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A Study on Nitrogen Fixing Ability of Soil Isolates and Quantitative Estimation of K Solubilization by Soil and Plant Isolates

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A B S T R A C T

The aim of present investigation was to study the nitrogen fixing ability and quantitative estimation of K solubilization by soil isolates, plant and soil isolates respectively. Only JS series (JS1 to JS5) of soil isolates showed vigorous growth on N free media, hence tested for quantitative estimation of nitrogen fixing ability of the cultures by Acetylene reduction activity (ARA) assay out of five isolates tested, the highest ARA was recorded with JS1 ($p<0.05$; 47.08 nmole ethylene/mg cell protein/h) which was statistically at par with JS2 ($p<0.05$; 41.94 nmole ethylene/mg cell protein/h). Isolate JS5 showed the lowest Acetylene reduction activity which was statistically at par with JS3 and JS4. Quantitative estimation of potassium released from mica in broth revealed highest K release by MER 4 ($p<0.05$; 1.2 µg/ml broth) isolate followed by OS 10 ($p<0.05$; 0.9 µg/ml broth) which was statistically at par with JS5 ($p<0.05$; 0.8 µg/ml broth). JS4 and JS1could release 0.6 and 0.4 µg K/ml broth respectively and were statistically at par to each other. K released by isolate MER 3 was lowest ($p<0.05$; 0.2 µg/ml broth) and was at par with control.

Introduction

Though a variety of nitrogen fixing bacteria like *Acetobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Derxia, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas* and *Zoogloea* have been isolated from the rhizosphere of various crops (Barraquio *et al.*, 2000; James *et al.*, 2000), interest in the beneficial nitrogen fixing growth promoting rhizobacterial-plant association has increased recently due to their potential use as biofertilizers (Vessey, 2003). Plant associated nitrogen-fixing bacteria have been considered as one of the possible alternatives for inorganic nitrogen fertilizer for promoting plant growth and yield (Ladha and Reddy, 2000). A wide range of bacteria namely *Pseudomonas, Burkholderia, Acidothiobacillus ferrooxidans, Bacillus mucilaginosus, B. edaphicus, B. circulans* and *Paenibacillus* sp. had been reported to release potassium in accessible form potassium-bearing minerals in soils (Sheng, 2006 and Lin *et al.*, 2002). These potassium solubilizing bacteria (KSB) were found to dissolve potassium, silicon and aluminum

from insoluble K-bearing minerals such as micas, illite and orthoclases, by excreting organic acids which either directly dissolved rock K or chelated silicon ions to bring K into the solution (Aleksandrov *et al.,* 1967; Ullaman *et al.,* 1996 and Bennett *et al.,* 1998). Inoculation with potassium solubilizing bacteria had been reported to exert beneficial effects on growth of wheat (Sheng and He, 2006). Similarly, inoculation of maize and wheat plants with *Bacillus mucilaginosus, Azotobacter chroococcum* and *Rhizobium* resulted in significant higher mobilization of potassium from waste mica, which in turn acted as a source of potassium for plant growth (Singh *et al.,* 2010).

For optimum crop production, soil solution and exchangeable K need to be replenished continually with K releasing by non-exchangeable K through weathering of K resources such as micas and feldspars (Sparks and Huang, 1985) or by addition of K fertilizers. Therefore, the application of K solubilizing bacteria is a promising approach for increasing K availability in soils cultivation of more K demand crops (Zahar *et al.,* 1984; Vandevivaea *et al.,* 1994 and Barker *et al.,* 1998). Hence, the present investigation was undertaken to study the nitrogen fixing ability of diazotrophic soil isolates and quantitative estimation of K solubilization by soil and plant isolates.

Materials and Methods

The present investigation conducted during r*abi* season of 2016-17 at Research Farm of ICAR-IISS, Bhopal to study the "The Study of Nitrogen fixing ability of soil isolates and quantitative estimation of K solubilization by soil and plant isolates". The material used and methods adopted during the course of experimentation in the field and laboratory are described in brief as under following heads.

Glasswares and media sterilization

All media were autoclaved at 15 psi (1.06 kg/cm^2) pressure for 20 minutes. Antibiotics, tryptophan stock were filter sterilized by using 0.22 µm disposable syringe filters. Glasswares used were sterilized in hot air oven for 2 hours at 180^{0}C.

Isolation of bacteria from soil

For isolation of aerobic nitrogen fixing bacteria, 5 g composite soil sample was added to flask containing 100ml Jensen's N-free broth. The flask was incubated at 30±2°C in incubator-shaker at 200 rpm for 48 hours. After 48 hours, 10 ml of inoculums was inoculated in fresh 100 ml Jensen's N-free broth and incubated as mentioned above. The process was repeated five times to eliminate all the other microbes not capable of fixing atmospheric nitrogen under aerobic condition. From last transferred flask, serial dilution was prepared up to 10^{-7}dilution and 100µl of inoculum was spread plated on Jensen's N-free Agar media.

Isolation of endophytic bacteria from maize root

Isolation of endophytic bacteria from maize root was done by washing it thoroughly in running water. The excised roots were surface sterilized by placing 10 grams fresh root in 5% sodium hypochlorite for 10 minutes and then in 70% ethanol for 2 minutes. The roots were thoroughly washed with sterile distilled water thrice. Ten ml of sterile water was added to the surface sterilized roots and shaken for 10 minutes vigorously to check the sterility of rhizoplane. From this suspension, 100 µl aliquot was spread plated on nutrient agar media. Further, the surface sterilized roots were crushed with sterilized mortar and pestle in 9 ml physiological saline (0.85% sodium chloride solution). From this sap,

serial dilution was prepared and 100µl inoculum was again spread plated on nutrient agar plates. Three replications were maintained for each dilution and the plates were incubated at 28°C for 48 hours. Morphologically distinct colonies were purified by repeated streaking on nutrient agar media and were used for further study.

Aerobic nitrogen fixation

Nitrogen free Jensen's agar media was used for detection of free living aerobic nitrogen fixers. Media was poured in sterilized petriplates.

After solidification, grids were prepared on the lower surface of petriplate. Isolates were washed in physiological saline by centrifugation to remove traces of nitrogen that it could have received from earlier media. All the isolates were spot inoculated in their respective grids and incubated at 28±2^{0}C for 2 days. Isolate showing growth were further streaked on Jensen's media to conform the nitrogen fixing ability.

Acetylene Reduction Activity (ARA) was used for determination of nitrogen fixing ability of the isolates. Ten ml of Jensen's broth was inoculated with 200µl of previously grown cultures showing growth on Jensen's agar plate. The inoculated tubes were incubated in an incubator at 28+2^{0}C for 4 days. After that cotton plugs were aseptically replaced with sterile subaseals. Ten percent of the airspace in the tube was replaced with acetylene and these were further incubated for 24 hrs.

At the end of incubation period, nitrogen fixation ability of the culture was determined by acetylene reduction activity (ARA) by the method of Hardy *et al.,* (1973) using a gas chromatograph (Nucon 5765 model) with FID detector having Porapak N column. The carrier gas was Helium. The operating conditions were: Column temperature: 75^{0}C, Injector temperature: 110^{0}C, and Detector temperature: 110^{0}C. Three replications per treatment and appropriate uninoculated controls were maintained. The results were expressed in terms of nmoles of ethylene/mg cell protein/h.

Potassium solubilization by bacterial isolates

For quantitative determination of potassium solubilization from mica, 100 ml of sterilized Aleksandrov media containing mica as mineral potash source were inoculated with 2 ml of bacterial suspension (approx 10^8 cfu/ml) positive for K solubilization based on plate assay in 250 ml Erlenmeyer flask. The flasks were and incubated at 30±2°C and 200 rpm for 7 days in an incubator-shaker (make-Kuhner). For each isolate three flasks were inoculated. After 7 days, each flask was checked for potassium release by flame photometry. The suspension was centrifuged at 10,000 rpm for 10 min and supernatant was retained. 1ml of supernatant was taken in 25 ml volumetric flask and volume was made to 25 ml with distilled water and mixed thoroughly. After that the solution was fed to flame photometer for estimating K (Hu *et al.*, 2006; Saiyad *et al.,* 2015).

Statistical analysis

Statistical analyses were carried out through one-way analysis of variance (ANOVA) and the mean of treatments were compared according to Fisher's multiple comparison tests. Least significant difference (LSD) was calculated at $p<0.05$ using statistical package of SAS. Multivariate analysis of PGP attributes was performed using Principle Component Analysis and clustering of isolates based on Euclidean distance method was calculated with SAS statistical package.

Results and Discussion

Nitrogen fixation

All the 32 isolates were spot inoculated on nitrogen free media in grid prepared on the plates. Except JS series cultures that were obtained through Enrichment Technique on N-free media, only few isolates showed growth on N-free media which upon restreaking on N-free media, failed to grow, indicating inability of these cultures to grow in absence of combined nitrogen (Table 1). Only JS series culture showed vigorous growth on N free media, hence JS series cultures were tested for quantitative determination of nitrogen fixing ability of the cultures by Acetylene reduction activity (ARA) assay. Out of five isolates tested, the highest ARA was recorded with JS1 ($p<0.05$; 47.08 nmole ethylene/mg cell protein/h) which was statistically at par with JS2 ($p<0.05$; 41.94 nmole ethylene/mg cell protein/h). Isolate JS5 showed the lowest Acetylene reduction activity which was statistically at par with JS3 and JS4 (Table 1).

Potassium solubilization ability

Quantitative estimation of potassium released from mica in broth revealed highest K release by MER 4 ($p<0.05$; 1.2 µg/ml broth) isolate followed by OS 10 ($p<0.05$; 0.9 µg/ml broth) which was statistically at par with JS5 ($p<0.05$; 0.8 µg/ml broth). JS4 and JS1could release 0.6 and 0.4 µg K/ml broth respectively and were statistically at par to each other. K released by isolate MER 3 was lowest ($p<0.05$; 0.2 µg/ml broth) and was at par with control. Decline in pH at the end of incubation period was also recorded with inoculation of isolates in media. The lowest pH was noted with MER4 (4.1) followed by JS4 (4.2), JS1 (4.3), JS5 (4.42), OS10 (4.7) and MER3 (5.32). Control broth could maintain neutral pH (Table 2).

Table.1 Acetylene reduction activity (ARA) of microbes isolated from soil

Isolates	ARA (n moles ethylene/mg protein/h)
JS1	47.08a
JS2	41.94a
JS3	22.92b
JS4	26.37b
JS5	20.72b
LSD (0.05)	6.2

Table.2 Quantitative estimation of potash solubilization by selected isolates

Isolates	K released (µg/ml broth)	pH
Control	0.1d	7.2
JS1	0.4c	4.3
JS4	0.6c	4.2
JS5	0.8b	4.42
OS10	0.9b	4.7
MER3	0.2d	5.32
MER4	1.2a	4.1
LSD(0.05)	0.21	

Growth on N-free Jensen's medium

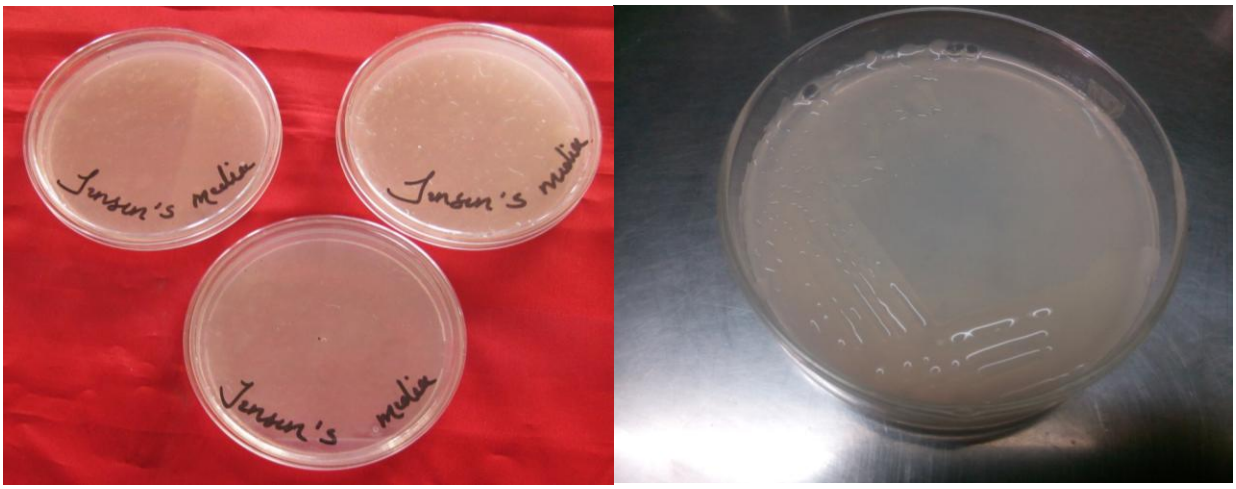


Fig.1 Acetylene reduction activity of cultures

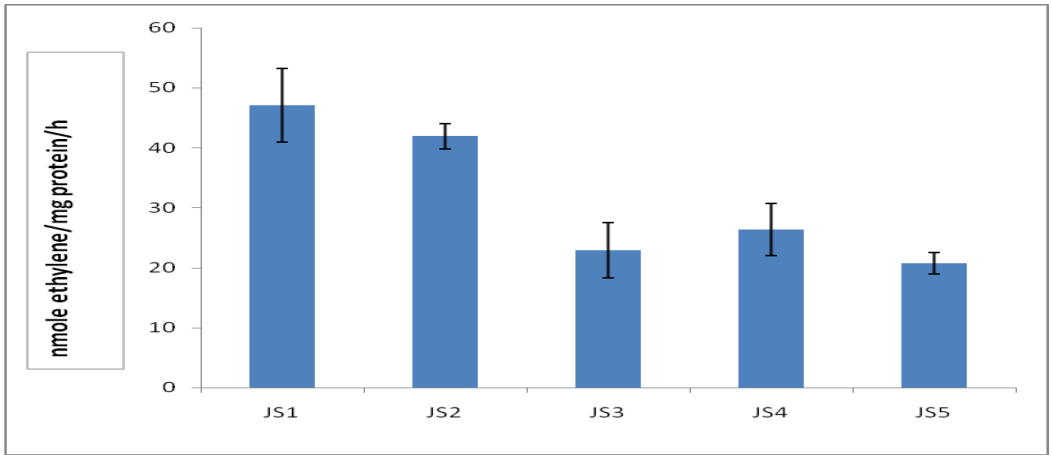
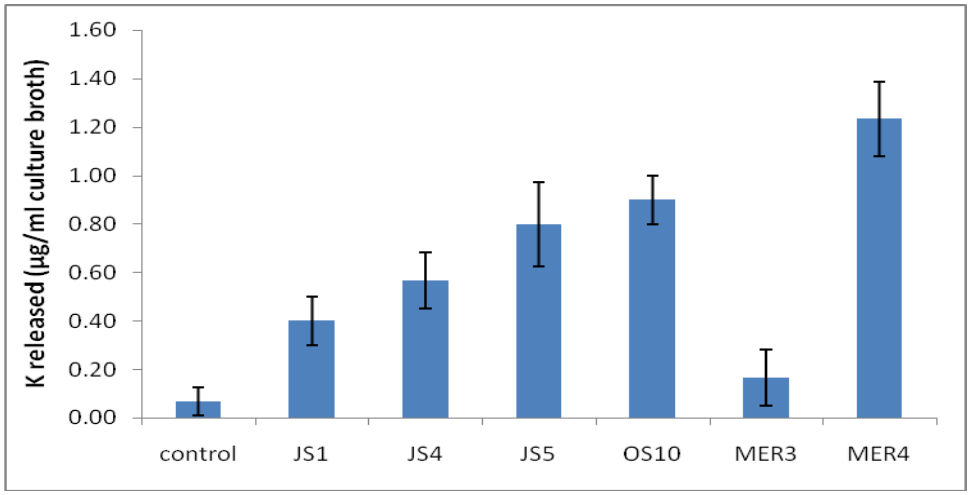


Fig.2 K released (µg/ml culture broth)



Over all, out of 32 isolates, only five isolates (JS1 to JS5) which was isolated by enrichment techniques were able to fix atmospheric nitrogen. Highest ARA was recorded with JS1 and JS 2 culture (Fig. 1). None of the other isolates were capable of nitrogen fixation. Some microorganisms fix atmospheric nitrogen to ammonia and make it available to the plant. Such microbes are found to possess enzyme nitrogenase for nitrogen fixation. Free living non-symbiotic aerobic nitrogen fixers are widely distributed in arable soils. Though non-symbiotic nitrogen fixation has a great agronomic significance, a major limitation that it faces is the availability of carbon and energy source for the energy intensive nitrogen fixation process (Saharan and Nehra, 2011) particularly under tropical soil which are inherently poor in organic matter content. The use of bio-fertilizer such as N_2 (nitrogen) fixing bacteria and beneficial micro-organism can reduce chemical fertilizer applications and consequently lower production cost.

Quantitative estimation also revealed 1.2µg/ml culture broth (having 10^7cells/ml) of K released, highest among all the positive isolates (Fig. 2). The drop in pH of media was suggestive of possible mechanism of K solubilization by the isolates.

A negative correlation (-0.70) between pH and K-released in media was observed indicating production of acids leads to solubilization of mineral and release of K in the media. Production of carboxylic acids like Lactic acid, Acetic acid and Gluconic acid in potash solubilizing media was also reported by Saiyad *et al.*, (2015), confirming our findings. In present investigation four endophytic isolates were obtained from maize roots. These isolates were caplable of mobilizing potash from mica and 24.227µg/ml (containing 10^7 cells/ml) in Potassium solubilizing media (MER3).

Conducted study concluded that out of five isolates tested, the highest ARA was recorded with JS1 ($p<0.05$; 47.08 nmole ethylene/mg cell protein/h) which was statistically at par with JS2 ($p<0.05$; 41.94 nmole ethylene/mg cell protein/h). Quantitative estimation of potassium released from mica in broth revealed highest K release by MER 4 ($p<0.05$; 1.2 µg/ml broth).

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