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ISOLATION AND CHARACTERIZATION OF POTASSIUM SOLUBILIZING BACTERIA FROM FOREST SOILS OF MEGHALAYA

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ABSTRACT

The use of microorganisms for improving nutrient availability for plants is an important practice and has become necessary for sustainable agriculture. In the present study, we have isolated potassium solubilizing bacteria from forest soils of Nongkhyllem Wild Life Sanctuary, Meghalaya and characterized them for other plant growth promoting traits. Four strains were isolated by screening 63 bacterial isolates based on the halo zone produced on Aleksandrov agar plates. Out of 4 isolates, all were positive for IAA production and two were positive for siderophore production while all the isolates were found negative for HCN production and P-solubilization. All the isolates could tolerate a temperature ranging from 15p C to 35p C, however two isolates *viz*. NKC-28 and NKC-35 could grow up to 45p C. When screened for salinity tolerance, NKC-20 could tolerate up to 12% NaCl, 15% KCl and 12% $MgCl_2$. Tests for tolerance of acidity/alkalinity showed NKC-20 and NKC-28 could grow in a pH range of 5 to 11 while rest of the two could tolerate pH 5 to pH 9. These potassium solubilizing bacteria may emerge as promising alternative to enhance the potassium uptake of important crops and thereby increasing the overall crop productivity.

INTRODUCTION

Potassium is one of the paramount nutrients required for higher and sustainable productivity of crops. It is a third important plant nutrient after Nitrogen (N) and Phosphorus (P). As a consequence, potassium deficiency is becoming one of the major constraints in crop production, especially in coarse textured soils. Even in fine textured soils the available fraction is low compared to total K in them, crops do respond to K fertilization in soils with high available K.

It is fourth most abundant nutrient constituting about 2.5per cent of the lithosphere. Potassium ions serve to activate certain enzymes especially those involved in photosynthesis, respiration and in starch and protein synthesis (Hopkins et al. 1995). Moreover, opening and closure of stomatal guard cells or daily changes in the orientation of leaves are affected by potassium concentration (Shehata and El-Khawas 2003). Potassium has two roles in the functioning of plant cells. It's found to have an inevitable role in the activation of enzymes which are fundamental to metabolic processes, especially the production of proteins and sugars (Johnston 1986). Potassium is available in four forms in the soil which are K ions (K+) in the soil solution, as an exchangeable cation, tightly held on the surfaces of clay minerals and organic matter, tightly held or fixed by weathered micaceous minerals, and present in the lattice of certain K-containing primary minerals (Ahmad 2009). A significant share of soil potassium occurs in unavailable form in soil minerals such as orthoclase and microcline (K-feldspars). Inoculation with bacteria, which can improve P and K availability in soils by producing organic acids and other chemicals, stimulated growth and mineral uptake of plants (Garcia et al. 2004, Girgis et al. 2008). These microorganisms are commonly known as potassium solubilizing bacteria (KSB) or potassium dissolving bacteria or silicate dissolving bacteria. Some research has been made about the use of potassium dissolving bacteria, known as "biological potassium biofertilizer (BPF)".These bacteria are capable of decomposing alumino silicate minerals and releasing a portion of the potassium contained therein (Basak and Biswas 2009). A detailed understanding of how bacteria affect mineral dissolution rates is essential to quantify mineral weathering on global element cycling (Xiufang et al. 2006). The microbe interactions in the rhizosphere are responsible for increasing plant health and soil fertility (Ahmad et al. 2008). PGPRs have been recently used increasingly worldwide in sustainable agriculture as biological fertilizer (Yildirim et al. 2011). The use of plant growth promoting rhizobacteria (PGPR), including potassium solubilizing bacteria (KSB) as biofertilizers, was suggested as a sustainable solution to improve plant nutrient and production (Vessey 2003). PGPR have been reported to directly enhance plant growth by a variety of mechanisms: solublization of minerals such as phosphorus, production of

siderophores, HCN and IAA test. The indirect mechanism involves biological control those provide significant evidenced by increases in seedling emergence, vigor, and yield (Ahmad et al. 2008). The potassium solubilizing bacteria is used as bio-inoculant (*Bacillus mucilaginosus* AS1.153) in China (Xiufang et al. 2006). No such efforts were made in our country.

About 72% of soils cultivated in India come under medium and low availability status category of potassium. Thus 72% of soils cultivated in India definitely require external potassium fertilizer supplementation (Ramamurthy and Bajaj 1969, Rehanul 2002). The aim of the study was to evaluate the potential of the direct application of K materials and co-inoculation with KSB for the improvement of K uptake and improve the plant growth under limited K soil conditions in an uncontrolled environment. The isolates were obtained from soil samples collected from Nong Khyllem Wild Life Sanctuary and Unsau Reserve forest in Meghalaya.

MATERIALS AND METHODS

a. Isolation of Potassium solubilizing bacteria

Soil samples were randomly collected from different locations of Meghalaya forest soils of Nongkhyllem Wild Life Sanctuary. The soil sample was collected from 7-8 inches under surface and brought to laboratory using sterile polythene bags. Forest soils have huge amount of microorganisms. The soil samples were serially diluted in distilled water (10t and 10u) and processed as per the standard technique (Benson 2002). The diluted samples were plated on Tryptic soya agar medium (United States Pharmacopeial Convention 2001). A total of 63 bacterial isolates were isolated. All isolates were screened in standard Alekshandro media (Parmar and Sindhu 2013); and incubated at 37Cp for 1 week to 10 days to obtain colonies exhibiting clear zone of potassium solubilizing bacteria (KSB).

b. Colony diameter, halozone and solubilization index of Isolated KSB:

Sterilized Alekshandro media was poured into sterilized Petri plates, after solidification of the media; 20µl of culture broth was inoculated on the Petri plates under aseptic conditions. The plates were incubated at 37Cp for 7 to 10 days. Then the ability of KSB to solubilize the insoluble potassium was studied by the determination of solubilization index: the ratio of the total diameter (colony + halozone) and the colony diameter (Edi- Premono et al. 1996).

Solubilization efficiency (%SE) = (Z-C/C) ×100

Z = Solubilization zone (mm)

C = Colony diameter (mm)

c. Plant growth promoting traits

The plant growth promotion traits of bacterial isolates were evaluated. Standard protocols were followed for the estimation of indole acetic acid (IAA), K- solubilization and P-solubilization, siderophore, hydrogen cyanide (HCN) and ammonia production according to Brick et al. (1991), Schwyn and Neilands (1987), Lorck (1948) and Dey et al. (2004), respectively. Siderophore production was confirmed by observing clear halo zone formation on Chrome Azurol S medium.

Assay for indole acetic acid (IAA) production

Quantitative analysis of IAA was performed using different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 mg/mL). Bacterial cultures were grown for five days in Jensen's broth. Fully grown cultures were centrifuged at 3000rpm for 10 min. The supernatant (1ml) was mixed with 4ml of Salkowski reagent (49ml of perchloric acid, 2 ml 0.5 M $FeCl_3$ solution and finally 100ml volume make up by DW). Development of pink color indicates IAA production. Optical density was taken at 530 nm with a spectrophotometer. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA obtained in the range of 10–100 mg/mL.

Assay for potassium solubilization

For the study of estimation of potassium solubilization, cultures were grown in the Aleksandrov liquid medium and incubated for 5 days at 30 p C. After incubation 5ml broth was centrifuged at 10000rpm for 10 min. and supernatant was collected and add 5ml of sodium cobalti nitrite solution (**Sol-A:** 25g cobalt nitrite, 12.5 ml glacial acetic acid, volume make up 50ml by Distilled Water. **Sol-B:** 120g $NaNO_3$ dissolved in 220ml DW. 210 ml Sol-B mix in Sol-A) incubate for 37p C at 45 min. then centrifuged (10000 rpm at 5min), orange yellow precipitate was obtained. 10 ml conc. HCl was added in the precipitate. Blue green color was developed. Optical density was taken at 623nm with a spectrophotometer. Concentration of potassium produced by cultures was measured with the help of standard graph of KCl obtained in the range of 10–100 mg/mL.

DNA extraction from isolates

Pure cultures of potassium solubilizing bacteria were grown in nutrient broth at 28± 2 p C for 24 h and pelleted cells from 2.0 ml broth were resuspended in 500 µl SET buffer

(75 mM NaCl, 25 mM EDTA and 20 mM Tris) with 50µl SDS(10%) and 5µl protinase K(10 mg mL^{-1}). Genomic DNA was extracted as described by Pospiech and Neumann (1995). Finally the washed DNA pellet was incubated at 37p C for 25–30 min. to remove ethanol completely, and then resuspended in 50µl TE buffer. The extracted DNA was checked on agarose gel and stained with ethidium bromide.

16S rDNA gene sequencing:

16S rDNA was amplified genomic DNA of strain using primers P16S F 5'-TGGCTCAGATTGAACGCTGGCGG -3'and P16SR 5'-GATCCAGCCGCAGGTTCCCCTAC -3'.PCR amplification was carried out in 25 µl reaction mixtures containing 10 pM of each primer, 50 ng of genomic DNA, 10X Taq DNA polymerase buffer(Genei, India), 3 U of Taq DNA polymerase (Genei, India) and 2.5 mM of each dNTP. Amplifications were performed with a Thermo cycler (Sigma) at 95 p C for 5 min, followed by 35 cycles of 1 min at 94 p C, 1 min at 52.7 p C and 1 min at 72 p C with a final extension at 72 p C for 10 min. A 5µl aliquot of each amplified product was electrophoresed on 1.2 % agarose gel along with 1 kb DNA ladder as marker in 1x TAE buffer at 55 V for 45 min, stained with ethidium bromide and visualized with a UV transilluminator (Biolog).

Results

Sixty one bacteria isolates were isolated from forest soil samples of Meghalaya region. Total of four bacterial isolates were screened for potassium solublization on Aleksandrov agar plates showed the development of sharp potassium solublization zones. Further selection is based on biochemical, PGPR activity, IAA quantification, and Potassium solubilization.

All isolates were positive in IAA but negative in phosphate solubilization and HCN production, in sedriphore rest of one (NKC-28) all isolates showed positive result. All isolates have shown significant PGPR activity. IAA production ranged from 0.229 to 0.458 Ng/mL. Among all isolates, NKC-28 produced maximum IAA (0.458 Ng/mL).

Ammonia production is another important trait of PGPR that indirectly influence the plant and microorganism growth. The potassium solubilization depends upon the cobaltinitrite with potassium, followed by dissolving the precipitate in concentrate HCl and reading the color intensity by spectrophotometer. The range of potassium estimation was 0.295 to 0.956 Ng.mL but NKC-20 showed maximum solubilization. The morphological and biochemical characteristics of the potassium solubilizing bacteria are tabulated below (Table 1). In our KSB 16S region was amplified in 1400 bp under 1.2 % agrose gel.

Table1. Morphological, Biochemical and PGP traits of isolates.

Test	NKC-13	NKC-20	NKC-28	NKC-35
Sedrophore	+	+	-	-
HCN-test	-	-	-	-
P-solubilization	-	-	-	-
IAA-production	+	+	+	+
Gram reaction	-	-	+	+
Cell Shape	Round lobate	Round lobate	Round irregular	Round irregular
Pigmentation	White	Yellow	Creamy white	Creamy white
Elevation	Convex	Convex	Flat	Flat
Texture	Non-translution	Shiny-colony	Translution	Translution
Size	Large	Small-medium	Large	Large
Margin	Entire	Entire	Undulate	Undulate
Motility	+	+	-	+
Urease	+	+	-	+
Nitrate reduction	-	-	-	-
Citrate utilization	+	-	+	+
Ammonia test	+	+	+	+

Contd...

Voges-Proskauer's Test	-	+	+	+
Methyl Red Test	+	+	+	-
Indole production	-	-	-	-
Starch Hydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Gelatin Liquefaction	+	+	+	+
Insuline hydrolysis	-	+	-	-
Arabinose	+	+	-	-
Xylose	+	+	+	+
Adonitol	-	-	-	-
Rhamnose	-	-	-	-
Cellobiose	+	-	+	+
Melibiose	+	-	+	+
Saccharose	+	+	+	+
Raffinose	+	-	+	+
Trehalose	+	+	+	+
Oxidase	+	+	-	+
Catalase	-	+	+	-
Range of growth				
pH-5	+	+	+	+
pH-11	-	+	+	-
Range of temperature (^{o}C)				
4 ^{o}C	-	-	-	-
15 ^{o}C	+	+	+	+
35 ^{o}C	+	+	+	+
45 ^{o}C	-	-	+	+
Tolerance to NaCl (%)	4%	12%	2%	6%
Tolerance to KCl (%)	5%	15%	1%	10%
Tolerance to $MgCl_2$ (%)	15%	15%	1%	5%
IAA-quantitative production	0.423	0.376	0.458	0.229
K- estimation test	0.601	0.956	0.433	0.295

Catalase activity was detected in most of the bacterial isolates that may be potentially very advantageous. NKC-20 showed maximum salt tolerance viz. 12%, 15% and 15% respectively in salts like NaCl, KCl and $MgCl_2$. All isolates gave positive result in 15-35Cp temperature. Only two isolates viz. NKC-28 and NKC-35 were positive at 45Cp temperature. All isolates grew in acidic condition (pH-5) but only two isolates NKC-20 and NKC-28 grew in alkaline condition (pH-11).

DISCUSSION

Microorganisms play important role in agriculture. They transform unavailable form of nutrient to available form in soil thereby increasing its availability to crops that enhance agricultural production. PGPR strains use one or more direct or indirect mechanisms to enhance growth and health of plants. These mechanisms can be active simultaneously or independently at different stages of plant growth. Although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion (Glick 1995, Lalande et al. 1989).

In present study, beneficial bacteria isolated from Meghalaya forest region were characterized by biochemical tests and were screened for different plant growth promoting activities. Four isolates showed zone of potassium solubilizing in Aleksandrov media. IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development. It has been reported that IAA production by PGPR can vary among different species and strains, and also influenced by culture conditions, growth stage and substrate availability (Mirza et al. 2001). All isolates shown positive result in IAA production but one isolate showed high level. All the isolates were able to produce ammonia. The quantification of potassium was based on precipitation of cobalt nitrite with potassium and followed by dissolving conc. HCl. The value was detected by spectrophotometer (Mahendre et al. 2014). NKC-20 showed maximum solubilization. All the bacterial isolates except one were able to produce catalase. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. A number of studies suggest that PGPR enhances the growth, seed emergence, crop yield, and contribute to the protection of plants against certain pathogens and pests (Dey 2004, Herman et al. 2008). The findings of present study may find application in the production of biofertilizer in areas where K^+ availability is limited or K^+ is fixed and is unavailable. However, identification of isolated KSB strains is required on the basis of molecular techniques. Complete spectrum of phytohormones and secondary metabolites produced needs further investigation.

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