academicJournals

Vol. 13(34), pp. 3450-3454, 20 August, 2014
DOI: 10.5897/AJB2013.13343
Article Number: 41B4CDB46834
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular identification of phosphate solubilizing bacterium (*Alcaligenes faecalis*) and its interaction effect with *Bradyrhizobium japonicum* on growth and yield of soybean (*Glycine max* L.)

Nandini, K.[1], Preethi, U.[1] and Earanna, N.[1,2]*

[1]Department of Biotechnology, University of Agricultural Sciences, GKVK, Bangalore-560065, India.
[2]Present address: Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bangalore-560065, India.

Received 3 October, 2013; Accepted 4 August, 2014

A phosphate solubilizing bacterium was isolated from the rhizosphere soil of upland rice and identified by 16S rRNA gene sequencing. The gene sequence showed 99% homology with *Alcaligenes faecalis*. Based on the gene sequence homology, it was identified as *A. faecalis.* Interaction effect of this bacterium on growth and yield of soybean was studied under glass house conditions by inoculating the bacterium either alone or in combination with *Bradyrhizobium japonicum* and *Bacillus megaterium* or both. The inoculated plants showed significantly taller plant height, more number of leaves, higher numbers of pods, plant dry weight and grain yield compared to un-inoculated ones (control). The triple inoculation was found superior compared to single as well as dual inoculations. Nitrogen and phosphorus content of the plant tissue was also higher in triple inoculation compared to others, which indicates their synergistic interaction in the rhizosphere of soybean.

Key words: *Alcaligenes faecalis*, *Glycine max,* phosphate solubilization, 16S rRNA gene sequencing.

INTRODUCTION

Next to nitrogen, phosphorus is the major plant nutrient required for early establishment and better growth of plant. Phosphorus also induces early maturity of the crop. Application of phosphate fertilizers significantly increased seed set, seed filling efficiency and kernel yield (Zehra, 2011). However, the phosphorus added to soil through phosphate fertilizers is fixed by soil minerals such as aluminum, iron and calcium, and form their respective phosphates eventually leading to phosphorus deficiency (Gyaneshwar et al., 2002). In such cases, a large fraction of soil microflora can dissolve insoluble phosphates in soil by secreting organic acids and make them available to plants as the pH of soil greatly influences phosphate solubilization.

Occurrences of phosphate solubilizing bacteria have been reported from different environmental niches

*Corresponding author. E-mail:earanna7@gmail.com, earanna7@yahoo.com.

(Castagno et al., 2011). Those isolated from alkaline soil showed tolerance to wide range of temperature and pH besides utilizing both organic and mineral phosphate to release absorbable phosphate ion to plants (Mohammad et al., 2009). Castagno et al. (2011) isolated different genera of phosphate solubilizing bacterium (PSB) through *Pantoea, Erwinia, Pseudomonas, Rhizobium* and *Enterobacter* from Salado river basin and characterized by 16S rRNA gene sequence analysis. *Aspergillus* and *Bacillus subtilis* have been found to be dominant species in the rhizosphere soil of beetle vine (Tallapragada and Seshachala, 2012). It is well known that 16S rRNA is a part of protein synthesizing machinery, which does not vary much from one organism to another. In molecular taxonomy, 16S rRNA gene sequencing technique is widely used for classifying bacteria isolated from different sources (Heilig et al., 2002; Woo et al., 2008; Patil et al., 2010; Naz et al., 2012).

Broader spectrum of phosphate solubilization and plant growth promotion resulted in the production of higher plant biomass (Panhwar et al., 2011). PSB applied with triple super phosphate increased plant height, number of tillers and mineral nutrient content in tissues of aerobic rice (Sarkar et al., 2012). Inoculation of PSB increased phosphorus uptake, growth and yield of upland rice (Panhwar et al., 2013). Soybean plants inoculated with *B. japonicum* together with pseudomonas strain (Phosphate solubilizer) resulted in 38% increased grain yield in pot culture experiments and 12% grain yield in field conditions (Aftab et al., 2010). Similarly, inoculation of *B. japonicum* increased phenolic compounds, organic acids, sterols and triterpenes in the aerial part of soybean (Carla et al., 2011; Luis et al., 2013). In this study, we isolated a phosphate solubilizing bacterium, from the root zone soil of upland rice, identified as *Alcaligenes faecalis* by 16S rRNA gene sequence analysis and explored its interaction effect with *B. japonicum* and *B. megaterium* on growth and yield of soybean.

MATERIALS AND METHODS

Isolation

Phosphate solubilizing bacteria were isolated from the root zone soil of upland rice by dilution plate method. Dilution (1:100) was made in sterile water and transferred 0.1ml on Pikovskays's medium dispensed in petri plates. These plates were incubated at 30°C for four days. The colonies forming clear zone around them were transferred on a fresh Pikovaskays's agar, purified and used for molecular identification.

Total genomic DNA isolation

Total genomic DNA was extracted by alkaline lysis method (Sambrook et al., 1989). The bacterial isolate was grown in Pikovakay's broth for 48 h at 30°C and 3 to 5 ml bacterial culture were pelleted with centrifugation at 12,000 rpm. The pellet was re-suspended in 650b µL of extraction buffer (10 mM Tris HCl pH 8.0, 20 mM EDTA and 250 mM NaCl) and incubated at 65°C for 30 min for lysis. To the extract, 100 µL of 5 M potassium acetate solution was added and placed on ice for 10 min for precipitation of protein and carbohydrates and clear supernatant was collected by centrifugation. DNA was precipitated by adding 0.6 volumes of chilled isopropanol and the DNA pellet was collected by centrifugation at 12,000 rpm. The pellet was washed twice with 70% ethanol, air dried and dissolved in 10 mM TE (10:1) buffer stored in aliquots at -20°C. The quality and quantity of the isolated DNA were checked with 0.8% agarose gel electrophoresis and spectrophotometrically.

Primer designing and PCR amplification

The primers were designed manually based on the already reported 16S rRNA sequences from the NCBI database (http://www.ncbi.nlm.nih.gov). A forward primer 5' GTTAGATCTTGGCTCAGGACGAACGC 3' and reverse primer 5' GATCCA GCCGCACCTTCCGATACG 3' were designed and used for the present study. The primers were custom synthesized by Sigma-Aldrich (Sigma, USA) and diluted accordingly for the polymerase chain reaction reactions. Annealing temperature for primer pair was standardized and PCR was performed in a 40 µL reaction volume containing 1X buffer with $MgCl_2$ (1.5 mM), dNTP's (200 µM), forward and reverse primers (0.5 µM each), *Taq* DNA polymerase (1 U Genei Bangalore) and template DNA (50 ng). Amplification was carried out with an initial denaturation at 96°C for 3 min followed by 35 amplification cycles consisting of 94°C for 1 min, 50°C for 30 s and 72°C for 1 min and a final extension step at 72°C for 10 min. Controls for PCR reactions were carried out with the same primers without providing template DNA. PCR products were separated on 1.0% agarose gel and documented using gel documentation system Hero Lab, Germany.

Cloning, plasmid isolation and sequencing

The PCR products were eluted from the gel using GenElute™ Gel Extraction Kit (Sigma, USA) and the eluted products were cloned into pTZ57R/T cloning vector using InsT/A clone PCR product cloning kit (MBI, Fermentas Life Sciences) after determining the appropriate vector: insert ratios. The ligation reaction was performed with 1.5 µL of 10X ligation buffer, 1 µL (50 ng) T/A cloning vector, 1 µL (5U) T4 DNA ligase in a 15 µL reaction volume at 16°C overnight. The ligated product was used to transform competent *Escherichia coli* (DH5α) cells using heat shock method (Sambrook et al., 1989) and plated on Luria Bertoni (LB) agar medium containing ampicillin (100 µg/ml) and X-gal, IPTG (50 µg/ml each). The recombinant colonies were initially screened by blue white selection, followed by colony PCR using M13 primers (Sambrook et al., 1989). Single positive colony was selected, inoculated in 3 ml LB broth containing ampicillin (100 µg/ml) and incubated overnight at 37°C. Cells were harvested by centrifuging at 12,000 rpm for 1 min and media was removed by aspiration, leaving the bacterial pellet as dry as possible. Plasmid was isolated using GenElute™ HP Plasmid MiniPrep Kit (Sigma, USA) following the manufacturer's protocol. The isolated plasmid was sequenced (SciGenom Labs Pvt. Ltd., India) using M13 forward and reverse primers.

Sequence analysis and homology search

Sequence results were analyzed with VecScreen online software from NCBI (nttp://www.ncbi.nlm.nih.gov) for removing the vector contamination. Forward and reverse primer sequences were checked against each other by generating the reverse complement of the "reverse" sequence using FastPCR Professional (Experimental test version 5. 0. 83) and aligning it with the "forward" sequence with the help of CLUSTAL W Multiple Sequence Alignment Program using the online software SDSC Biology Workbench (San Diego Supercomputer Center). The full length gene homology

Table 1. Influence of *A. fecalis, B. megaterium* and *B. japonicum* on growth, yield, nitrogen and phosphorus content of soybean.

Bacterial culture	Plant height (cm)	Number of pods	Number of leaves	Dry weight of plant		Dry weight (g) of seeds	Nitrogen content (mg/plant)		Phosphorus content (mg/plant)	
				Shoot	Root		Shoot	Root	Shoot	Root
Control	27.66^{c}	28.00^{b}	8.66^{d}	2.92^{c}	0.47^{c}	4.35^{d}	4.14^{c}	0.60	0.83^{b}	0.08^{b}
Alcaligenes fecalis	43.33^{b}	40.66^{a}	12.66^{bc}	5.85^{b}	1.06^{bc}	7.53^{bc}	8.45^{b}	0.84	1.50^{b}	0.12^{ab}
Bacillus megaterium	41.00^{b}	40.66^{a}	10.33^{cd}	5.72^{b}	1.28^{abc}	6.68^{c}	8.40^{b}	0.73	1.63^{b}	0.13^{ab}
Bradyrhizobium japonicum	46.33^{ab}	41.00^{a}	12.00^{bc}	6.44^{b}	1.29^{abc}	6.63^{c}	8.28^{b}	0.84	1.49^{b}	0.17^{ab}
A.faecalis + B.meagterium	40.66^{b}	39.00^{ab}	12.00^{bc}	6.14^{b}	2.12^{a}	8.21^{bc}	9.49^{b}	1.36	1.83^{b}	0.26^{ab}
A.faecalis + B. japonicum	44.33^{b}	40.66^{a}	13.66^{b}	6.75^{b}	1.79^{ab}	9.83^{ab}	10.07^{b}	0.88	1.76^{b}	0.18^{ab}
A.faecalis+ B. meagterium+ B. japonicum	53.00^{a}	45.33^{a}	21.66^{a}	10.87^{a}	2.15^{a}	10.64^{a}	16.99^{a}	1.67	3.24^{a}	0.34^{a}
LSD	6.80	11.26	2.70	1.77	0.89	2.20	1.83	NS	0.96	0.20

Means with same superscript along the column do not differ significantly at p=0.05 level by DMRT. NS, Non significant.

search was performed with Centre for Biotechnology Information (NCBI) (nttp: //www.ncbi.nlm. nih.gov/BLAST/) (Altschul et al., 1990).

Interaction effect of P solubilizers with *B. japanicum* on growth and yield of soybean

A. faecalis and *B. megaterium* along with *B. japanicum* were used in the green house experiment to explore their interaction effect on growth and yield of soybean. *A. faecalis* and *B. megaterium* were grown in Pikovakay's broth and the *B. japanicum* was on the yeast extract mannitol medium on a rotary shaker at 30°C for four days. Culture having appropriate population (~10^{7}-10^{8}cells/ml) was used for inoculation. The red sandy loam soil was mixed with a recommended quantity of farm yard manure (FYM) and filled in polyculture bags of 10"×16"size (4kgs/bag) and watered one day prior to sowing. The bacterial cultures (10 ml each) were inoculated as per treatment allocation given in Table 1. The vegetable soybean seeds were sown and allowed for germination. After germination, two plants per bag were maintained in each treatment. Observations for growth (plant height, number of leaves and number of pods) were recorded on 90th day, then the crop was harvested, dried in hot air oven at 60°C for five days to obtain constant weight and observation for plant biomass was recorded. The seeds were separated from pod and dry weight was recorded. Total nitrogen content of the plant was estimated by Micro Kjheldhal method and phosphorus content was estimated by vanadomolybdate yellow colour method (Jackson, 1973). The data obtained were statistically analyzed by analysis of variance (ANOVA) using MSTAT-C soft ware and the means were separated by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Isolation of agriculturally important microorganisms from different ecological niche is advantageous in efficient strain screening, which can be used for biofertilizers production. Formation of clear zone around the colony of bacteria is an indication of phosphate solubilization when grown on Pikovskay's agar (Mahantesh and Patil, 2011). PSB was isolated from the root zone of upland rice and the bacterium formed clear zone around the colony on Pikovskay's agar indicating phosphate solubilization. *A. faecalis* isolated from Dehradun valley soil samples solubilized phosphates (Shruti and Pathak, 2012). Further, the bacterium was identified by 16SrRNA gene sequence analysis. The genes encoding 16S rRNA in prokaryotes and 18S rRNA in eukaryotes are most widely used in molecular phylogenetics as these genes are universally distributed, functionally constant, sufficiently conserved and have adequate length (Madigan et al., 2009). Thus, the 16s rRNA gene sequence has emerged as a preferred genetic technique for the identification of poorly described strain (Farrelly, 1995; Goto et al., 2000; Clarridge, 2004). In this study, the primers designed yielded approximately 1.5 kb product which was separated on 1% agarose gel and cloned into T/A cloning vector (pTZ57R/T). The recombinant bacterial colonies obtained after transformation were confirmed through colony PCR, as well as, with isolated plasmid (Figure 1). The sequence analysis (BLASTn) showed 99% homology with earlier reported *A. faecalis*. Hence, the bacterium was confirmed as *A. faecalis*. Jimenez et al. (2011) reported similar BLAST analysis for characterizing free nitrogen fixing bacteria of the genus *Azotobacter* from soil samples.

Use of PSB as biofertilizers has currently increased phosphorus uptake in plants and

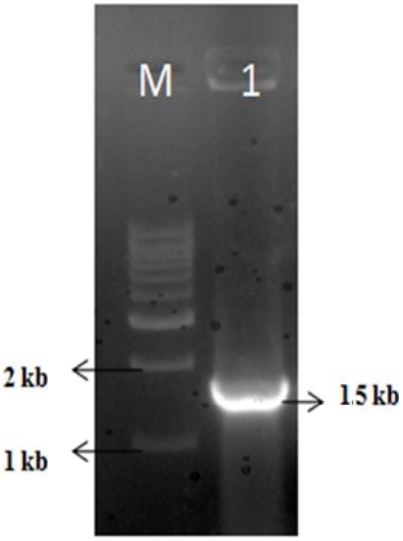

Figure 1. Amplification of 16S rDNA of *Alcaligenes faecalis* isolated from the rhizosphere soil of upland rice (M: marker, lane 1: 16S rDNA).

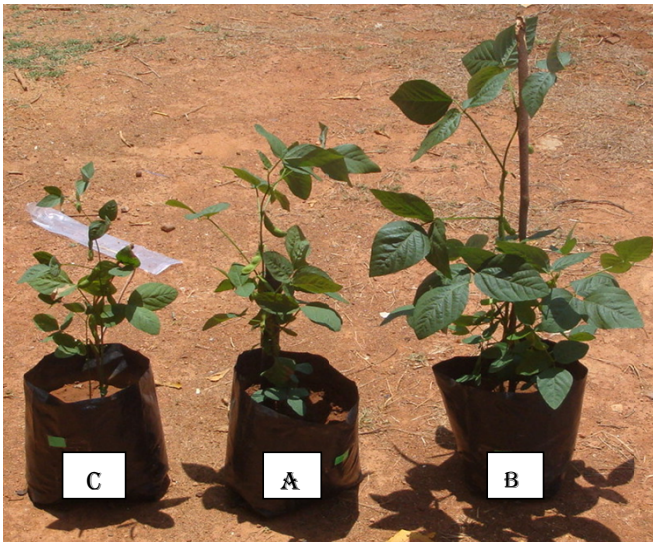

Figure 2. A. Treated with *A. faecalis*; **B.** treated with *A. faecalis* and *B. japonicum;* **C.** control.

improved yields in several crop such as *Lens culinaris Medic,* rice, sugarcane, *Lotus tenui* (Monika et al., 2009; Sarkar et al., 2012; Sundara et al., 2002; Castagno et al., 2011). Inoculation of PSB to aerobic rice had increased phosphorus uptake by plants and resulted in higher plant height and yield (Panhwar et al., 2011). In our study, inoculated treatments showed significantly increased growth in terms of plant height, number of leaves, number of pods, plant dry weight and grain yield compared to uninoculated plants. Inoculation of *A. faecalis* alone significantly increased the plant height compared to *B. megaterium*. Co-inoculation of *A. faecalis* +*B. megaterium* + *B. japonicum* significantly increased growth and yield of soybean (Table 1 and Figure 2) compared to single as well as dual inoculations. This indicates that the new isolate of *A. faecalis* is an efficient PSB compared to *B. megaterium*. Phosphate solubilizing bacteria alone or in combination with nitrogen fixing bacteria could promote growth and yield of plants (Castagno et al., 2011). Sarkar et al. (2012) reported significantly taller plant height and more number of tillers per plant due to PSB inoculation along with triple super phosphate to rice plants.

Nitrogen and phosphorus content in the tissue of soybean plant was found significantly higher in inoculated plants (Table 1) compared to un-inoculated ones. The highest was obtained from triple inoculation. However, nitrogen content of root did not vary significantly over the control. Similarly, highest shoot and root phosphorus content was observed in the triple inoculation treatment (Table 1) whereas; others were on par with the control plants. This indicated that the supplement of nitrogen by *B. japonicum* through biological nitrogen fixation and the availability of soluble phosphorus in rhizosphere of the plant is due to PSB inoculation (Qureshi et al., 2012) which resulted in increased growth and yield of soybean plant. Besides, bacteria involving better scavenging of soluble phosphorus would enhance plant growth through biological nitrogen fixation (Alia et al., 2013). This study suggests that the *A. faecalis* is an efficient PSB and interacts synergistically with *B. japonicum* to promote growth and yield of soybean crop.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Aftab Afsal, Asghari Bano, Mussarat Fathima (2010). Higher soybean yield by inoculation with N-fixing and P-solubilizing bacteria. Agron. Sustain. Dev. 30: 487-495.

Alia, Aftab Afzal, Shahida N Khokhar, Bhushra Jabeen Saeed A Asad (2013). Phosphate solubilizing bacteria associated with vegetables roots in different ecologies. Pak. J. Bot. 45: 535-544.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol., 215: 403-410.

Carla Couto, Luis R. Silva, Patrilcia Valentao, Encarna Velazquez, Alvaro Peix, Paula B. Andrade (2011). Effects induced by the nodulation with Bradyrhizobium japonicum opn Glycine max (soybean) metabolism and antioxidant potential. Food Chem. 127: 1487-1495.

Clarridge III JE (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clin. Microbiol. Rev. 17(4): 840-862.

Castagno LN, Estrella MJ, Sannazzaro AI, Grassano AE, Ruiz OA (2011). Phosphate solubilization mechanism and invtro plant growth promotion activity mediated by Pantoea eucalypti isolated from Lotus tenuis rhizosphere in the Salado River Basin (Argentina). J. Appl. Microbiol. 110:1151-65.

Farrelly V, Rainey FA, Stackebrandt E (1995). Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. 61: 2798-2801.

Goto K, Omura T, Hara Y, Sadaie Y (2000). Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. J. Gen. Appl. Microbiol. 46: 1–8.

Gyaneshwar P, Naresh KG, Parekh LJ, Poole PS (2002). Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245: 83-93.

Heilig HGHJ, Zoetendal GE, Elaine EV, Philippe M, Akkermans ADL, Willem M de V (2002). Molecular Diversity of *Lactobacillus spp*. and other Lactic Acid Bactria in the Human intestine as determine by specific amplification of 16S Ribosomal DNA. Appl. Environ. Microbiol. 68: 114-123.

Jackson ML (1973). Soil chemical analysis, prentice hall (India) pvt Ltd. New Delhi. pp. 239-241.

Jimenez DJ, Montana JS, Martinez MM (2011). Characterization of free nitrogen fixing bacteria of the genus *Azotobacter* in organic vegetable-grown colombian soils. Braz. J. Microbiol. 42: 846-858.

Luis R Silva, Maria J Pereira, Jessica Azevedo, Rebeca Mulas, Encarna Velazquez, Fernando Gonzalez-Andres, Patricia Valentao, Paula B Andrade (2013). Inoculation with *Bradyrhizobium japonicum* enhances the organic and fatty acids content of soybean (*Glycine max* (L.)Merrill) seeds. Food Chem. 141:3636-3648.

Madigan MT, Nartinko JM, Dunlap PV, Clark DP (2009). Microbial Genomics: In: Brock Biology of Microorganisms, Pearson Education, Inc., San Francisco, USA. pp. 343-397.

Mahantesh P, Patil CS (2011). Isolation and Biochemical characterization of phosphate solubilizing microbes. Int. J. Microbiol Res. 3: 67-70.

Mohammad Ali Malboobi, Parviz Owlia, Mandana Behbahani, Elaheh Sarokhani, Sara Moradi, Bagher Yakhchali, Ali Deljou, Kambiz Morabbi Heravi (2009).Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates. World J. Microbiol. Biotechnol. 25: 1471-1477.

Monika K, Dheeraj V, Zia ul-Hasan, Umesh KD (2009). Effect of PSB (Phosphate Solubilizing Bacteria) morphological on characters of *Lens culinaris Medic.* Biol. Forum 1(2):5-7.

Naz I, Asghari B, Bushra R, Simab P, Mazhar I, Ambrin S, Farah Y (2012). Potential of *Azotobacter vinelandii* Khsr1 as bio-inoculant. Afr. J. Biotechnol. 11:10368-10372.

Panhwar QA , Radxiah O, Zaharah AR, Sariah M, Razi I Mohd (2011). Role of phosphate solubilizing bacteria on rock phosphate solubility and growth of aerobic rice. J. Environ. Biol. 32: 607-612.

Panhwar QA, Radziah O, Naher UA, Zaharah AR, Razi MI, Shamshuddin J (2013). Effect of phosphate-solubilizing bacteria and oxalic acid on phosphate uptake from different P fractions and growth improvement of aerobic rice using 32P technique. Aust. J. Crop Sci. 7:1131-1140.

Patil MM, Ajay pal, Anand T, Ramana KV (2010). Isolation and characterization of Lactic acid Bacteria from curd and cucumber. Indian J. Biotechnol. 9:166-172.

Qureshi MA, Ahmad ZA, Akhtar N, Iqbal A, Mujeeb F, Shakir M A (2012). Role of phosphate solubilizing bacteria (PSB) in enhancing Pavailability and promoting cotton growth. J. Anim. Plant Sci. 22: 204-210.

Sambrook J, Fritsol EF, Maniatis T (1989). Molecular Cloning- A Laboratory Manual, 2nd Ed. Cold Spring Harbor, New York.

Sarkar A, Islam T, Biswas GC, Alam S, Hossain M, Talukder NM (2012). Screening for phosphate solubilizing bacteria inhabiting the rhizoplane of rice grown in acidic soil in Bangladesh. Acta Microbiol. Immunol. Hung. 59: 199-213.

Shruti Agrawal KM, Pathak RK (2012). Phosphate Solubilization by *Alcaligenes faecalis* over *Pseudomonas fluorescens*. Agric. Sci. Res. J. 2: 92-94.

Sundara B, Natarajan V, Hari K (2002). Influence of Phosphorus Solubilizing Bacteria on the changes in soil available phosphorus and sugarcane and sugar yields. Field Crop Res. 77:43-49.

Tallapragada P, Seshachala U (2012). Phosphate-solubilizing microbes and their occurrence in the rhizospheres of Piper betel in Karnataka, India. Turk. J. Biol. 36: 25-35.

Woo PCY, Lau SKP, Teng JLL, Tse H, Yuen K (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin. Microbiol. Infect. 14: 908–934.

Zehra EKIN (2011). P- solubilizing bacteria and phosphorus fertilizer applications to sunflower improves seed set, seed filling efficiency and concentration of macro and micro nutrients of seeds. Turk. J. Field Crops 16: 183-189.