 2005) [21]. It undergoes various modifications in the soil and precipitates with other elements, which finally affects their availability to roots for absorption. The Indian soils are low in zinc content; however, the total zinc content is considerably high, but it gets fixed in unavailable forms such as smithsonite ($ZnCO_3$), sphalerite (ZnS), zincite (ZnO), franklinite ($ZnFe2O_4$), willemite (Zn_2SiO_4), and hopeite ($Zn_3(PO_4)_2 \cdot 4H_2O$). Consequently, large amount of zinc fertilizers is required to be added to the soil to meet out the requirement of zinc to the plants. The strategy to improve the availability of zinc to the crops to avoid zinc fertility constraints in TBP region requires practical, ecologically sound, efficient, and cost-effective alternatives. It is admirable that zinc mineralizing and solubilizing bacteria can aid in resolving the major issue. Zinc-solubilizing microorganisms can make available the zinc from inorganic and organic sources that can be utilized by plants to increase the zinc content. Some bacterial species of the genera *Acinetobacter*, *Bacillus*, *Gluconacetobacter*, and *Pseudomonas* have been reported (Sharma *et al.*, 2011) [20].
The present study was conducted with an objective including the selection of effective zinc solubilizing bacterial isolates with multiple beneficial traits and development of inoculants with commercialization potential, a major challenge hindering the bio-inoculants production technology. Such an isolate will increase the bioavailability of zinc to plants, promote the nutrient recycling and increase the quantity of zinc in the crop which could considerably increase the growth and yield, simultaneously contributing to the ecosystem functioning.

Material and methods

Zinc solubilizing bacteria were isolated from collected soil samples by serial dilution followed by plating on TRIS minimal agar medium containing 0.1% ZnO. The media plates were incubated at room temperature for 3 days and the colonies exhibiting clear zones were selected, purified by four-way streak plate method.

Screening for zinc solubilization (plate assay)

The isolated zinc solubilizing isolates were spot inoculated on to the TRIS minimal agar plates and incubated in 30 ºC for 3 days and the observations were noted. The diameter of zone of solubilization was measured and expressed in centimeter and the selected isolates were preserved on agar slants for further use.

$$\text{Solubilization efficiency} = \frac{\text{Solubilization diameter}}{\text{Diameter of colony growth}} \times 100$$

After analyzing the results of the plate assay, isolates which showed the better results were subjected to further experimental studies such as quantitative estimation (broth assay) and PGPR characterization.

Quantitative estimation of zinc solubilization (Broth assay)

Analysis of insoluble zinc solubilization in a liquid medium by the bacterial culture was determined quantitatively by following the protocol of Fehmida *et al.,* (2002) [6] using Atomic absorption Spectrophotometer (AAS). The selected bacterial isolates were grown in 100 ml of mineral salts medium broth supplemented with insoluble source of zinc oxide at 0.1% concentration and they incubated at the temperature of 28 ºC in an orbital shaker at 120 rpm. The cultured broth was collected at 7 days interval, filtered through Whatman No. 42 filter paper and centrifuged at 10,000 rpm for a period of 10 minutes. The culture supernatant was fed directly to the Atomic Absorption Spectroscopy for the determination of soluble zinc content. The amount of zinc solubilized was obtained by subtracting the soluble Zn of the inoculated sample from the un-inoculated control and expressed as ppm culture.

Elucidation of beneficial traits

The bacterial isolates showing efficacy in Zinc solubilization were further used in elucidating the mechanisms of growth promotion, such as siderophore production, production of IAA, phosphate solubilization, organic acid production as given below.

Indole acetic acid (IAA) production

IAA production potential of bacterial isolates was tested in nutrient broth supplemented with 0.1 concentration tryptophan at 28 ^{0} C. The culture broth after 3 days of incubation was centrifuged at 5,000 rpm for 5 min and concentration of IAA was analyzed by spectrophotometric method using Salkowaski's reagent. One ml of supernatant was mixed with 1 ml of Salkowaski's reagent (2 ml of 0.5 M $FeCl_3$ + 98 ml 35 percent HCl) and the intensity of red colour developed within 30 min was read at 530 nm using spectrophotometer. The concentration was evaluated by using a standard curve prepared by standard solution of indole acetic acid.

Siderophore assay

Siderophores assay was conducted by referring to the CAS shuttle assay of Payne (1994) [11]. The culture extract (0.5 ml) was mixed with 0.5 ml of CAS reagent. The colour obtained was measured using the spectrophotometer at 630 nm after 20 min of incubation. The blank was prepared using uninoculated broth medium. The Siderophore content in the aliquot was calculated by using the following formula.

$$\text{Siderophore unit (\%)} = \frac{\text{As} - \text{Ar}}{\text{As}} \times 100$$

Where, As =Absorbance of the sample at 630 nm (CAS reagent) and
Ar =Absorbance of the reference

Phosphate Solubilization

For this test sterilized Pikovskaya's agar medium was poured as a thin layer on to the sterilized petri plates and the plates were incubated for a period of 24 hr. After incubation, bacterial isolates were spot inoculated on the Pikovskaya's plates (Pikovskaya, 1948) [12] and incubated at 28 ± 1 °C for 4 days. Formation of translucent white hallow zone around the colony was scored as a positive result for phosphate solubilization.

$$\text{PSE (Phosphate Solubilization Efficiency)} = \frac{\text{Z}}{\text{C}} \times 100$$

Where, Z- Clearance zone including bacterial growth
C- Colony diameter

Organic acid production

One ml of every isolate that was 24 hours old culture was inoculated to 25 ml of TRIS minimal broth and incubated at a temperature of 28 ± 2 °C for a period of 10 days. The broth culture was then centrifuged at 10,000 rpm for a period of 10 minutes. The supernatant that was obtained was concentrated to approximately to 1/10th of the original volume in a water bath at 60 °C. The concentrated sample was utilized for the determination of organic acid by paper chromatography in comparison with standard organic acids (Gaur, 1990) [7].

Results and discussion

Screening for zinc solubilization

The cultures were placed at the center of the TRIS minimal agar plates. After 3 days of incubation at 28 ± 2 $^{\circ}$C, plates were seen with clear solubilization zone. The solubilization zones were ranging from 8 mm to 21 mm. The isolate MZSB-8 has shown the maximum solubilization zone of 21 mm followed by MZSB-6 (19 mm). The solubilization efficiency ranged from 384.6% to 109.85%. The maximum solubilization efficiency was shown by MZSB-8 with an efficiency of 384.6% followed by MZSB-6 (295.48%) and minimum solubilization efficiency was found in MZSB-26 (109.85%). Such observation was made earlier that among the insoluble zinc compounds ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$ found to solubilize readily (Saravanan *et al*., 2006 and Sarathambal *et al*., 2010) [18, 17].

Quantitative estimation of zinc solubilization

In the broth assay, the isolates were tested for their efficiency to solubilize Zn using the Atomic Absorption Spectroscopy. The total available zinc was recorded highest on the 21st day (17.675 to 12.447 ppm) followed by 14th (13.434 to 7.232 ppm) and 7th day (3.090 to 1.630 ppm) as in table 6. Among the isolates MZSB-8 released maximum amount of Zn from zinc oxide (17.675 ppm) followed by MZSB- 6 (16.920 ppm), MZSB-3 (14.530 ppm), MZSB-4 (13.620 ppm), MZSB-9 (13.630 ppm), MZSB-7 (13.593 ppm), MZSB-2 (13.290

ppm), MZSB-1 (13.130 ppm), MZSB-5 (12.650), MZSB-10 (12.447 ppm) and reference strain about 15.217 ppm on 21st day. The increase in the amount of zinc content was progressive from 7th to 14th and 21st days after incubation. The results agree with the findings of Desai *et al.* (2012) [5]. They reported that *Pseudomonas sp.* was capable of solubilizing ZnO in appreciable amounts. Thus, the potentiality of bacteria to release Zn largely depends on various parameters like nature of the zinc compounds, carbon source, temperature, pH, the variable concentration of sodium chloride and glucose (Shahab and Ahmed, 2008) [19]. The variability among the isolates indicates the significance of isolation of zinc solubilizing bacteria from different locations and exploration of possible mechanisms of the zinc solubilization. Similarly, Venkatakrishnan *et al.* (2003) [23], Azadeh *et al.* (2012) [3] showed the solubilization of Zn by the isolates.

Elucidation of plant growth-enhancing traits

Plant root colonizing rhizobacteria can also function as detrimental, deleterious rhizobacteria (DRB) or beneficial, Plant Growth Promoting Rhizobacteria (PGPR). PGPR colonize the roots of both monocots and dicots, thus influence the plant growth by various direct as well as indirect mechanisms. Alterations of root architecture indicate the production of phytohormones and other sequestering molecules by PGPR that cause enhancement and proliferation of the roots and root hair cells.

Indole Acetic Acid (IAA) production

When the isolated zinc solubilizing bacteria were grown in culture medium supplemented with tryptophan, the isolates produced IAA as detected by the Salkowaski's reagent under spectrophotometer. All the isolates produced IAA. It ranges between 4.340 µg ml^{-1} and 8.21 µg ml^{-1} as in table 3. A significantly higher concentration of IAA was observed from MZSB 8 (8.21 µg ml^{-1}) followed by MZSB 6 (7.92 µg ml^{-1}) when compared to reference strain which had 7.18 µg ml^{-1}. Lowest observations were recorded in MZSB 2 (5.44 µg ml^{-1}). IAA which plays important role in apical dominance, phototropism, gravitropism, prevention of leaves and fruit abscission and induction of adventitious root system. Similar results were observed by Mohite (2013) [10]; Madhuri (2011) [9] and Sadaf *et al.* (2009) [16] with different isolates.

Table 1: Screening of Zinc oxide solubilizing bacteria on TRIS minimal medium by plate assay method

S. No	Isolates	Zinc oxide solubilization		Solubilization efficiency (%)
		Solubilization zone (mm)	Culture diameter (mm)	
1	Reference strain	18.43	7.27	253.50
2	MZSB 1	13.40	6.53	205.20
3	MZSB 2	11.63	5.93	196.12
4	MZSB 3	12.83	6.47	198.29
5	MZSB 4	16.75	7.47	224.23
6	MZSB 5	10.70	5.47	195.61
7	MZSB 6	19.00	6.43	295.48
8	MZSB 7	14.60	7.46	195.71
9	MZSB 8	21.00	5.46	384.61
10	MZSB 9	10.33	5.43	190.23
11	MZSB 10	10.33	5.46	189.19
12	MZSB 11	10.73	6.46	166.09
13	MZSB 12	11.73	8.46	138.65
14	MZSB 13	11.08	6.46	171.51
15	MZSB 14	9.27	5.46	169.78
16	MZSB 15	8.50	4.46	190.58
17	MZSB 16	14.13	9.46	149.36
18	MZSB 17	15.26	11.30	135.04
19	MZSB 18	15.93	9.46	168.39
20	MZSB 19	16.66	9.46	176.10
21	MZSB 20	10.03	6.76	148.37
22	MZSB 21	14.13	8.43	167.61
23	MZSB 22	8.16	5.46	149.45
24	MZSB 23	10.13	7.66	132.24
25	MZSB 24	13.00	10.46	124.2
26	MZSB 25	11.13	8.50	130.94
27	MZSB 26	7.36	6.70	109.85
28	MZSB 27	12.10	7.46	162.19
29	MZSB 28	14.20	9.46	150.10
30	MZSB 29	10.33	7.43	139.03
31	MZSB 30	14.96	10.43	143.43
32	MZSB 31	10.73	7.43	144.41
33	MZSB 32	15.93	9.43	168.92
34	MZSB 33	15.03	8.43	178.29
35	MZSB 34	9.167	6.06	151.27
36	MZSB 35	10.01	7.43	134.72
37	MZSB 36	14.33	8.43	169.98
38	MZSB 37	10.30	5.43	189.68
39	MZSB 38	11.33	6.43	176.20
40	MZSB 39	15.16	9.43	160.76
41	MZSB 40	11.36	6.43	176.67

Table 2: Quantification of Zinc oxide solubilization capacity under *in vitro* conditions with Atomic absorption spectrophotometer (AAS)

S. No.	Isolate	Amount of total available zinc (ppm)		
		7 DAI	14 DAI	21 DAI
1	MZSB1 (YRG-2)	2.310	8.500	13.130
2	MZSB2 (MLB-1)	2.490	9.830	13.290
3	MZSB3 (KLM-3)	2.610	9.890	14.530
4	MZSB4 (KVT-1)	1.920	9.850	13.620
5	MZSB5 (GVT-2)	1.630	8.400	12.650
6	MZSB6 (MNT-1)	3.056	12.170	16.920
7	MZSB7 (NMV-2)	2.075	8.945	13.593
8	MZSB8 (MNV-1)	3.090	13.434	17.675
9	MZSB9 (GVT-4)	1.825	9.727	13.630
10	MZSB10 (TNP-1)	1.834	7.232	12.447
11	Reference strain	2.406	10.439	15.217
12	Control	0.200	0.200	0.200

Siderophore production

All the isolated zinc solubilizing bacterial formed orange halo zone surrounding the colony on dark blue coloured Chrome Azurol S agar plates indicating the production of siderophore. Further, the siderophore production potential of these isolates was determined quantitatively based on the CAS shuttle assay of Payne (1994) [11]. The values range between 53.33% and 76.733% as in table 3. The isolate MZSB-8 produced significantly higher siderophore of 76.733% followed by isolate MZSB- 6 that produced 72.367% and these were significantly superior to the reference strain (67.80%). The results obtained were in agreement with Sreedevi *et al*. (2014) [22], who obtained maximum siderophore production of 94, 88 and 83% for *Pseudomonas* 1, *Pseudomonas* 2 and *Pseudomonas* 3 isolates, respectively. Similar results were also reported by Bruno *et al*. (2015) [4]; Veronique *et al*. (2015) [24]; Sabrina *et al*. (2014) [15]; Anand and Kulothungan (2010) [2].

Phosphate solubilization

Ten bacterial isolates were able to solubilize phosphate on Pikovskaya's media containing tricalcium phosphate in the range of 4.46 mm to 12.3 mm which is depicted in Table 9. Among ten bacterial isolates MZSB-8 showed the highest solubilization zone (12.3 mm) followed by MZSB-6 (10.3 mm) and which have solubilization efficiency of 332.4% and 251.2% respectively when compared with the reference strain which had solubilization zone of 9.3 mm and solubilization efficiency of 206.6%. Production of organic and inorganic acids and release of phosphatase enzyme by the isolates hydrolyse the phosphate from tricalcium phosphate in the media, which loses the white colour to become colourless and results in formation of hallow zone around the colonies.

Organic acid production by the isolates

The organic acids profile of the ten isolates of zinc solubilizers were analysed by descending chromatography. All the isolates found to produce one or the other organic acid tested. It was noticed that the gluconic acid was observed to be most common organic acid produced by all the ten isolates and reference strain. The other organic acids produced by the isolates include malic acid by five strains (MZSB-1, MZSB-2, MZSB-7, MZSB-8, MZSB-9), succinic acid by two strains (MZSB-9, MZSB-10) citric acid was two strains (MZSB-1, MZSB-3), oxalic acid by eight strains (reference strain, MZSB-1, MZSB-2, MZSB-3, MZSB-6, MZSB-7, MZSB-8, MZSB-9) and tartaric acid by three strains (reference strain, MZSB-4, MZSB-5, MZSB-6). The mechanism for solubilization of insoluble zinc compounds was by the action of organic acids like gluconic acid (Fehmida *et al*., 2002) [6].

Table 3: Production of indole acetic acid by zinc solubilizing bacteria

S. No.	Strains	IAA (µg ml^{-1})	Siderophore Production (%)
1	Control	0	1.79
2	Reference strain	7.18	67.80
3	MZSB1 (YRG-2)	4.81	55.67
4	MZSB2 (MLB-1)	5.44	61.47
5	MZSB3 (KLM-3)	6.56	63.33
6	MZSB4 (KVT-1)	5.54	60.07
7	MZSB5 (GVT-2)	5.67	64.53
8	MZSB6 (MNT-1)	7.92	72.367
9	MZSB7 (NMV-2)	4.58	62.867
10	MZSB8 (MNV-1)	8.21	76.733
11	MZSB9 (GVT-4)	4.340	53.333
12	MZSB10 (TNP-1)	4.76	58.067

Table 4: Screening for phosphate solubilization by Zinc solubilizers on Pikovosky's medium

S. No.	Isolate	Phosphate solubilization		Solubilization efficiency (%)
		Solubilization zone (mm)	Culture diameter (mm)	
1	Reference strain	9.3	4.5	206.6
2	MZSB1 (YRG-2)	6.4	4.6	139.1
3	MZSB2 (MLB-1)	7.3	5.6	130.3
4	MZSB3 (KLM-3)	5.4	4.1	131.7
5	MZSB4 (KVT-1)	7.4	5.5	134.5
6	MZSB5 (GVT-2)	7.2	4.5	160.0

7	MZSB6 (MNT-1)	10.3	4.1	251.2
8	MZSB7 (NMV-2)	7.4	4.7	157.4
9	MZSB8 (MNV-1)	12.3	3.7	332.4
10	MZSB9 (GVT-4)	7.5	4.3	174.4
11	MZSB10 (TNP-1)	4.46	3.2	139.3

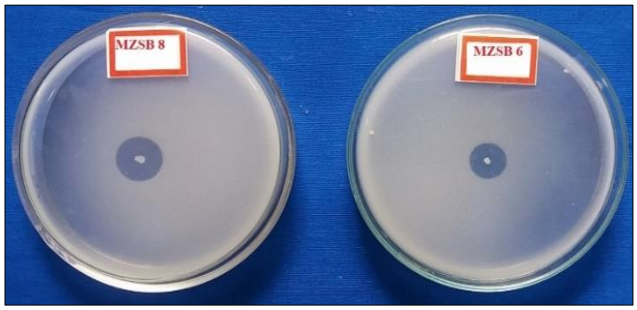

Fig 1: Zn solubilization by the efficient isolates *viz*., MZSB 8 and MZSB 6.

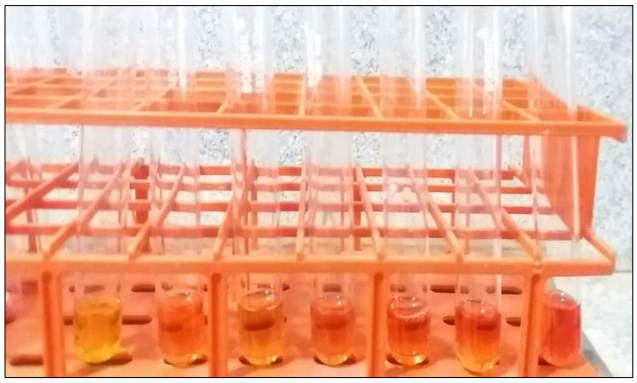

Fig 2: IAA production test: zinc solubilizing bacteria with Salkowski's reagent.

Conclusion

Zinc deficiency in plants could impair their biological activities, thus developing efficient zinc solubilizing bio-inoculants would be of higher significance. In our experiment, we came up with two efficient bacteria which can solubilize greater amount of unavailable form of zinc. They also possess plant health influencing traits, which can help in increasing the plant growth and yield.

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