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Paenibacillus glucanolyticus, a promising potassium solubilizing bacterium isolated from black pepper (*Piper nigrum* L.) rhizosphere

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Abstract

A bacterium possessing high ability to solubilize potash was isolated from the rhizosphere of black pepper. On the basis of biochemical and 16S rDNA sequence analysis, the bacterium was identified as *Paenibacillus glucanolyticus* strain IISRBK2. The optimal medium composition and cultural conditions for the isolation of *P. glucanolyticus* were sucrose 5.0 g, Na_2HPO_4 2.0 g, $MgSO_4.7H_2O$ 2.0 g, $FeCl_3$ 0.005 g, $CaCO_3$ 0.1 g and wood ash 1.0 g at pH 7.5 at 30°C. The strain was also evaluated for plant growth and potassium (K) uptake of black pepper in soil artificially treated with 0.5,1 and 1.5g K kg^{-1} soil in the form of wood ash. In this study, wood ash was used as a source of K which contained 53.1 g Kg^{-1} K of which 4.5% was in insoluble form. Inoculation with strain *P. glucanolyticus* was found to increase tissue dry mass (ranging from 37.0% to 68.3%) of black pepper in 1g K kg^{-1}wood ash amended soil. In the soil treated with 0.5 -1.5 g K kg^{-1}, K uptake in live bacterium inoculated black pepper plants increased by 125.0-184.0% compared to uninoculated control.

Keywords: black pepper, potassium solubilizer, potassium uptake, rhizosphere

Introduction

Potassium (K), one of the seventeen chemical elements required for plant growth and reproduction, is often referred to as the "the regulator" since it is involved with over 60 different enzyme systems in plants. Besides its potential to resist drought and disease (Cakmak 2005; Billore *et al*. 2009), it helps in the production of starch, controls root growth and regulates the stomata movement in plant cells and also contributes to quality. Although, soils commonly hold over 20000 ppm of total K, plants can use only the exchangeable K on the surface of the soil particles and that dissolved in the soil water which often amounts to less than 100 ppm and comprise only 0.1 to 2% of the total K (George & Michael 2002). Among the nutrient deficiency syndromes, K deficiency becomes more problematic because K decreases easily in soils due to crop uptake, runoff, leaching and soil erosion (Sheng & Huang 2002). So

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replenishing the soil through extraneous application of various nutrients in the form of fertilizers of various kinds was found to be an essential requirement. However, there are soil microbes that facilitate easy availability of K to crops by solubilizing unavailable form of soil K. Sheng (2005) used sucrose minimal salt medium with illite as K source for the isolation of *Bacillus edaphicus* strain NBT from rhizosphere soil of cotton. Further, Wu *et al*. (2005) used the same medium with glass powder for the growth of K solubilizer *B. mucilaginous*. The present study is an attempt to isolate K solubilizer from rhizosphere soil of black pepper in order to develop an effective biofertilizer package.

Materials and methods

Collection of soil sample

Soil samples for the isolation of N fixers, P solubilizers and K solubilizers were collected from various black pepper and cardamom tracts in Kerala and Karnataka during 2005 to 2006. Several diverse habitats in different areas were selected for the isolation of indigenous strains. These habitats included the plant rhizosphere, protected areas and reserve forests.

Preparation of media

For the isolation of K solubilizer, Sucrose minimal salt medium (SMSM) was modified and used. The basal medium contained sucrose 5.0 g, Na_2HPO_4 2.0 g, $MgSO_4.7H_2O$ 2.0 g, $FeCl_3$ 0.005 g, $CaCO_3$ 0.1 g. The modified composition is given in Table 1. Illite 1.0 g was supplemented with varying quantities of wood ash (0.5–2.5%). Wood ash was used as a source of K which contained 53.1g kg^{-1} K of which 4.5% is in insoluble form. The physico chemical properties of wood ash used in the study are given in Table 2. Since addition of ash increases the pH of the media, care was taken to maintain the pH at 7.5 using 0.1 M HCl. The media was sterilized at 121°C for 20 min. at 120 lb.

Table 2. Physico- chemical properties of wood ash

pH	12.8
Ec (dSm^{-1})	9.8
OC (%)	1.3
P (g kg^{-1})	15.7
K (g kg^{-1})	53.1
Ca (g kg^{-1})	290.8
S (g kg^{-1})	6.9
Mg (g kg^{-1})	19.4

Isolation of K solubilizers

Isolation from the fresh soil sample was done using dilution plate technique in the modified media. Nutrient agar was also included as a check. The plates were incubated at 30°C for 72 h. The organisms grown in each media were selected and purified and numbered by giving prefix BK (B for black pepper and K for potash solubilizer).

Specificity of isolates

The isolated colonies were grown as broth culture in all the combinations of modified

Table 1. Modified composition of sucrose minimal salt media (Basal media)

Ingredients	Modified composition					
	Conc.	mc1	mc2	mc3	mc4	mc5
Sucrose (g)	5.0	5.0	5.0	5.0	5.0	5.0
Na_2HPO_4 (g)	2.0	2.0	2.0	2.0	2.0	2.0
$MgSO_4$ (g)	2.0	2.0	2.0	2.0	2.0	2.0
$FeCl_3$ (g)	0.005	0.005	0.005	0.005	0.005	0.005
$CaCO_3$ (g)	0.1	0.1	0.1	0.1	0.1	0.1
Ash (g)	1.0	0.5	1	1.5	2.0	2.5
Agar (g)	15.0	15.0	15.0	15.0	15.0	15.0

SMSM for 72 h. The colony count was estimated at intervals of 24, 48 and 72 h. The isolates were cross streaked on different media such as N-free malic acid medium (Nfb), Pikovskaya agar and Nutrient agar besides modified combinations of SMSM *viz*., Mc1, Mc2, and Mc3 in order to confirm whether there was any specificity towards modified SMSM (Thakuria *et al*. 2001). This was tested in comparison with already available standard isolates of P solubiliser (PB21a), N fixer (*Azospirillum* sp.P_1AR_6) and *Pseudomonas aeruginosa* (IISR6) from the IISR repository. The growth of each of the isolate was cross-checked by inoculating in these media.

Quantitative estimation of K content

The K content of the inoculated culture broth and uninoculated control was estimated by incubating on an orbital shaker at 150 rpm for 72 h at 30°C. The available K content was measured both by filtration and digestion method using atomic absorption spectrophotometer (AAS 240FS). The pH of the culture broth was also measured.

Preliminary characterization

Based on specificity study and *in vitro* ability for K solubilization, the isolates were selected for *in vivo* assay and routine bacteriological tests were performed for preliminary characterization of the isolates such as gram reaction, endospore production, and biochemical tests like indole, citrate, oxidase, catalase and starch. Antibiotic sensitivity assays were also performed for Ampicillin, Chloramphenicol, Kanamycin, Gentamicin, Rifampicin, Streptomycin, Nalidixic acid and Aztreonam.

Evaluation of growth promotion and K uptake in black pepper by K solubilizer

Evaluation of growth promotion and K uptake by isolated K solubilizers were conducted using black pepper cuttings raised in sterilized potting mixture consisting of soil: farm yard manure: sand (1:1:1) in polythene bags of size 15 × 21 cm. Uniform, healthy cuttings of variety Sreekara were used for the experiment. Different concentrations of wood ash individually and with the isolated organisms were used to study the K release by the organisms and its effect on growth promotion and K uptake by the plant. The experiment was designed in CRD with five replications with seven treatments including control. The treatments were (1) Control (plants as raised in potting mixture) (2) Potting mixture supplemented with wood ash 0.5% (3) Wood ash 1.0% (4) Wood ash 1.5% (5) Potting mixture supplemented with wood ash 0.5% + BK1 (6) Wood ash 1.0% + BK2 and (7) Wood ash 1.5% + BK3. For inoculation, the isolates were grown separately in their respective media *viz*., Mc1, Mc2 and Mc3. Cells were harvested by centrifugation at 5000 rpm for 10 min, washed with sterile distilled water and again pelleted by centrifugation. Inoculum was prepared by suspending pelleted cells in sterile distilled water. The final inoculum contained a cfu of 10^7 mL^{-1}. Inoculation of the plants were done immediately before planting by dipping roots for 15 minutes and then drenching the soil with respective inoculum @ 50 mL $plant^{-1}$. The plants were irrigated once in two days and were grown for four months. Growth parameters such as height of the plant, number of leaves and root length were recorded. Fresh weight of the plant and dry weight were recorded and these plants as well as soil were analyzed for N, P and K content and soil pH following standard methods (Bremner 1996; Jackson 1973). The microbial population in the rhizosphere was also estimated. The rhizosphere colonization by K solubilizers in all the treatments was recorded at monthly intervals for up to three months.

Identification of potential K solubilizers

Identification of potential K solubilizer was done using Biolog as well as by 16SrDNA sequencing. The isolates to be identified were grown on agar medium and then suspended in inoculating fluid at recommended cell density (90–98%). Then the cell suspension was inoculated into the GEN III micro plate using multichannel pipette, @100µL per well and incubated at 30°C for 72 h. During incubation, the increased respiration causes reduction of tetrazolium dye forming purple colour. After incubation, the phenotypic fingerprint of the

purple wells was compared with Biolog. Genomic DNA from the bacteria was isolated and amplification of 16s rDNA gene was performed with universal primer set pA (Fp) (5′- AgAgTTTgATCCTggCTCAg -3′) and pH (Rp) (5′- AAggAggTgATCCAgCCgCA -3′) (Woese 1987; Stackebrandt & Goebel 1994). DNA sequencing was performed and sequences were subjected to BLAST analysis and nucleotide sequence similarities were determined with the NCBI data base.

Results and discussion

Isolation and enumeration

On dilution plating, growth appeared in different media combinations and nutrient agar up to 10^{-4} dilution (Fig 1). The total microbial count was very less in all the modified media combinations when compared to nutrient agar where considerably large population of bacteria and fungi were observed. Bacterial growth was completely absent in Mc4 and Mc5 media containing 2 and 2.5% of wood ash, whereas fungal colonies were present. Mucoid, greyish, entire, slow growing and raised bacterial colonies appeared in Mc1, Mc2 and Mc3 (Fig 1).

Specificity of the isolates

When the isolates were grown in broth solutions of all the media combinations (Mc1, Mc2 and Mc3) the colonies appeared only after 72 h. The isolates showed very clear specificity in growth and multiplication. The isolate, IISRBK1 was able to grow and multiply only in Mc1 and so also the other isolates ie. BK2 in Mc2 and BK3 in Mc3. In general, these isolates showed specificity towards the source of isolation. Cross streaking studies on the efficacy of isolates to grow on other selective medium showed that the K solubilizers can grow only in their source media other than nutrient agar. *Pseudomonas aeruginosa* IISR6 and Pb21a were unable to grow on different SMSM combinations. *Azospirillum* sp. P_1AR_6 grew on Mc1. However, the time taken by the isolate to appear into well recognizable colonies was much longer when grown in the cross medium.

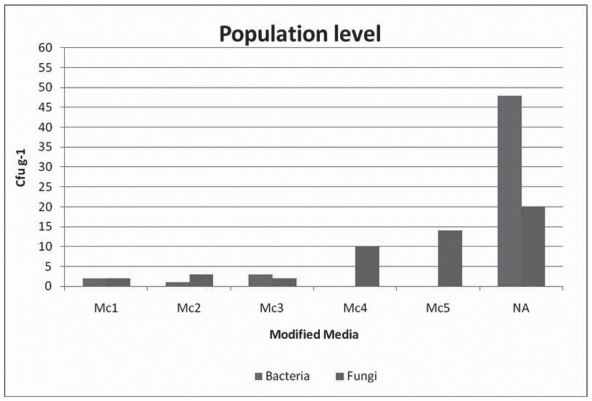

Fig 1. Population of bacteria and fungi in modified media composition supplemented with wood ash

Quantitative estimation of K content

K content in the media with different isolates were estimated by analyzing the released K using both filtration and digestion method. Estimation of K content showed that the isolates when grown in the corresponding media showed maximum K solubilization after 72h of growth. In filtration method, the K content of the inoculated broth showed less K content and decreased pH with all the three isolates when compared to digestion method (Table 3). In digestion method, the inoculated broth showed higher K content as compared to control. The decrease in K content of inoculated broth in filtration method was due to the exclusion of bacterial cells by filtration, which mobilized part of the K available in the broth. But in digestion method, bacterial cells were also digested resulting in increased K content (Table 3). This explains the K utilization by the bacterial cells.

Evaluation of growth promotion and K uptake in black pepper by K mobilizers

The three isolates *viz*., BK1, BK2 and BK3 showing high specificity towards modified SMSM (Mc1, Mc2 and Mc3) and increased K solubilization were further evaluated under *in vivo* conditions.

The *in vivo* assay of these isolates with black pepper cuttings showed that these isolates released insoluble K from ash into the soil and enhanced the K content of the soil and therefore plant growth. The pH of the inoculated mixture

Table 3. *In vitro* study on K mobilization by selected isolates in the respective media

Treatments	K content (mg L^{-1})
FiltrationMc1 + BK1	165
Mc1	237
Mc2 + BK2	213
Mc2	315
Mc3 + BK3	256
Mc3	328
DigestionMc1 + BK1	205
Mc1	205
Mc2 + BK2	255
Mc2	245
Mc3 + BK3	328
Mc3	315
CD (P<0.05)	8.0

with addition of ash was low, but the K content was high. Conversely, the pH of the uninoculated mixture with addition of ash was high, but the K content was low (Table 4). The ash contained predominately sodium and potassium carbonate, sodium and potassium chloride, silica and calcium carbonate. The potential availability of the nutrients to plants in the ash was high with the possible exception of P, and followed the order K > Mg > Ca > P (Eriksson 1998).

The dry matter of the plants after four months ranged from 3.31 to 5.57 g $plant^{-1}$. The total dry matter content and number of leaves and root length were higher in BK2 isolate grown in 1.0% ash (Table 5). Significant increases in soil N and K were also observed in BK2 isolate. High K content in the plant and increased K uptake were also recorded with the same isolate (Table 5). The isolate also showed maximum

Table 4. Soil nutrient status and pH as affected by K solubilizers in black pepper rhizosphere

Main Treat	Sub Treat	Soil			
		N	P	K	pH
		(kg ha^{-1})			
Ash	0.5	88.6	12.4	283.3	6.56
	1.0	101.3	8.17	381.6	7.07
	1.5	89.3	16.5	402.3	7.17
Ash+organisms	0.5 + BK1	122.6	19.1	530.0	5.89
	1.0 + BK2	148.6	11.7	633.6	5.96
	1.5 + BK3	133.6	12.6	503.0	6.03
Control		92.0	12.5	223.3	6.14
CD (P<0.05)		7.2	0.6	55.4	NS

Table 5. The influence of K solubilizers on biomass and NPK uptake of black pepper

Main Treat	Sub Treat	Dry weight	N	P	K
		g $plant^{-1}$		mg $plant^{-1}$	
Ash	0.5	4.11	135	11.97	60.83
	1.0	4.95	166	14.97	79.10
	1.5	4.50	128	13.30	77.83
Ash + organisms	0.5 + BK1	5.03	134	12.87	82.37
	1.0 + BK2	5.57	117	14.23	114.2
	1.5 + BK3	4.52	100	11.42	83.4
Control		3.31	96.0	7.90	43.7
CD (P<0.05)		0.44	14.7	1.21	7.30

colonization in the rhizosphere soil (Table 6). But no significant difference could be noticed with respect to soil P and plant N and P.

Table 6. The colonization of K solubilizers in black pepper rhizosphere during different growth periods

Main treatment	Sub treatment	K solubilizers		
		30 days	60 days	90 days
Ash	0.5	1.66	8.33	3.33
	1.0	2.33	6.00	4.33
	1.5	0.67	0.00	0.00
Ash+organisms	0.5 + BK1	7.33	8.00	10.0
	1.0 + BK2	10.0	11.3	11.3
	1.5 + BK3	5.00	6.00	9.33
Control		0.00	0.00	0.00
CD (P<0.05)		1.08	0.54	1.21

It was also found that the incorporation of ash to media/soil at variable rates of 0.5%, 1.0% and 1.5% had no deleterious effect either on bacterial population or on black pepper cuttings. A parallel study to quantify the amount of wood ash required to isolate rhizosphere K-solubilizers revealed that the maximum population was found in 1.0% ash followed by 0.5% (Table 6). The population decreased when the percentage of ash increased. The fungal population predominated in 2.0% ash.

Identification of potential K solubilizers

The isolates were gram positive, irregular rods, indole, citrate and oxidase negative. The isolate BK2 showed positive reaction to endospore production, catalase and starch. In addition, the isolate also showed positive reaction to chemical sensitivity assays such as 1.0% sodium chloride, 1.0% sodium lactate, and potassium tellurite and resistance to antibiotics Nalidixic acid and Aztreonam. The phenotypic fingerprint, when compared with Biologs extensive species library, showed 99.0% similarity with *Paenibacillus glucanolyticus*. Based on biochemical, 16S rDNA analysis and Biolog micro station system, the isolates BK1, BK2 and BK3 were identified as *Arthrobacter globiformis*, *Paenibacillus glucanolyticus* and *Microbacterium esteraromaticum* respectively.

Several studies have indicated the effect of K solubilizers on growth of crops under various conditions. K solubilizers mediated increase in K uptake has been recorded by Chandra & Singh (1999) and Chandra *et al*. (2002). Similarly, Nayak (2001) studied the effect of K mobilizer on brinjal and recorded increased K uptake and increased plant biomass in K mobilizer treated plants as compared to control. Sheng (2005) examined the plant growth promoting effects and nutrient uptake of K releasing strain *Bacillus edaphicus* NBT on cotton and rape. Similarly the application of biofertilizer containing N fixer (*Azotobacter chroococcum*), P solubilizer (*Bacillus megaterium*) and K solubilizer (*Bacillus mucilaginous*) and AM fungi (*Glomus mosseae*) significantly increased the growth of *Zea mays.* Microbial inoculum not only increased the nutritional assimilation of plant, but also improved soil properties, such as organic matter content and total N in soil (Wu *et al*. 2005). Studies on the effect of K solubilising bacteria *Frateuria aurantia* on brinjal showed significant increase in yield, plant height and K uptake (Ramarethinam & Krishan 2006). There are also reports where soil inoculation with P or K solubilizing bacteria significantly increased N, P and K uptake in pepper and cucumber plants (Han *et al*. 2006).

Based on biochemical, Biolog and 16S rDNA analysis, the isolate showing maximum K solubilizing property was identified as *Paenibacillus glucanolyticus.* Earlier reports on K solubilization by *Paenibacillus* species do exist. Hu *et al*. (2006) isolated K solubilising strains from the soil of Tianmu Mountain (China) and characterized them phenotypically and

phylogenetically. They shared a 16S rDNA gene sequence similarity of more than 99.1% with *Paenibacillus* sp. and was also proved to be very efficient in promoting the shoot and dry matter of wheat plants (Peres *et al*. 2008).

The present study, showed that *Paenibacillus glucanolyticus* IISRBK2, obtained from black pepper rhizosphere soil significantly improved plant growth and K uptake. However, the persistence of plant growth promoting activity under field condition needs to be investigated.

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