

Evaluation of the Efficiency of Plant Growth Promoting Rhizobacteria and Its Effect on Germination of *Solanum Melongena* L. Seeds

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ABSTRACT: Some of the microorganisms have the potential to be used as biofertilizer by fixing atmospheric nitrogen, solubilizing P and K from their complex minerals. The aim of the present investigation was to study the nitrogen fixing, P and K solubilizing ability of bacteria isolated from rhizosphere region of nutmeg plants. Among the bacteria isolated 4 PSB, 4 KSB and 5 NFB were taken for this study. PSB 1 and KSB 1 showed maximum solubilization zone [4.32 ± 0.079 mm and 4.14 ± 0.052 mm] in Pikovskaya and Aleksandrov agar plates respectively. Nitrogen fixing bacteria were isolated in DF – NH_4SO_4 medium and were tested for ACC deaminase activity. NFB 1 was found to be better in ACC deaminase production and showed good growth in DF – NH_4SO_4 medium. Among PSB and KSB isolates, PSB 1 and KSB 1 was affective in solubilizing P [0.55 ± 0.007 mg/ml] and K [1.46 ± 0.005 ppm] respectively. The most efficient isolates and their combination were tested to determine their effect on seed germination of *Solanum melongena* L.. A combination of NFB, PSB and KSB showed high level [97%] of seed germination. The above results strongly recommend the use of rhizobacteria in agriculture industry in place of chemical fertilizer.

KEYWORDS: Phosphorous and potassium solubilizing bacteria, biological nitrogen fixation, ACC deaminase, clear zone index, tricalcium phosphate, plant growth promoting rhizobacteria

Abbreviations : P – Phosphorous, K – Potassium, PSB- Phosphorous solubilizing bacteria, KSB –Potassium solubilizing bacteria, NFB- Nitrogen fixing bacteria, PKV Agar- Pikovskaya Agar, ADV Agar- Aleksandrov Agar, TCP- Tricalcium phosphate, ACC: 1- aminocyclopropane 1-carboxylate, OD- Optical density.

I. INTRODUCTION

Nitrogen, Phosphorous and Potassium are the major essential macronutrients for plant growth and development. Nitrogen is the most abundant extremely unreactive gas in Earth's atmosphere (Frank et al.,2003). Atmospheric nitrogen cannot be directly assimilated by the plants. It becomes available only through biological nitrogen fixation carried out by prokaryotes (Claudine et al.,2009).

The amount of phosphorous in the soil is quite high but most of this P is insoluble and therefore not available for the plants. Also a major portion of soluble inorganic P applied to soil as chemical fertilizer is immobilized soon after application and becomes unavailable to plants. Soil microorganisms play an important role in releasing P from inorganic and organic forms through solubilization and mineralization (Hilda and Fraga, 1999).

In soil K exists mainly in three forms:- soil minerals, non exchangeable and available form (Goldstein, 1994). Soil microorganisms have been reported to play a key role in the K cycle and therefore KSB present in the soil could be useful to make K available for plant uptake (Groudev, 1987). Rhizosphere is the term used to designate soil immediately surrounding roots under the influence of the plant (Claudine Franch et al.,2009). Nitrogen fixing

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microorganisms are generally active in plant root zone soil (Bagali Shrimant, 2012). High proportion of PSB is also concentrated in the rhizosphere and are metabolically more active than from any other sources (Vazquez et al., 2000).

The aim of the present study was to screen the soil isolates for nitrogen fixing, P and K solubilizing properties and evaluate their effect on germination of *Solanum melongena L.* seeds. Inoculation of such bacteria to crop fields instead of chemical fertilizer can prevent environmental pollution and increase crop yields.

II. RELATED WORKS

Schoebits et al., 2013 investigated the efficiency of rhizobacteria, *P. fluorescens* and *Serratia sp.*, in immobilized form on solubilisation of rock P and their assimilation by wheat plants. P solubilisation of 89 and 93 µg/L was reported with immobilized bacteria and around 64% P uptake by wheat plants after 60 days of growth was observed with *P. fluorescens*.

Potassium solubilizing bacteria isolated from soil, rock and mineral samples and their effect on solubilization from microcline, orthoclase and muscovite mica minerals and ground nut plant growth were reported by Sugumaran and Janarthanam 2007, *Bacillus mucilaginosus* MCRC_{p1} showed maximum solubilisation (4.29 mg/L) in muscovite mica. Dry matter and oil content of ground nut plant were increased 125 % and 35.41% repeatedly through the inoculation of MCRC_{p1} bacterium.

The effect of phosphate and potassium solubilizing bacteria on mineral uptake, soil availability and growth of egg plant were studied by Han and Lee, 2005. Inoculation of *B. megaterium* (PSB) and *B. mucilaginosus* (KSB) in conjunction with amendment of its respective rock P and K materials increased the availability of P and K in soil, enhanced N, P and K uptake and promoted growth of egg plant.

The role of rhizobacteria having different ACC Deaminase activities in increasing ACC upon inoculation in etiolated pea seedlings through highly ethylene specific classical "triple" response bioassay were investigated by Shaharoon et al., 2006. According to this study, the inoculation with rhizobacteria containing ACC Deaminase could be used to decrease ACC thereby decreasing the inhibitory effects of ethylene both under normal and stress conditions.

Muhammed Iqbal Hussan et al., 2013 reported the impact of PGPR, *Burkholderia sp.*, *Bacillus sp.*, *Pseudomonas sp.* and *Flavobacterium sp.* on growth and yield in maize. All strains showed increased growth and yield of maize when compared with control treatments.

Endophytic bacteria isolated from *Piper nigrum* were screened for various plant growth promoting properties and studied for their growth promoting effect on *Vigna radiata* seedlings. *Klebsiella sp.* and *Enterobacter sp.* were identified and reported with excellent growth promoting properties (Jasim et al., 2013)

III. MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected randomly from the rhizosphere region of nutmeg plants, Kerala. The samples were air dried, powdered, mixed and packed in a polythene bag. This sample was used for this study.

Isolation and screening of PGPR

Isolation was carried out by serial dilution and spread plate technique on selective medium.

PSB were isolated by plating serially diluted soil sample on Pikovskaya Agar medium:- Dextrose 10g, NH₄SO₄ 0.5g, KCl 0.2g, MgSO₄ 7 H₂O Trace, MnSO₄ 0.1g, Yeast extract 0.5g, Agar 15g, Distilled water 1L containing 5g /L insoluble TCP [Pikovskaya, 1948] and KSB on Aleksandrov agar medium:- Glucose 5g, MgSO₄ 7 H₂O 0.005g, FeCl₃ 0.1g, Ca₂CO₃ 2g, Ca₃PO₄ 2g, Distilled water 1L containing 2g/L mica powder [Hu et al., 2006]. The plates were incubated at room temperature for 5 days and observed for clear zone formation.

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Bacteria able to fix atmospheric dinitrogen were isolated on DF salt minimal medium :- KH_2PO_4 4 g, Na_2HPO_4 6g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, H_3BO_3 10 μg , MnSO_4 10 μg , ZnSO_4 70 μg , CuSO_4 50 μg , MoO_3 10 μg , Glucose 2 g, Gluconic acid 2g, citric acid 2g, Agar 12 g, Distilled water 1L [Dworkin and Foster, 1958] supplemented with 2g/L ammonium sulphate as sole nitrogen source. The bacterial growth in the media after incubation indicates nitrogen fixing ability of the isolates by producing ACC deaminase. The isolates obtained were purified by further streaking on fresh plates and stored at refrigerator for further use.

PSB and KSB isolates were screened for P and K solubilizing property by spot test method on their selective medium. The plates were incubated at room temperature and observed for solubilization zone regularly during 5 days. The solubilization index was calculated according to the ratio of total diameter (colony + halo zone) to colony diameter [Edi-Premono et.al., 1996].

Quantitative assay of P and K Solubilization

The isolates showing zone of solubilization in both PVK and ADV plates were further examined for their ability to release inorganic P from TCP and K from mica in broth medium. Overnight culture (1ml) of each isolates were inoculated separately into 50ml of PVK broth (for PSB) and ADV broth (for KSB) and incubated for 12 days at room temperature. The samples were analyzed at every 3 days interval and compared with a set of uninoculated controls. The samples were centrifuged at 10000 rpm for 10 minutes to separate the cell mass and insoluble P and K.

The available P content in the supernatant was estimated by Phospho molybdate yellow colour method [Bureau of Indian standards, third edition]. To 0.1 ml aliquote of the supernatant, 2.5 ml Barton's reagent was added and volume was made upto 5 ml by with de-ionized water. The absorbance of the resultant yellow colour was read after 10 minutes at 470 nm in UV/Visible spectrophotometer. Intensity of yellow colour is directly proportional to P concentration. A calibration curve was prepared by using suitable volumes of standard KH_2PO_4 solution (0.022 g/dL) and the total soluble P in the samples were calculated from the curve.

The microbial release of K from mica in the samples were detected by Flame photometer described by Sugumaran Janarthanam, 2009 using KCl (2 ppm solution) as the standard.

ACC Deaminase Assay

NFB isolates were assayed for ACC deaminase activity by inoculating into vials containing DF – NH_4SO_4 solution and shaken at 125 rpm for several days. The bacterial growth was monitored at every one day interval until 4 days by measuring optical density at 600 nm. The average value of OD at 0.05 or more indicated the ACC deaminase activity of the isolates [Edi Husen et al., 2005]

Effect of inoculation of rhizobacterial isolates on seed germination of *Solanum melongena* L.

The most efficient NFB, PSB, KSB isolates and their combination were tested to determine their effect on seed germination. *Solanum melongena* L. seeds were selected for the present study. The seeds were surface sterilized in 2% sodium hypochlorite for 3 minutes and then rinsed 5 times with sterile distilled water. The seeds were placed in sterile petriplates containing filter paper [Whatman no 1] wetted with 5 ml overnight culture of microorganism. The plates were kept in dark for 3 days and subsequently subjected to light condition for another 3 days under room temperature. Nutrient solution was added accordingly during the exposure to light. The seed germination was calculated as :

$$\text{Germination rate (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100 \quad [\text{Krishnaswami and Sheshu, 1890}]$$

Statistical Analysis

The results were analysed using the software SPSS Version 16. A two way mixed ANOVA was performed to compare the results. Post hoc analysis was done by using Bonferroni correction to determine the significant difference between

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all the pair wise comparisons ($P < 0.001$). Fisher exact test was conducted for comparing seed germination frequency among groups ($P = 0.006$).

IV. EXPERIMENTAL RESULTS AND DISCUSSIONS

Isolation and screening of PGPR

The production of clear zone around the colonies of the microorganism in PVK and ADV agar plates is an indication of the presence of PSB and KSB respectively. Such organisms were isolated and purified. Among the isolates, 4 PSB and 4 KSB were selected and named as PSB1, PSB2, PSB 3,PSB4 and KSB1, KSB2, KSB3 and KSB4.

The PSB and KSB isolates were screened for the P/K solubilizing character on selective media and solubilisation index was calculated (Table 1 and 2). PSB 1 and KSB 1 showed highest solubilisation index of 4.32 ± 0.079 mm and 4.14 ± 0.052 mm in their selective media. The results obtained were found to be statistically significant when compared with the control ($P < 0.001$). The formation of halo zone on PKV and ADV plates should only be considered as a primary screening test for P and K solubilisation. A quantitative assay was performed to detect P and K solubilisation in broth.

Table 1. Solubilization index of PSB upto 5 days

Isolates	Solubilization index				
	1 Days	2 Days	3 Days	4 Days	5 Days
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
PSB 1	1.00 ± 0.000	2.44 ± 0.070	24.15 ± 0.053	4.26 ± 0.052	4.32 ± 0.079
PSB 2	1.00 ± 0.000	2.42 ± 0.079	2.76 ± 0.070	3.05 ± 0.053	3.16 ± 0.052
PSB 3	1.00 ± 0.000	2.03 ± 0.068	3.01 ± 0.088	3.45 ± 0.053	4.00 ± 0.082
PSB 4	1.00 ± 0.000	2.09 ± 0.088	3.10 ± 0.082	3.23 ± 0.082	4.04 ± 0.052

Table 2 : Solubilization index of KSB upto 5 days

Isolates	Solubilization index				
	1 Days	2 Days	3 Days	4 Days	5 Days
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
KSB 1	1.00 ± 0.000	1.98 ± 0.042	3.50 ± 0.082	3.96 ± 0.052	4.14 ± 0.052
KSB 2	1.00 ± 0.000	1.28 ± 0.244	2.05 ± 0.053	2.54 ± 0.052	2.89 ± 0.099
KSB 3	1.00 ± 0.000	1.29 ± 0.251	2.03 ± 0.048	2.80 ± 0.082	3.15 ± 0.053
KSB 4	1.00 ± 0.000	1.21 ± 0.099	1.74 ± 0.052	2.15 ± 0.053	2.94 ± 0.052

Quantitative assay of P and K Solubilization

The microbial solubilisation of P and K were confirmed by detecting soluble P and K in PKV and ADV liquid media containing TCP and mica respectively. The maximum phosphorous solubilized from TCP by the PSB isolates were in the range of 0.24 ± 0.006 mgml^{-1} to 0.55 ± 0.007 mgml^{-1} . P solubilisation was maximum at 9 days of incubation. At 12 days of incubation, soluble P content in the broth was reduced. This may be due to the auto accumulation of P inside the isolates. PSB 1 showed highest level of P solubilisation (Table 3).

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Table 3 . P solubilized from TCP by PSB isolates at 3, 6, 9 and 12 days of incubation

Isolates	P solubilized (mg/ml)			
	3 Days	6 Days	9 Days	12 Days
Control	0.01 ± 0.000	0.01 ± 0.000	0.01 ± 0.003	0.01 ± 0.003
PSB 1	0.19 ± 0.007	0.24 ± 0.007	0.55 ± 0.007	0.50 ± 0.005
PSB 2	0.11 ± 0.008	0.22 ± 0.005	0.51 ± 0.009	0.43 ± 0.011
PSB 3	0.19 ± 0.088	0.17 ± 0.010	0.46 ± 0.011	0.37 ± 0.0015
PSB 4	0.12 ± 0.009	0.18 ± 0.009	0.24 ± 0.006	0.21 ± 0.010

The KSB isolates were found to be efficient in solubilizing K from mica on ADV broth. Highest K solubilisation was reported with KSB 1 (4.32± 0.079 ppm) at 12 days of incubation (Table 4). The ability of bacteria to release K largely depends on the nature of the mineral compounds

Table 2 . K solubilized from mica by KSB isolates at 3, 6, 9 and 12 days of incubation

Isolates	K solubilized (ppm)			
	3 Days	6 Days	9 Days	12 Days
Control	0.11 ± 0.008	0.11 ± 0.005	0.10± 0.005	0.11 ± 0.007
KSB 1	1.21 ± 0.009	1.31 ± 0.013	1.46± 0.005	1.56± 0.007
KSB 2	0.53± 0.009	0.75± 0.007	0.89± 0.008	1.07± 0.076
KSB 3	0.61± 0.008	0.67± 0.008	0.75± 0.005	0.86± 0.005
KSB 4	0.84± 0.007	0.95± 0.005	1.08± 0.045	1.21 ± 0.021

The results obtained were found to be statistically significant when compared with the control (P < 0.001).

ACC Deaminase Assay

The average OD values of 0.05 and more was considered as positive result. All the isolates produced ACC deaminase. Average OD value was more than 0.05 for all isolates after 48hrs of incubation.

Pseudomonad producing ACC deaminase has been known to promote plant growth by lowering ethylene biosynthesis in higher plants (Edi Husen et al., 2009). ACC deaminase enzyme is considered as one of the most important/beneficial character of PGPR, because it promotes plant growth and fights against plant pathogens (Cattelan et al., 1999). Rhizobacteria containing ACC Deaminase have been reported to decrease the inhibitory effects of ethylene both under normal and stress condition due to their ACC Deaminase activity.

Seed Germination Frequency

Effect of PGPR isolates and their combination were tested for seed germination efficiency. Seed Germination Frequency was high with combination of isolates.

Fisher exact test was performed for comparing seed germination frequency among isolates, P = 0.006. Statistically there is a significance between seed germination frequency and isolates when compared with control (Table 5).

Table 5 :determination of seed germination frequency

Isolates	% of seed Germination
Control	60
PSB	70
KSB	70
NFB	80
PSB+KSB+NFB	97

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V. CONCLUSION

The natural character of soil is spoiled by the repeated application of chemical fertilizer. The microorganisms located in the rhizosphere region of soil have the ability to solubilize bound form of soil minerals. Nitrogen fixing phosphorous and potassium solubilizing bacteria were isolated from rhizosphere soil of nutmeg plants. The selected isolates were screened for their ability to be used as biofertilizer. PSB 1 and KSB 1 were found to be most effective in solubilizing P and K respectively. ACC deaminase assay was reported positive for NFB isolates. The most efficient isolates and their combination showed significant effect on the germination of *Solanum melongena L.* seeds. The present study strongly suggests that by applying these microorganisms to the agricultural field, it is possible to increase the soil fertility, crop yield and reduce the usage of chemical fertilizer, thereby keeping our environment clean and safe.

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