Bioprospective potential of a novel *Bacillus* strain isolated from the rhizosphere of *Azadirachta indica* Linn.

P. GIRI , J. BASU, H. SAHA, K. BISWAS AND P. BHOWMIK

J. Mycopathol. Res. 60(1) : *111-116, 2022;*
ISSN 0971-3719
© Indian Mycological Society,
Department of Botany,
University of Calcutta,
Kolkata 700 019, India

This article is protected by copyright and all other rights under the jurisdiction of the Indian Mycological Society. The copy is provided to the author(s) for internal non-commercial research and educational purposes.

J. Mycopathol. Res. **60(1)** : 111-116, 2022; (ISSN 0971-3719)
© Indian Mycological Society, Department of Botany,
University of Calcutta, Kolkata 700 019, India

Bioprospective potential of a novel *Bacillus* strain isolated from the rhizosphere of *Azadirachta indica* Linn.

P. GIRI [1]*, J. BASU,[2] H. SAHA[1], K. BISWAS[3] AND P. BHOWMIK[4]
[1]Department of Botany, Dum Dum Motijheel College, Kolkata -700074
[2]Department of Microbiology, Dum Dum Motijheel College, Kolkata-700074
[3]Department of Physics, Dum Dum Motijheel College, Kolkata-700074
[4]Department of Microbiology, Techno India University, Kolkata-700091

Received : 14.12.2021 Accepted : 01.02.2022 Published : 28.03.2022

Low phosphate availability associated with high salinity in the soil is a great constrain on the crop productivity. Having adverse rhizospheric effect along with the ability to grow in salt enriched soil, *Azadirachta indica* might be a suitable host harbouring microbial wealth of high adaptogenic capabilities in its rhizosphere. This article deals with the occurrence of a most efficient phosphate solubilising bacterium (PSB) in the rhizosphere of the *Azadirachta indica* which is identified as *Bacillus* sp. (Strain Bac 196; Gene Bank Accession No. KX641579.1).The bacterium is unique because of its high slat tolerance ability apart from its high phosphate solubilising efficiency. Thus, there is a sufficient potentiality for exploitation of this strain in organic farming not only to the crops of salt enriched soil but also to the other conventional crops subject to proper soil amendment.

Key words: Colony forming unit, phosphate solubilisation, rhizosphere, salt tolerance, soil amendment

INTRODUCTION

Azadirachta indica (Family : Meliaceae), commonly known as margosa tree or neem is an economically important plant because of its various medicinal properties. This plant has the ability to grow in a wide variety of agroclimatic regions. The rhizoecological niche of this plant is unique because of the occurrence of the microbial community with less diversity (Biswas *et al*. 2016). The existence of various stress inducing parameters might be the causal factors for less diversity in the microbiota prevailing in the rhizosphere of the plant. The secondary metabolites producing isolates growing in the rhizosphere exert antagonistic role that reduce the microbial load in such ecological niche (Pandey and Singh, 2013). Such antagonistic microbial population have been reported to have the ability of controlling phytopathogens (Zuhaib *et al*. 2019).

The surviving microbiota in the rhizosphere of margosa tree, therefore, must have some properties for averting the available stressors. Isolation of phosphate solubilising microbes from such stress tolerant microbial population would be a promising biotechnological strategy to recover unavailable P fixed to soil particles. As the plant can grow in a soil having wide range of salinity, therefore, therhziosphere might contain efficient phosphate solubilising microbes which would have high degree of salt tolerance. Though, stress tolerant phosphate solubilising fungal strain *Talaromyces funiculosus* has been reported from the neem rhizosphere, but no bacterial strain with such valuable potential have so far been reported from the same ecological niche (Kanse *et al*. 2015). Our study is therefore aimed to isolate and identify the bacteria having both salt tolerance and phosphate solubilising efficacy. Efforts have been made to correlate the rhizosphere soil parameters and occurrence of phosphate solubilising bacteria keeping in view applying such phosphate solubilising bacteria (PSB) to other crops through proper soil amendments.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples were collected from the rhizosphere of *Azadirachta indica* growing in eight different

*Correspondence: pranabgiri1973@gmail.com

districts of West Bengal, India. Total twenty two different sites of different districts are selected for collection of soil samples. Samples of the soil adhering to the root surface of the plant were collected 7.5 cm below the surface using soil borer. Gloves were worn to prevent cross contamination between samples and human associated microbes. Collected soil samples were preserved in sterile plastic packets with lockmouth. In the laboratory, soil materials are sieved aseptically through 2mm mesh.One part of each soil sample was used for isolation and quantification of bacteria and the other part was used for analysis of different soil parameters.

Microbiological Analysis

To isolate bacteria from collected soil sample 1 gm of soil was dissolved in 50 ml of water in 250 ml conical flask. The conical flask was kept in a shaking condition overnight to disperse the microbial load from soil particles. To measure cultivable bacterial density 1.0 ml of supernatant of each sample was diluted tenfold in a sterile water solution (9ml). The serial dilution in respect of each soil sample was continued until it reaches upto 10^{-6}. Then 1.0 ml of aliquot of each sample of 10^{-6} dilution was inoculated into a petriplate following pour plate method using nutrient broth as culture medium. The plates were incubated at 37^0C for 48 hours in a BOD incubator. The CFUs (Colony Forming Units) are counted and segregated on the basis of morphology, colour, diffusible pigment and growth pattern. The colonies were isolated and maintained in Nutrient Broth agar slants using separate code for each type. The quantification of bacteria per ml of suspension was measured using the following formula:
No. of bacteria/ml = No. of CFU x dilution factor.

Screening of Phosphate Solubilizing Bacteria (PSB)

The phosphate solubilising efficiency of the bacterial isolates was determined through streak plate technique. Modified Pikovskaya (PVK) medium (Rao, 1963) supplemented with insoluble tri calcium phosphate was used as medium for screening of phosphate solubilising bacteria. The Petri Plates with medium were inoculated in the form of a streak with a loopful of inoculum of the individual isolates and incubated at 37^0C for 12 days. Solubilisation was detected by the formation of a clear zone surrounding the growth. The extent of solubilisation was measured from the width of the clear zone formed.
The width of the clear zone = The diameter of the clear zone – the diameter of the growth in the form of streak.

Determination of salt tolerance of the most efficient PSB

Glass tube test was performed (Chen *et al*. 2018) to determine the biofilm formation ability of the isolate in different salt concentration. A culture tube containing 10 ml of liquid medium was inoculated with 100µl of isolate and incubated at 37^0C for 17 h. The medium was then removed and the cells were stained with 2 ml if 1% Crystal violet at room temperature for 30 min. Clear water was used to wash the CV staining solution until colourless water was obtained. The cells were dried at room temperature. The junction of the liquid level and colour of the glass tube wall was visually observed and result was recorded.

Analysis of the Rhizosphere Soil Sample

The soil characters like soil texture, pH, nitrogen, phosphorus and organic carbon content of different locations were measured using standard methods of Walkelyand Black (1934), Subiahand Asija, (1956),Klute (1986) andNag (2015).

Identification of Bacterial Isolate

The colony is sub-cultured and DNA is isolated from the culture. The isolated DNA is subjected to quality check and quantification. The 16S amplicon is then generated using the primers 8F and 1492R.The amplicon was sequenced by capillary electrophoresis using the Sanger Big Dye Termination Chemistry (BDT v3.1 Cycle Sequencing Kit) generating the forward and reverse sequences on ABI 3500 Genetic Analyzer. A consensus sequence of the 16S region is generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence is BLASTed against the database of NCBI gene bank. The first ten sequences are selected based on maximum identity score.These sequences were aligned using Clustal W (multiple alignment software programs). A Distance matrix was then generated using MEGA 7. The phylogenetic tree is constructed using MEGA 7 (Tamura *et al.* 2004).

RESULTS AND DISCUSSION

A total 42 bacterial cultures differing in colonial morphology and diffusible pigments were isolated in pure form from 22 soil samples collected from eight different districts of West Bengal. Results as shown in Table 1 indicate that the total bacterial counts of the rhizosphere soil of various locations are not significantly different. The microbial diversity of the rhizosphere soil samples analysed is also low. This finding corroborates with the observation made by Jyotirmayee *et al*.(2021) who showed that the morphological diversity of the colonies produced by the bacteria present in the rhizosphere soil sample of margosa tree is not as much of the other medicinal plants. All the 42 bacterial isolates were screened for phosphate solubilisation and only 36 (85%) isolates showed different degree of solubilisation. On the basis of the performance of the phosphate solubilising isolates on solid medium, they have been categorized as most efficient, potent, moderate and weak. Among the PSB only 5.58% isolates were potent and only one isolate was most efficient (Table 2 & Fig.1). The most efficient PSB isolate was coded as APC. Although the rhizosphere of the plant has high percentage of PSB but the quantity of efficient PSB is very low. Therefore, role played by the most efficient PSB in terms of increasing phosphate availability to the host plant is very important. Though report on potential plant beneficial rhizosphere fungal isolate to circumvent phosphate deficiency is available (Pitchurajan *et al*. 2020) but very few reports on bacterial isolate are available so far (Omkar, 2012). In term of agricultural sustainability such isolate could play a pivotal role (Vejan *et al*. 2016).

The most efficient PSB isolate is a Gram positive, spore forming, rod shaped bacterium having amylase producing ability (Fig. 2). It can exhibit a fast growth rate while cultivating in nutrient agar medium (Fig. 3). A consensus sequence of 16s r RNA gene of the isolate consisting of 1272 bp has been developed on the basis of forward and reverse sequence generated through capillary electrophoresis (Fig. 4). The gene sequence is then blasted against NCBI gene bank data base (Fig. 5). Based on nucleotide homology and phylogenetic analysis (Fig. 6) the isolate was proved to have homology with *Bacillus* sp. strain Bac. 196 (Gene Bank accession number: KX 641579.1). The specific identity of the isolate, however, could not be determined during the course of present study. As margosa tree cultivation has been prioritized in our state because of its high medicinal value, the application of this strain in biofertilizer formulation to enhance the growth of this plant should be emphasized.

One important finding has come up through our investigation is that the identified efficient phosphate solubilising *Bacillus* strain can retain its biofilm forming ability upto 25% of NaCl concentration (Fig. 7). It reveals the high salt stress tolerant potentiality of the strain. So, the unique strain having greater extent of phosphate solubilising and salt stress tolerant capacity could be applied in the form of biofertilizer to the host species for its sturdy growth (Dey *et al*. 2021). But question may arise, is there any possibility for the application of the same biofertilizer to other non-host crops? The answer of this question depends upon the rhizosphere soil parameters that are correlated with the occurrence of the PSB including this novel unique strain. The statistical correlation analysis presented in the Table 3 reveals that the occurrence of the PSB in the rhizosphere of margosa tree is significantly correlated with the total bacterial count (TBC), total nitrogen, organic matter, availability of magnesium, calcium, potassium, and sand. Thus any biofertilizer formulation based on this strain could effectively be applied to different non host crops, if the soil parameters are adequately amended. Detail investigations including field trials are required to reach a specific conclusion in this regard.

CONCLUSION

In conclusion, it could be said that the rhizosphere of *A. indica* is the harbour of different types of phosphate solubilising bacteria of which one *Bacillus* strain is the most efficient phosphate solubilizer. Apart from phosphate solubilising potentiality, the strain also can tolerate high salt stress. These dual characters of the strain have raised the possibility of the exploitation of the same in the formulation of effective biofertilizer to achieve fast and sturdy growth of the host plant. There is every possibility is for getting optimum benefits by the application of such biofertilizer in other non-host crops subject to the proper soil amendments.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Pradeepta Gupta Roy, Principal, Dum Dum Motijheel College for his

Table 1: Isolation and screening of Phosphate solubilising bacterial isolates from the rhizosphere soil collected from eight different districts of West Bengal.

Locality	No. of soil samples analyzed	Number of isolates	No. of phosphate solubilising isolates	Total bacterial count(TBC) per gram of soil
Kalimpong	2	5	4	$16x10^6$
Cooch Behar	2	6	5	$14x10^6$
Malda	3	5	4	$14x10^6$
Midnapore (East)	3	6	5	$15x10^6$
South 24 Parganas	4	6	6	$16x10^6$
North 24 Parganas	3	5	4	$17x10^6$
Purulia	2	4	3	$15x10^6$
Birbhum	3	5	5	$14x10^6$
TOTAL	22	42	36	$121x10^6$

Table 2: Categorization of phosphate solubilising bacterial isolates

Type of isolates[a]	No. of isolates
Most efficient	01(2.77)[b]
Potent	02(5.58)
Moderate	16(44.44)
Weak	11(30.55)
Non solubiliser	06(16.66)
Total	36

Note: Grouping of the isolates was made on the basis of the width of the halo zone formed. Measurement was taken after 12 days of incubation at 37^{0}C.
[a] Most efficient solubilizer = halo zone -14mm, Potent solubilizer = halo zone e" 9mm but < 14 mm, Moderate solubilizer = halo zone 6-8 mm, Weak solubilizer = halo zone < 6 mm.
[b] Figures in parenthesis denote % of total isolates

kind support and help to do the experimental works availing all the infrastructural facilities of the College.

Fig. 1 : The extent of phosphate solubilisation by the most efficient isolate (*Bacillus* sp.)

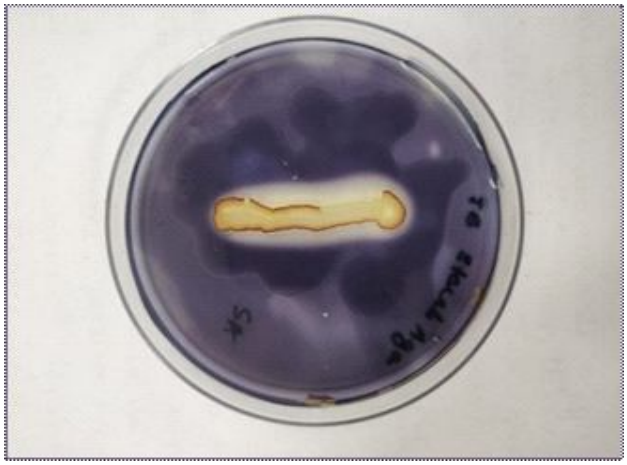

Fig. 2 : Amylase production ability of the most efficient PSB (*Bacillus* sp.)

Fig. 3 : Vigorous growth habit of the most efficient PSB

Table 3: Multiple correlations between the number of PSB and the physicochemical characteristics of rhizosphere soil sample of *A. indica*

	PSB	TB	pH	OM	P	N	CEC	Mg	Ca	K	Sand
PSB	1.00										
TB	0.800**	1.00									
pH	-0.346	-0.05	1.00								
OM	0.903**	0.036	0.152	1.00							
P	0.031	0.061	-0.052	-0.064	1.00						
N	0.856**	0.53**	-0.031	0.822**	-0.022	1.00					
CEC	0.003	0.04	-0.005	0.030	0.031	-0.105	1.00				
Mg	0.53**	0.03	0.210	-0.035	-0.253	0.124	-0.025	1.00			
Ca	0.822**	-0.231	-0.352	0.532	0.423	0.041	0.352	0.112	1.00		
K	0.741**	-0.42	-0.032	-0.421	0.003	0.0421	-0.523	0.003	-0.231	1.00	
Sand	0.551**	-0.003	-0.230	-0.123	-0.152	0.231	-0.321	-0.523	-0.33	-0.023	1.00

** indicates a significant correlation at (P< 0.01)
PSB - Phosphate solubilising bacteria, TB - Total Bacteria, OM - Organic matter, CEC - Cation Exchange Capacity, P - Available phosphate

Fig. 4 : Forward primer chromatogram, reverse primer chromatogram and development of consensus sequence of the 16s r DNA of the most efficient PSB

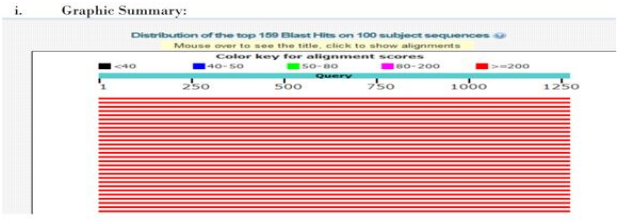

Fig. 5 : BLAST Data: Analysis from NCBI website (GenBank)

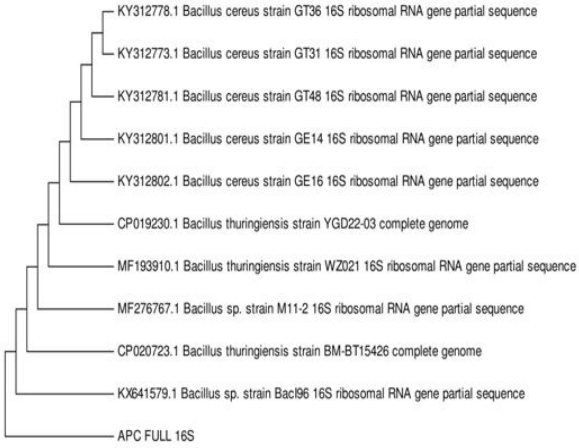

Fig. 6 : Evolutionary analyses were conducted in MEGA7. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1272 positions in the final dataset.

Fig. 7 : Biofilm forming ability of the most efficient PSB (*Bacillus* sp.) upto 25% NaClconcentration

REFERENCES

Biswas, K., Basu, J., Ghosh, A., Giri, P. 2016. Study of the rhizospheric bacterial population of *Azadirachta indica* (Neem) of North 24 Parganas district of West Bengal forbio-prospective consideration. *Inter. J. Exp. Res. Rev.* **6**:62-66.

Chen, X.P., Ali, L., Wu, L.Y., Liu, C., Gang, C.X., Huang, Q.F., Ruan, J.H., Bao, S.Y., Rao, Y.P.,Yu, D. 2018. Biofilm formation plays a role in the formation of multidrug-resistant *Escherichia coli* toward nutrients in microcosm experiments. *Front. Microbiol.* **9**:367.

Dey, G., Banerjee, P., Sharma, R.K., Maity, J.P., Etesami, H., Shaw, A.K., Huang, Y.H., Huang, H.B., Chen, C.Y. 2021. Management of phosphorus in salinity-stressed agriculture for sustainable crop production by salt-tolerant phosphate-solubilizing bacteria-A review. *Agronomy*, **11** :1552.

Jyotirmayee, B., Mahapatra, S., Mahalik, G. 2021. Comparative analysis of rhizospheric bacteria associated with four medicinal plants. *Asian J. Biologic. Life Sci.* **10**:353

Kanse, O.S., Whitelaw-Weckert, M., Kadam, T.A.,Bhosale, H.J. 2015. Phosphate solubilization by stress-tolerant soil fungus *Talaromycesfuniculosus* SLS8 isolated from the Neem rhizosphere. *Ann.Microbiol.* **65** :85-93.

Klute, A. 1986. Water retention: laboratory methods. *Methods of soil analysis: Part 1 Physical and Mineralogical Methods* **5**:635-662.

Nag, A. 2015. Analytical and Instrumental TechniquesIn*: Agriculture, Environmental And Food Engineering*. PHI Learning Pvt. Ltd.

Omkar, S. 2012. Isolation and characterization of phosphate solubilising bacteria from rhizospheric soil samples. *Online Inter.Interdisc. Res. J.* **2**:28-29.

Pandey, A.,Singh, A. 2013. A comparative study on secondary metabolites producing microbes isolated from rhizosphericand non-rhizospheric region of *Azadirachta Indica* and *Oscimumtenuiflorum*. *Inter. J. Pharmaceut. Res. Allied Sci.* **2**:36-48

Pitchurajan, K., Aishwarya, M., Sujitha, S., Devi, K.S., Mohan, V. 2020. Screening and Selection of Potential Plant Beneficial Rhizosphere Fungi from Medicinally Important Plant Species in Tamil Nadu, India*. J. Acad.Indust. Res.***8** :153-159.

Rao Sundara, W.V.B. 1963. Phosphate dissolving organisms in the soil and rhizosphere. *Ind. Jour. of Agr. Sci.*, **33**:272-278.

Subiah, B. V., Asija,G. L. 1956. A rapid procedure for the determination of available nitrogen in soil. *Curr. Sci.* **25**: 259-260.

Tamura, K., Nei, M., Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. National Acad. Sci.* **101**:11030-11035.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., Nasrulhaq Boyce, A. 2016. Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. *Molecules*, **21**: 573.

Walkley, A., Black, I.A. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil science*, **37**:29-38.

Zuhaib, M., Ashraf, S., Musheer, N., Ali, M. 2019. Role of Rhizospheric Microbes in the Management of Phytopathogens. In *Plant Health Under Biotic Stress* , Springer, Singaporepp. 83-98.