Physiological and biochemical characterization of phosphate utilizing and zinc tolerant endophytic bacteria for indigenous rice variety of Jharkhand.

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Abstract

Plant growth-promoting bacteria with the ability to tolerate heavy metals have importance both in sustainable agriculture and phytoremediation. The present study reports on the isolation and characterization of mineral phosphate-solubilizing (MPS) and Zinc tolerant endophytic bacteria associated with three different indigenous rice i.e *Arsunga Gora (AG) Kala Jira (KJ) and Karhani(KH)*. Out of 24 bacterial isolates, 5 isolates, namely R2, R12, P16, R17 and R23, with high MPS activity were selected and subjected to the assessment of MPS activity under ZnSO4 stress conditions. MPS activity by the selected isolates was observed at concentrations of as high as >1.1 M ZnSO4. Significant improvement in plant growth was observed on bacterization of seeds (rice) with all of the five selected isolates. R23 was the best in terms of phosphate-solubilizing activity and its response to various stresses. The ability of R23 and other isolates to exhibit MPS activity at high ZnSO4 concentrations suggests their potential as efficient biofertilizer for growing plants in metal (ZnSO4)-contaminated soil.

Keywords: Diazotrophic bacterial endophytes, plant growth promoting activity, *p*-solubilization, indigenous rice varieties, Zinc tolerant.

Introduction

Phosphorus is the second most limiting nutrient for plant growth. A major portion of the phosphorus present in soil is usually in the immobilized form and thus unavailable for assimilation by plants [1]. Only 1–5% of the total soil phosphorus is available in a soluble form that can be utilized by plants [2]. This necessitates the application of phosphate (P) fertilizer for attaining high growth and yield of plants. However, extraneously added phosphorous fertilizers get converted into salts in soils and become insoluble by forming a complex with cations like Ca, Al and Fe [3]. Moreover, excessive application of fertilizers leads to economic and environmental problems. Therefore, application of microorganisms capable of mobilizing P from the bound form is considered to be a potential biofertilizer for sustainable agriculture in a cost-effective and eco-friendly manner [4–6]. Several bacterial genera including *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobac terium*, *Erwinia* and *Klebsiella* are known to possess P solubilization activity [7]. The basic mechanisms considered for P solubilization are mediated by organic acid (chiefly gluconic acid) production, ammonium ion assimilation or phytase activity [7]. The P solubilization ability of microorganisms has been employed for improving crop yield in agriculture and horticulture [8]. In order to exploit P-solubilizing bacteria as biofertilizer, bacteria should possess the ability to compete with other members of the soil microflora and to tolerate local stress. Exploration of P-solubilizing bacteria with the above properties will be of immense importance in sustainable agriculture practice. The composition of soil and other environmental factors can severely affect plant growth-promoting (PGP) bacteria [9]. Toxic levels of heavy metals including zinc in the soil restrict the growth of both plants and associated bacteria by altering their cellular metabolism. Although the availability of zinc in the soil is essential for supporting cellular growth, high concentrations of zinc can lead to an inhibition of the growth and maintenance of microbial cells. A decrease in the metabolic activity of bacteria and other organisms in the presence of zinc has been reported in previous studies [10]. However, certain bacteria have evolved one or several mechanisms to tolerate the uptake of heavy metal ions, which enables them to survive in metal contaminated area. The role of PGP bacteria capable of showing enhancement of plant growth in certain metal-contaminated soils has been demonstrated in the recent years [11]. Many areas in Jharkhand and other states of India are heavily contaminated with toxic amounts of zinc due to mining and other human activities, which affects the soil microflora as well as plants growing in that region [10]. Moreover, the soil of Jharkhand faces other abiotic stresses like salt and high temperature, which restrict vegetation in these areas. In the present study, we report on the isolation and characterization of P-solubilizing bacteria associated with the *Oryza sativa* which grows in soil rich in zinc compounds. Application of such bacteria will be of great importance in agricultural practice and also in bioremediation of soil.

MATERIALS AND METHODS

Sources of Endophytic Diazotropic Bacteria for Phosphate solubilization

Indigenous rice varieties Arsunga Gora, Kala Jira and Karhani were chosen for isolation of endophytic diazotrophic bacteria. The selection of these rice varieties for isolation of endophytic bacteria and their important features of the selected varieties and their cultivation pattern are given in Table 1.

Place	Location of the rice field	Latitude and longitude	Rice variety	Land type	Importance
State-Jharkhand Country- India	Village- kolang District- Gumla	22.95^{0}N 84.57^{0}E at an average elevation of 620m	Arsunga Gora, Kala Jira, Karhani.	All the varieties are upland variety but can be grown in low land also and the varieties selected are mostly sown by broadcasting method.	Oldest known rice which can be stored up to 50 years without the use of any other preservatives Used in the preparation of tribal drink Hadia which is used to cure jaundice. The Hadia prepared from Karhani is known as medicinal Hadia

Table 1. The Endophytic bacteria were isolated from indigenous rice grown by the indigenous people of Kolang Village of Gumla District, Jharkhand and, important features (based upon the TK of tribal farmers of the region) of the selected varieties and their cultivation pattern are given. (All the information are based on the local tribal knowledge)

Isolation of putative endophytic diazotropic bacteria for *P* solubilisation

Endophytic bacteria were isolated from different parts of selected rice plant such as root and employing standard microbiological methods. Method of Barraquio et al., (1997) was used for isolation (Yadav and Dadarwal 1997). The roots/stems of selected plants were thoroughly washed with tap water to remove all the soil particles before subjecting them to surface sterilization. One gram of roots/stems was taken and washed in triple distilled water (TDW) with sterilized glass beads by vigorous shaking for 3 hr. The plant materials were then dipped in 70% (v/v) alcohol for 3 min followed by three times washings with TDW to remove alcohol. Then they were treated with chloramine-T 1% (w/v) for 15 min and washed thrice with TDW. The last wash was stored and 100 µl of it was plated on NA (nutrient agar) to ensure complete surface sterilization The surface sterilized roots and culms were crushed separately in mortar with pestle and suspended in 1 ml PBS (phosphate buffer saline). The suspension was diluted to 10-10 and 100 µl from each dilution was spread on nitrogen-free JNFb- solid agar-agar medium to enrich N2-fixing population only. Colonies showing distinct morphological characters were selected and re-cultured on JNFb- agar medium. All the isolates showing growth on JNFb- solid medium were also grown in semi-solid (0.15% agar-agar) JNFb- medium to test the pellicle formation. Appearance of pellicle formation, if any, was observed after 48 hr of growth at 30^{0}C.

Screening and analysis of P solubilization activity

Isolates were spot-inoculated on National Botanical Research Institute's phosphate (NBRIP) growth medium containing (g/l): glucose, 10; Ca3(PO4)2, 5; MgCl2 · 6 H2O, 5; MgSO4 · 7 H2O, 0.25; KCl, 0.2, (NH4)2SO4, 0.1; and agar-agar, 20 [12]. They were grown at 30 °C for 4 d, followed by observation for the appearance of a clear zone around the colonies. A clear halo zone was scored as positive test for MPS. Quantitative estimation of P released by bacterial isolates was made by the *ortho*-phosphomolybdate method as described by Ames [13]. Different concentrations of K2HPO4 were used to prepare a standard. P solubilization was measured in terms of mg/ml/OD. The optical density (OD600) of the cultures was measured after adding 0.5 volumes of 1 N HCl to dissolve the residual insoluble P against a blank identically treated. The pH of the medium after P solubilization was measured after 4 d of incubation. The initial pH of the medium was adjusted to 7.0.

Evaluation of the minimum inhibitory concentration of ZnSO4

In order to test the toxicity of various concentrations of ZnSO4 on the growth of five selected isolates, the minimum inhibitory concentration (MIC) for each isolate was tested prior to the test for P solubilization activity in the presence of ZnSO4. The standard strain of *Escherichia coli* was subjected to the above test as negative control. For testing toxicity, selected isolates were grown in nutrient broth medium supplemented with 0.1–1.5 M ZnSO4 for 48 h and thereafter the OD of the culture was read at 600 nm in a spectrophotometer.

Results

Isolation of plant-associated P-solubilizing bacteria

Before isolation and characterization of bacteria, a physicochemical analysis of the rhizospheric soil was made. Analysis of the soil showed a pH at around 5.7 and the value of the Zn content was 8.2 µg/g soil. Based on morphological features 24 endophytic isolates. P-solubilizing activity, of 24 endophytic isolates were found to be positive for P solubilization activity. The results of the quantitative estimation of the P solubilization activity ranged between 0.606 and 3.828 mg P/ml in all the 25 isolates. The highest activity was recorded in isolate R23 (3.828 mg P/ml) followed by R16 (2.936 mg P/ml of culture) (Table 2). Five isolates exhibiting high MPS activity, namely R2, R12, R16, R17 and R23, were selected for further studies. Assuming P solubilization to result from the production of organic acid by the bacterial isolates, the pH of the medium was measured after growing the selected isolates in NBRIP medium for 4 d. The pH of the medium after growth in medium supplemented with tricalcium phosphate ranged between 2.8 and 4.5. Isolate R5 showed the highest drop in the pH of the medium; the lowest drop in pH was observed for R23.

Table 2. Test for P solubilization in different isolates.

Serial no.	Bacterial isolates	Phosphate utilization (µg/ml)	Serial no.	Bacterial isolates	Phosphate utilization (µg/ml)
1	R1	803.00 ± 0.78	13	R13	728.00 ± 0.23
2	R2	1845.00 ± 0.08	14	R14	682.00 ± 0.59
3	R3	704.00 ± 0.54	15	R15	814.00 ± 0.21
4	R4	903.00 ± 0.70	16	R16	2936.00 ± 0.14
5	R5	606.00 ± 0.06	17	R17	2572.00 ± 0.63
6	R6	901.00 ± 0.23	18	R18	1043.00 ± 0.17
7	R7	805.00 ± 0.83	19	R19	926.00 ± 0.62
8	R8	865.00 ± 0.28	20	R20	824.00 ± 0.49
9	R9	1132.00 ± 0.85	21	R21	798.00 ± 0.06
10	R10	1005.00 ± 0.59	22	R22	923.00 ± 0.19
11	R11	793.00 ± 0.08	23	R23	3828.00 ± 0.37
12	R12	2153.00 ± 0.32	24	R24	729.00 ± 038

The P solubilization test was made following growth of cultures in NBRIP medium. Free phosphorous was estimated after 4 days of growth.

Effect of stress conditions on P-solubilizing activity

The P-solubilizing activity of the selected isolates was assessed in the presence of ZnSO4, at 30ºC. Before conducting the test for MPS activity, the toxicity of ZnSO4 to various isolates was tested. The MIC of ZnSO4 for all the isolates ranged between 1.3 and 1.5 M. Increasing concentrations of ZnSO4 in the medium affected P solubilization to varying degrees. There was 30–73% inhibition of the MPS activity in different isolates (Fig. 1). Out of five isolates, R23 showed the highest P solubilization at a concentration of 1.5 M ZnSO4.

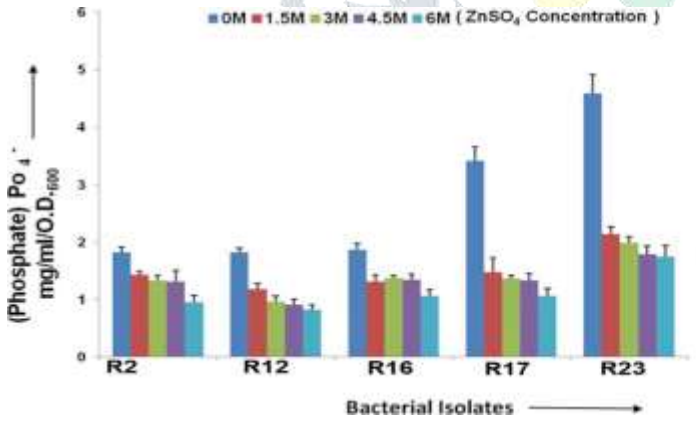

Figure 1. Effect of ZnSO4 on P solubilization (MPS) activity. Release of phosphate was estimated after 4 d of growth at 30 °C. Results are means ± SD of three experiments conducted separately in triplicate.

Discussion

Knowing that a quantitative estimation of the P solubilization activity by using the plate method is not accurate [4, 8], we used the phosphomolybdate method to avoid any ambiguity. From the results, it is evident that the MPS activity of the selected five isolates is very high (up to 3.828 mg/ml. The lower activity in the endophytic isolates may be due to the fact that these bacteria are not exposed directly to insoluble phosphates and thus have less expression of enzymes responsible for the P solubilization process. Since most of the P-solubilizing bacteria exhibit MPS efflux of H^+, a decline in the pH of the medium following P mobilization is expected to occur. This fact is supported by our results as a pH decrease was evident for all the isolates. The role of organic acids in MPS has been demonstrated in earlier studies [4]. However, a direct correlation between the rate of P solubilization activity and the drop in pH has not been reported. We also did not find a sharp decrease in the pH of the medium in the case of one isolate, i.e. R23. This indicates the

possibility of additional or alternative mechanism(s) of MPS activity, such as ammonium ion assimilation or phytase activity [7]. The importance of PGP bacteria in phytoremediation deserves special attention for reclamation of soil [14]. Among several heavy metals, zinc toxicity in soil is of great concern due to its toxic effect at higher concentrations [10]. In this study, there was a decrease in the level of P solubilization activity, but significant activity was present even at a very high concentration of ZnSO4 (1.5M). Detailed studies are required for understanding the mechanism(s) involved in conferring resistance to zinc in these isolates. The effect of zinc on P solubilization has been demonstrated in a few studies. Rajkumar *et al.* [15] reported P solubilization by *Bacillus weihenstephanensis* at 200 mg/l ZnSO4. It is presumed that zinc-tolerant phosphate-solubilizing bacteria will have the potential to stimulate plant growth in regions contaminated with zinc. Additionally, bacteria tolerant to zinc may also show resistance to other heavy metals, such as Pb, Cd and Ni, as reported in some recent studies [16, 17].

Conclusion

In conclusion, the present study reports the isolation of certain novel bacteria with PGP properties. P solubilization activity by certain isolates at high concentrations of ZnSO4 is a new finding of this study. The ability of these bacteria to tolerate metal contamination will be useful for growing plants at metal-contaminated sites. The efficiency of these bacteria to colonize roots would be beneficial for plant health as well as in phytoremediation of soil. Further studies are required to study the mechanism of zinc tolerance in these isolates. The stress-tolerant strains may serve as suitable candidates for developing microbial formulations for the growth of plants in desert-like areas that experience diverse types of abiotic stress.

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