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Isolation and Molecular Characterization of a Potential Zinc Solubilizing Bacteria for Sustainable Agriculture

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Abstract—*Zinc is one of the eighth essential trace elements required for the normal growth and reproduction of crop plants. Most of the zinc in soil exists in unavailable forms held in iron and manganese oxides. Further the major essential macronutrients for plant growth and development are also present in unavailable forms in the soil. Soil contains plant growth promoting rhizobacteria that are responsible for mineral solubilization and thus making them avaible. The present investigation was conducted to isolate a potential bacteria that can solubilize Zn, P and K besides fixing atmospheric nitrogen. The zinc solubilizing bacteria (ZSB) were isolated from rhizospheric soil . The ZSB were further tested for P and K solubilization on medium containing TCP and Mica powder. Since the solubilization of zinc might limit the growth of the bacteria at higher level, therefore the ability of selected isolate to tolerate solubilized zinc was determined under different concentrations of soluble zinc ($ZnSO_4$). The isolate showing maximum Zn solubilization (60.43%) and having PSE (30%) and KSE(13%) was molecularly characterized as Bacillus sp by 16 S rDNA sequencing.*

1. INTRODUCTION

Most of the zinc in soil exists in unavailable forms. Soil contain 2-25 ppm of exchangeable and organic zinc with a larger portion held in iron and manganese oxides and other non available forms. Crops generally take up less than 0.5lb/a of zinc, yet when zinc is deficient, crop yields are reduced markedly. It is required in relatively small concentrations in plant tissues (5-100/kg).In severely deficient zinc soils, wheat germination is poor and Zinc can substantially improve seed germination and seedling vigor. Zinc concentrations in the grain is inherently very low and the major reason for widespread occurrence of zinc deficiency problems in crop plants is due to low solubility of zinc in soils rather than a low total amounts of zinc. Therefore, the addition of zinc fertilizers has become a common practice in modern agriculture. However, 15-20% of the soluble inorganic zinc applied to soil as fertilizer is rapidly immobilized by the iron and aluminium ions in acidic soils and by calcium ions in calcareous soils soon after application, thus becoming unavailable to plants . Zinc plays a very beneficial role in proper metabolism and growth functions such as auxin metabolism, which influences the activities of dehydrogenase and carbonic anhydrase, synthesis of cytochrome and the stabilization of ribosomal fractions.. Zinc is transferred in the form of Zn2+ in plants and is an essential nutrient that has particular physiological functions in all living systems, such as the maintenance of structural and functional integrity of biological membranes and facilitation of protein synthesis and gene expression, enzymes structure, energy production and Krebs cycle. The use of ZSB as bioinoculants has gained momentum in the recent years as they play a vital role in maintaining the soil nutrient status, structure and sustains the production base. However information on the isolation and characterization of zinc solubilizing bacteria having simultaneously P solubization , K solubilization and nitrogen fixing capacity is scanty.Isolation of a bacterium that can provide essential macronutrients- NPK to the plant along with the essential micronutrient. Zn to the plant may prove a boon to the agriculture.

2. MATERIALS & METHODS

2.1 Collection of rhizosphere samples and isolation of zinc solubilizing bacteria

Zinc solubilizing bacteria (ZSB) were isolated rhizosphere samples collected from a wheat grown in Faridabad district of Haryana. Intact root systems along with adhered soil from the plants were collected. The samples were stored in plastic bags at low (4ºC) temperature until processed. ZSB were isolated by dilution plating using appropriate dilutions on Bunt & Rovira (BR) medium containing zinc oxide. The probable isolates showing halozone around colonies were picked up initially and rechecked for Zn-solubilization on above medium.

2.2 Determination of zinc solubilization activity of ZSB isolates

Zn-solubilization by ZSB isolates was assessed on Bunt and Rovira media. The culture broth (40µl) of the isolates having

approximately 10^8 cfu/ml was spotted on the BR plates for determination of the Zn-solubilization efficiency. The plates were incubated at 30ºC for 2 days and diameter of colony as well as halozone was measured. Zn-solubilizing efficiency (ZSE) was calculated as: ZSE (%) = Z-C/C x 100 where, Z = halozone diameter, C = Colony diameter. The pH of the ZSB culture filtrates and the uninoculated samples was also determined after filtering the culture using Whatman No.1 filter paper.

2.3 Determination of Zinc tolerance by ZSB isolates

The ability of selected isolate to tolerate solubilized zinc was determined under in vitro condition in nutrient broth containing different concentrations of soluble zinc ($ZnSO_4$). The nutrient broth was prepared and splitted in 10 ml aliquots in test tubes. ZnSO4 was incorporated into the broth in such a way that the final concentration of zinc was 10, 20,30,40,50, 100,150 and 200 mg kg-1. These solutions were divided in 10 ml quantities in test tubes, sterilized at 15 psi for 15 min and inoculated with 0.1 ml of ZSB. An uninoculated control was also maintained. The total ZSB population was assessed by plating on nutrient agar media. The growth of bacteria in the zinc containing medium indicated their tolerance to zinc. The highest concentration at which poor growth was observed was taken as tolerance level.

2.4 Screening of the ZSB isolates for P solubilization and K solubalization zone

P-solubilization by ZSB isolates was assessed on PVK media and K solubalization on modified Aleksandrov medium. (5.0 g Glucose, 0.5 g $MgSO_4.7H_2O$, 0.1g $CaCO_3$, 0.006 g $FeCl_3$, 2.0 g Ca_3PO_4, 3.0 g insoluble mica powder as potassium source and 20.0 g agar) . The culture broth of the isolates having $\simeq 10^8$ cfu/ml was spotted on the PVK plates for determination of the P-solubilization efficiency. The plates were incubated at 30ºC for 2 days and diameter of colony as well as halozone was measured. solubilizing efficiency was calculated as : SE (%) = Z-C/C x 100 where, Z = halozone diameter, C = Colony diameter. Detection of potassium solubilization by different rhizobacterial isolates was based upon the ability of solubilization zone formation.

2.5 Estimation of growth regulators produced by ZSB

All the ZSB isolates were subjected to qualitative analysis for the production of Indole acetic acid (IAA) and Gibberlic acid (GA). The overnight grown cultures of ZSB isolates were spotted on Luria agar plates supplemented with 0.06 per cent sodium dodecyl sulphate and one per cent glycerol. The spotted plates were overlaid immediately with sterile disc of Whatman No.1filter paper. After 4 days of incubation period, the filter paper discs were removed from the plates and treated with Salkowaski reagent. Bacteria producing IAA were identified by the formation of characteristic red halo around the colony on filter paper. Salkowski reagent consisted of a mixture of 15 ml 0.5 M FeCl3, 500 ml distilled water,and 300 ml of concentrated H,S04 sp. gr. 1.84. The paper discs after treatment with Salkowaski's reagent were viewed under UV light. The spots giving typical green fluorescence were taken as positive for GA production.

2.6 Molecular characterization of PSB

Molecular characterization of PSB was done using 16S rDNA specific primers .

3. RESULT, OBSERVATIONS & CONCLUSION

3.1 Isolation

3.1.1 Isolation and screening of zinc isolubilizing bacteria from rhizosphere of wheat

A total of 5 samples were collected .The samples were serially diluted and count of total bacteria, ZSB and types of ZSB were determined using Bunt and Rovira medium. The probable isolates showing halo zone around the colonies were assumed to be zinc solubilizers. A total 10 ZSB were finally selected. All the 10 ZSB isolates spotted on BR medium plates keeping approximately same cell number in the culture broths and incubated at 30±2ºC for 2 days. Their colony as well as halo zone diameter was measured and Zn-solubilization efficiency (ZSE) was calculated. Zn solubilization by isolates varied from 7.14% - 60.43% (Table 3.1)

3.1.2 Zn tolerance for the selected isolates

Zinc is a nutrient at low concentration but toxic at higher concentration. The solubilization of zinc might limit the growth of the bacteria at higher level. Unless the cultures tolerate a higher level of zinc its solubilization may not continue. Therefore the ability of selected isolate to tolerate solubilized zinc was determined under *in vitro* condition in nutrient broth containing different concentrations of soluble zinc (ZnSO4) table(3.2) showed that most of the isolates were able to grow upto 100 ppm of ZnSO4.

3.1.3 Screening of isolates for other growth promoting attributes

The isolates were screened for the solubilization of P, K, Nitrogen fixation, IAA and GA production. The results showed that the PSE varied from 4 – 60% with the reduction in ph. The highest P solubilization (60%) was observed by ZSB2 Similarly KSE varied from 4%- 20% with some strains showing no solubilization at all. Some of the strains produced IAA and GA, however most of the strains showed little or no production.

3.1.4 Molecular characterization of the selected isolate

The isolate showed 98% homology to *bacillus thuringensis*

3.2 Conclusion

The amount of available Zn is very low in soil inspite of high level of total Zn. Application of zinc containing fertilizers to

the soil is essential to maintain adequate level of soluble Zn for better plant growth. However, only 15 to 20 per cent is utilized by the crops and rests of it get fixed in the soil. The fixed Zn is released very slowly and is not readily available to plants. Enhancing Zn availability to crops through inoculation of zinc solubilizing bacteria (ZSB) holds promise in the present situation as zinc fertilizers are quite expensive, cause pollution, get fixed in the soil. The ZSB are known to mobilize the unavailable Zn in the rhizosphere and make it available to crops. These bacteria have been isolated and enumerated from different sources such as tannery (Fasim et al), rhizosphere of plant etc. The seed inoculation studies have shown positive effects of these microorganisms on crop yield. Extensive studies have been done on the isolation of ZSB and their role in crop production, but the information available on isolation of bacteria that can simultaneously provide the major macronutrients is scanty. The present investigation was aimed at the isolation of zinc solubilizing bacteria isolated from the rhizosphere of wheat. Having a bacterial strain that can provide micro as well as macronutrients will certainly strengthens the crop improvement strategy with ZSB as bioinoculants.

In the present investigation ZSB were isolated by dilution plating on BR medium and evaluated for Zn- solubilization on solid medium containing zinc oxide. In the past also, efforts were made to identify zinc solubilizers with varying abilities. The demonstrated variation in the ability of solubilizing given zinc sources could be due to metabolic activity of a given strain which is in agreement with observations of Sadaf and Nuzhat(2008). There are different mechanisms of solubilization which have been identified including proton excretion, production of organic acids and other chelating metabolites (Agnihorti, 1970). Organic acid production by microbial strains has been reported to be a major mechanism of solubilization (Nguyen et al., 1992; Fasim et al., 2002). The zinc solubilization in our studies could be due to production of organic acids, like gluconic acids that is augmented by the fall in pH of culture media noted in all cases. Zinc phosphate solubilization by a strain of *P. fluorescens* was investigated by Di Simine et al. (1998) and it was observed that supplementing medium with zinc sulphate resulted in more gluconic acid production. Most of the isolates decreased the pH of the medium which could be assigned to the release of organic acids by ZSB (Mishra, 1985 and Nahas, 1996).

The isolate that showed maximum zinc solubilization was identified as *Bacillus thuringiensis* on the basis of the 16 S rDNA sequencing using primers BAC 27F and BAC1378R. This strain showed TCP solubilization, K solubilization, and were also able to solubilize insoluble Zn sources as well as able to fix atmospheric nitrogen . However, the isolate showed little IAA production. Desai et al 2012 isolated Azotobacter , Azospirillum , Bacillus and Pseudomonas strains from diverse crop production systems and evaluated for solubilization of ‘Zn’ and ‘Pi’ in vitro from insoluble zinc (ZnO, $ZnCO_3$) and phosphorus [tri-calcium phosphate (TCP)], respectively. After 15 days of incubation, 15 strains solubilized zinc under solid and liquid culture conditions. *Pseudomonas* strain released highest available 'Pi' and solubilization of Zn and Pi corresponded with fall in pH of the medium. *Azospirillum* strains; *Bacillus* strains; and *Pseudomonas* strains solubilized both Zn and P sources showing their ability to supplement both essentials.

Table: 3.1 Zn-Solubilization efficiency(ZSE) on Bunt and Rovira medium (containing zinc oxide), P- solubilization efficiency(PSE) of the isolates on PVK media, K-solubilization efficiency(KSE) of the isolates on Aleksandrov medium containing mica powder, Screening of the isolates for the production of IAA and GA.

Isolate No.	ZSE (%)	PSE (%)	KSE(%)	IAA Production	GA production	pH for Zn	pH for p
ZSB1	35.00	9.50	4.16	+	+	5.10	5.67
ZSB2	30.20	60.38	0.00	-	-	6.73	4.40
ZSB3	60.43	14.39	0.00	+	-	4.35	6.40
ZSB4	43.66	22.20	6.66	+	-	5.50	6.28
ZSB5	34.29	12.50	10.00	+	-	5.36	5.67
ZSB6	9.50	0.00	5.33	-	+	4.90	3.10
ZSB7	10.00	4.29	11.10	-	-	5.60	6.25
ZSB8	15.38	56.66	20.00	-	-	4.80	5.40
ZSB9	0.00	5.11	9.50	-	-	6.80	5.72
ZSB10	7.14	11.10	13.33	-	+	5.95	6.27

Table 3.2: Zinc tolerance ability of isolated bacterial strains

Conc of Zinc	Name of isolates									
SNO.	ZSB1	ZSB2	ZSB3	ZSB4	ZSB5	ZSB6	ZSB7	ZSB8	ZSB9	ZSB10
10Mm	+	+	+	+	+	+	+	+	+	+
20Mm	+	+	+	+	+	+	+	+	+	+
30Mm	+	+	+	+	+	+	+	+	+	+
40Mm	+	+	+	+	+	+	+	+	+	+
50Mm	+	+	+	+	+	+	+	+	+	+
60Mm	+	+	+	+	+	+	+	+	+	+
70Mm	+	+	+	+	+	+	+	+	+	+

80Mm	+	+	+	+	+	+	+	+	+	+
90Mm	+	+	+	+	+	+	+	+	+	+
100Mm	+	+	+	+	+	+	+	+	+	+
150Mm	-	-	+	-	-	-	-	-	-	-
200Mm	-	-	+	-	-	-	-	-	-	-

+/- Presence or absence of growth

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