ANUL LIV **2009**

S T U D I A
UNIVERSITATIS BABEŞ-BOLYAI

CHEMIA

SPECIAL ISSUE

2

Desktop Editing Office: 51ST B.P. Hasdeu Street, Cluj-Napoca, Romania, Phone + 40 264-405352

CUPRINS – CONTENT – SOMMAIRE – INHALT

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- Science Citation Index Expanded (also known as SciSearch®)
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- Journal Citation Reports/Science Edition

STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, LIV, Sp.Iss. 2, 2009

APPLIED MICROBIAL TECHNOLOGY: SOLUBILIZATION OF INORGANIC PHOSPHATE AND PRODUCING SIDEROPHORE BY ISOLATED NITROGEN FIXING BACTERIA

ÉVA LASLO[a], ÉVA GYÖRGY[b], GYÖNGYVÉR MARA[b], ÉVA TAMÁS[a], ISTVÁN MÁTHÉ[b], BEÁTA ÁBRAHÁM[b], SZABOLCS LÁNYI[b]

ABSTRACT. The benefic microbes play an important role in the transformation, mobilization and solubilization of the nutrient content of the soils. Their nitrogen fixing ability to in symbiosis with legume has a central role in the nitrogen-cycle of most natural ecosystems. A total of 30 bacteria were isolated from different leguminous plants nodules and rhizospheric soils. The morphological and biochemical characterization of these isolates was made and they was screened for their plant-growth promoting factors: tricalcium phosphate mobilization capability and siderophore productivity by agar plate method. Some A of these bacteria were classified genotypicaly.

Keywords: leguminous plant, inorganic phosphate, siderophore production.

INTRODUCTION

Rhizosphere bacteria that colonize plant roots may enhance the plant growth, the development and crop yield by different ways e.g. by enhancing the bioavailability of soil phosphates for plant roots. They increase phosphorus mobilization in soil and produce ferric ion specific chelating agents, which capture ferric oxides by converting them into water soluble forms available for absorption for the roots [1].

Phosphorus is an important element for plant growth because it stimulates growth of young plants, promotes a vigorous start and hastens maturity. Consequently, plant growth is diminished, maturity is delayed and yield reduced when an inadequate supply of phosphorus is present [2, 11].

Approximately 70-80% of phosphorus found in cultivated soils is inorganic. The solubilising ability of a microorganism is related to its organic acid production that binds cations such Al^{3+}, Ca^{2+}, and Fe^{3+} from mineral phosphates and releases soluble phosphorus, enhancing plant nutrition [6].

[a] *Universitatea POLITEHNICA, Bucureşti, Facultatea de Chimie Aplicată şi Ştiința Materialelor; Spl. Independenței nr. 313, Sector 6, RO-060042 Bucureşti lasloeva@sapientia.siculorum.ro*

[b] *Universitatea SAPIENTIA Cluj-Napoca, Facultatea de Ştiințe Sociale şi Tehnice, Piața Libertății nr. 1, RO-530104 Miercurea-Ciuc*

In neutral or alkaline soils, the production of acids decreases the pH of the rhizosphere, favourising thus the solubility of calcium phosphates and apatites. The production of organic acid leads to acidification of microbial cells and their surroundings, consequently enhance the release of phosphate ions from the by H^+ substitution for Ca^{2+} [7].

Insoluble sources of inorganic phosphorus in liquid broth are solubilised by bacteria either by lowering the pH or by enhancing the cation chelation by phosphates. Chelation involves the formation of two or more coordinative bonds between phosphate containing molecules and metal ions, results a ring structured complexes. Chelation by an organic acid ligand occurs via oxygen contained in hydroxyl and carboxyl groups [5].

The proton release is thought to be associated with cation assimilation, such as ammonium ion (NH_4^+). H^+ excretion accompanying NH_4^+ assimilation is responsible for P-solubilization.

A major mechanism for mineral phosphate solubilization in Gram-negative bacteria is the direct oxidation of glucose to gluconic acid. Gluconic acid biosynthesis is carried out by the glucose dehydrogenase enzyme and the co-factor, pyrroloquinoline quinine [9].

Iron is a limiting bioactive metaliv element in soil and is essential for plant growth. Rhizobacteria have to develop some strategies to acquire iron [4]. To sequester and solubilize ferric iron, many microorganisms synthesize and utilize siderophores that are relatively low molecular weight, ferric-ion specific chelating agents [8]. In case when microorganisms grow under iron-deficient conditions, they synthesize and excrete siderophores to sequester and solubilize iron from the environment [12]. Chemically, most siderophores belongs to hydroxamates or catechols [3].

Generally, the aim of our study is the development of plant-growth promoting inoculants. In the present article we discuss the cell morphology, biochemical characteristics and molecular categorization of the isolated nodule- and rhizobacteria, as well as we present data on the inorganic phosphate solubilization and siderophore production.

RESULTS AND DISCUSSION

In this assay 30 isolates from different leguminous plants and their rhizosphere from Ciuc Mountains were characterized through morphological analysis, biochemical tests, molecular restriction fragment polymorphism, and were screened *in vitro* for their beneficial traits like siderophore production, and inorganic phosphate solubilization.

All of the isolated bacteria were found to be Gram-negative and three of the isolates were spore formulating.

A lot of bacteria have the capability to utilize oxygen from nitrate as electron acceptor in anaerobic respiration, a reaction controlled by the nitratase enzyme. Twenty three from the isolated bacterial strains possess nitrate reducing capability.

Results of the oxygen utilization test on thyoglicolate agar showed, that nine of the isolates were strictly aerobic, two facultative anaerobic, three microaerophilic and 16 bacterial isolates aerobic and facultative anaerobic.

The ability of carbohydrate consumption, gelatin liquefaction and detection of cytochrome-oxidase activity gives essential information in the identification process of the bacteria [14, 17]. From the carbohydrate utilization's point of view al the examined isolates are able to oxidize the glucose, and only three isolates possess lactose oxidizing capability. In case of seven bacterial isolates the liquefaction of gelatine was detected, evidencing the gelatinase exoenzyme presence. Based on oxidase-test, we can assume that in case of 16 isolated strains the cytochrome c oxidase was present. Two of the isolated and characterized bacteria have similar biochemical and morphologic properties with the symbiotic nitrogen-fixing bacteria [16]. These two cultures belong to the same group on the basis of the PCA (Fig.1). A number of 20 bacterial isolates from the 30 studied cultures were found to be positive for the studied beneficial traits, the siderophore production and tricalcium phosphate solubilization. The plant growth promoting traits of PGPR rhizobacteria are reported in literature [4, 9].

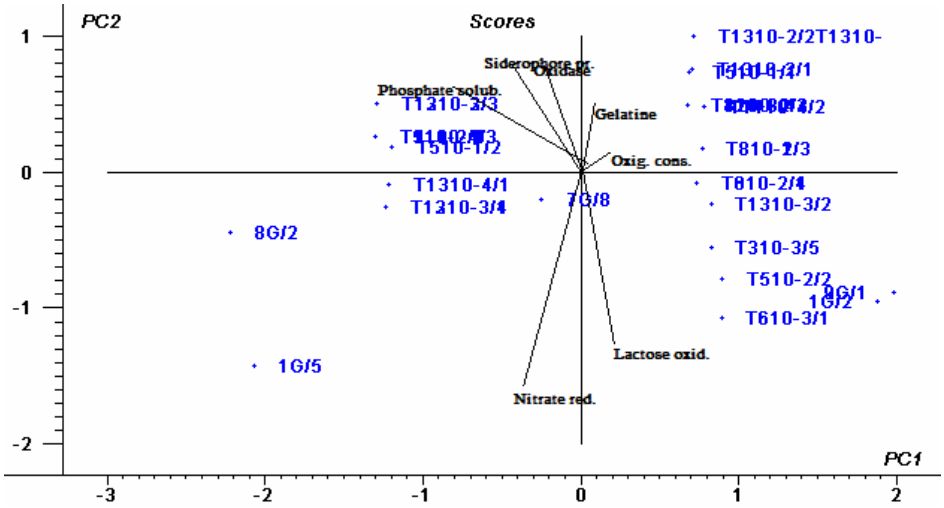

Figure 1. Classification of the isolates on the basis of examined properties using principal component analysis.

Four isolates originated from the nodules of *Trifolium medium* L.*, Anthyllis vulneraria* L. and from rhizospheric soil of *Onobrychis montana* DC. showed negative results for inorganic phosphate solubilization and siderophore production. Five strains from the cultures isolated from rhizospheric soil gave positive results for only one for the assayed plant- growth promoting properties. The 16S rDNA characterization was realized for 14 strains. The restriction patterns of 11 isolates for the two restriction enzymes are shown in Fig 2. and Fig 3.

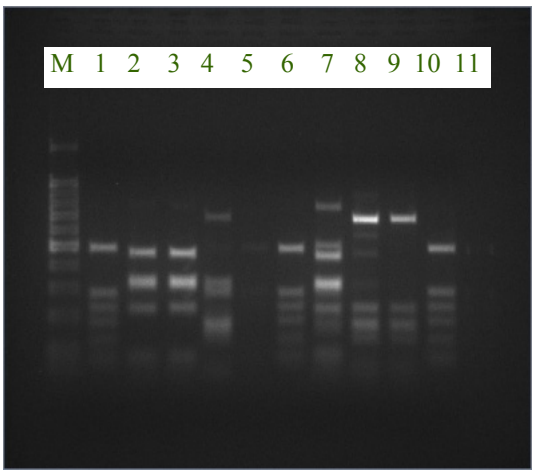

Figure 2. Restriction patterns of the 16S rDNA region of selected strains after digestion with *Hae III* (1: 9G/1, 2:T410^{-3}/4, 3:T1310^{-4}/1, 4:8G/2, 5:T1310^{-2}/1, 6:T610^{-3}/1, 7:7G/8, 8:1G/5, 9: T810^{-2}/1, 10: T1210^{-3}/4, 11:T510^{-2}/1)

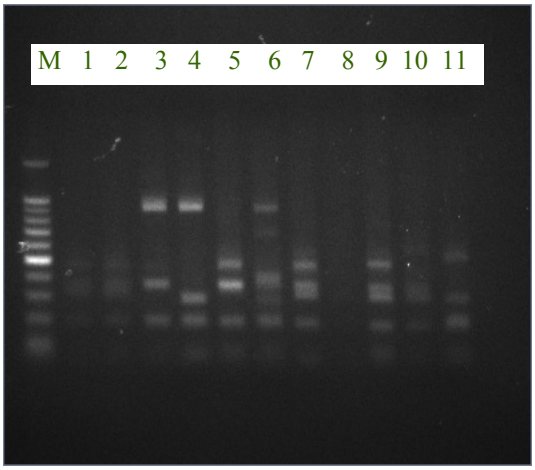

Figure 3. Restriction patterns of the 16S rDNA region of selected strains after digestion with Taq I (1: 9G/1, 2:T410^{-3}/4, 3:T1310^{-4}/1, 4:8G/2, 5:T1310^{-2}/1, 6:T610^{-3}/1, 7:7G/8, 8:1G/5, 9: T810^{-2}/1, 10: T1210^{-3}/4, 11:T510^{-2}/1)

On the basis of the cluster analysis (Fig.4.) of bacterial strains three different groups can be distinguished. The isolates are not identical but they show genetic similarity. Isolates originated from the rhizosphere of the same plant show low genetic similarity [16].

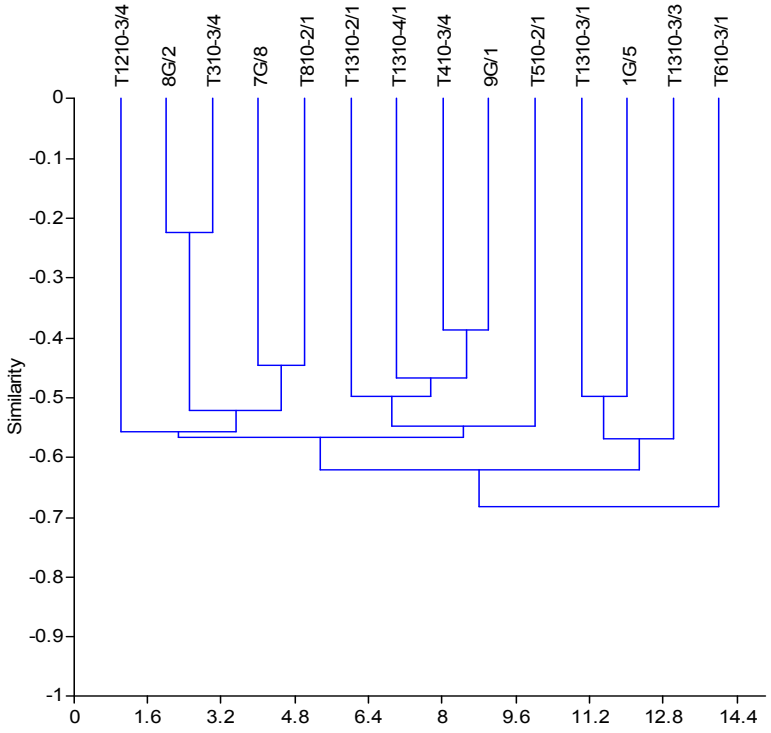

Figure 4. Genetic divergence of the isolated bacterial strains based on 16S ribosomal DNA digestion fragments.

To identify the distinguished strains with the RFLP analysis a further sequence analysis of the 16S rDNA would be necessarily.

CONCLUSIONS

On the basis of our results 2 isolates had similar characteristics with *Rhizobium sp.* isolated from the rhizosphere soil of *Lathyrus transsilvanicus* (Spreng.) Fritsch and *Cytisus hirsutus* L.

Siderophore-producing and inorganic-phosphate solubilizing bacteria are good candidates for plant growth promotion, especially in neutral to alkaline soils. A number of 20 bacterial isolates from the 30 cultures were

found to be positive for the two beneficial traits as the siderophore production and tricalcium phosphate solubilization. The results show that rhizospheric phosphate utilizing and siderophore producing bacteria could be a promising source for plant growth promoting agent in agriculture, because can improve the nutrient availability. The16S rDNA characterization showed a great genetic diversity of our isolates. These may be an important source for the development of bacterial biofertilizers and decrease the amount of chemical fertilizers.

EXPERIMENTAL SECTION

Isolation of rhizobacteria

Bacteria were isolated from four leguminous plant roots nodule and from seven plant rhizosphere soil from Ciuc Mountain locations. Two of the cultures originate from *Anthyllis vulneraria* L.*,* one from *Trifolium pannonicum* L.*, one from Trifolium medium* L. nodules and five from *Cytisus hirsutus* L., three from *Lathyrus transsilvanicus* (Spreng.) Fritsch four from *Trifolium montanum* L., two from *Onobrychis montana* DC*. ssp. transilvanica*, three from *Medicago lupulina* L.*,* one from *Trifolium repens* L.*,* and three from *Vicia sepium* L. rhizosphere soil.

The nodules were disinfected to destroy the microbes on the surface of it in a 6% sodium hypochlorite solution, then washed in sterilized water and smashed in a sterile mortar, homogenized with 9 ml distilled water. From the homogenized extract that contains the rhizobia an amount of 0.1 ml was spread on the surface of yeast extract mannitol agar (YEM medium). The YEM medium composition: 10.00 g/l mannitol, 0.50 g/l K_2HPO_4, 0.20 g/l $MgSO_4 \cdot 7H_2O$, 0.10 g/l NaCl, 1.00 g/l yeast extract, 0.20 g/l $CaCl_2 \cdot 2H_2O$, 0.01 g/l $FeCl_3 \cdot 6H_2O$, 20.00 g/l agar, 25.00 μg/ml bromothymol blue. The pH of the media is between 6.7–7.0.

From the soil samples serial dilutions were prepared, and a volume of 0.1 ml was spread on YEM medium. The inoculated Petri-dishes were incubated 48 hour at the temperature of 28 °C.

The isolates were maintained on YMA agar slants, with the following composition in distilled water: 10.0 g/l mannitol, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.4 g/l yeast extract, 15.0 g/l agar.

The strain designations are composed as follows: the numbers before the liters indicating the nodules (G) or after the number the rhizosphere soil samples (T) refer to the plant species**:** 1-*Anthyllis vulneraria* L., 3-*Medicago lupulina* L., 4-*Trifolium repens*, 5-*Trifolium montanum* L., 6-*Onobrychis montana ssp. transilvanica* DC., 7-*Trifolium pannonicum* L., 8-*Trifolium alpestre* L., 9-*Trifolium medium* L., 11-*Vicia sepium* L., 12-*Lathyrus transsilvanicus* (Spreng.) Fritsch, 13-*Cytisus hirsutus* L.. The other numbers refer to the isolation characteristics of the strain [10].

Biochemical characterization of rhizobacteria

Bacterial isolates were characterized biochemically following the lactose and glucose fermentation capability, oxidase test, nitrate reduction capability and evidencing of oxygen utilization by bacterial cultures on thioglycolate agar and morphologically by Gram's reaction and spore formulation ability [10].

Siderophore production

Siderophore production was detected by the universal method of Schwyn and Neilands (1987). This assay is based on a competition for iron between the ferric complex of an indicator dye, chromeazurol S (CAS), and a chelator, or siderophore produced by microorganisms. The iron is removed from CAS by the siderophore, which apparently has a higher affinity for iron (III). The positive reaction results in a color change of CAS reagent (usually from blue to orange) [11].

Phosphate solubilization

Evaluation of tricalcium phosphate solubilization of isolated strains was assessed using Pikovskaya's agar containing $Ca_3(PO_4)_2$. Each bacterial culture was spot inoculated in the centre of the plate. After incubation for 48 hour at the temperature of 28 °C a clear zone around the colony indicate inorganic phosphate solubilization [13].

The isolated bacteria were classified on the basis of the examined characteristics with Principal Component Analysis (PCA). PCA is a multivariate statistical method where the data are transformed to a new coordinate system to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data set [15].

Genotypic classification

Each strain was inoculated on YMA broth at 28°C and 150 rpm overnight. Bacterial total DNA was extracted using PROMEGA DNA isolation Kit according to manufacturer instructions. 16S rDNA amplification was carried out through PCR amplification having the following programme: 3 min 94 °C initial denaturation followed by 25 cycles of 30 s at 94 °C initial denaturation, 25 s at 50 °C annealing, 2 minutes at 72 °C, elongation, and 7 minute at 72 °C final elongation. The used primers were the following: 27f 5' AGAGTTTGATCMTGGCTCAG3' and 1492 r5'TACGGYTACCTTGTTAC GACTT3'.

The obtained 16S rDNA fragments were further digested with *Taq I* and *Hae III* restriction enzymes [18]. The fragments were analyzed with agarose (1.5 %) gel electrophoresis in Tris-acetate-EDTA buffer containing 3 μl of

ethidium bromide. The amplification patterns were observed with BioRad UV Transilluminator and documented with GelDocXR software.

ACKNOWLEDGMENTS

Authors would like to thank Ministry of Education, Research and Innovation for financing the present study by the National Plan for Research, Development and Innovation through which this study could be realized.

REFERENCES

1. B. Susana, M. Rosas, J.A. Rovera, *Development in Plant and Soil Sciences* **2007**, *102*, 125.
2. J. Sawyer, J. Creswell, *Phosphorus Basics*, **2000**, *31*, 182.
3. L. Raaska, L. Viikari, T. Mattila-Sandholm, Journal *of Industrial Microbiology*, **1993**, *11*, 181.
4. M. Chaiharn, S. Chunhaleuchanon, A. Kozo, S. Lumyong, *K Science and Technology Journal*, **2008**, *8*, 1.
5. M. A. Whitelaw, *Advances in Agronomy,* **2000**, *69*, 99.
6. E. Nahas, *Development in Plant and Soil Sciences* **2007**, *102*,111.
7. H. Rodríguez, R. Fraga, *Biotechnology Advances,* **1999**, *17*, 319.
8. J. Neilands, The Journal of Biological Chemistry, **1995**, *270*, 45.
9. P. Illmer, F. Schinner, *Soil Biology and Biochemistry,* **1995**, *27*, 3.
10. S. Dunca, E. Nimițan, O. Ailisiei, M. Ştefan, "*Microbiologie aplicată*", Tehnopress, Iaşi, **2007**.
11. M. Chaiharn, S. Lumyong, *World Journal of Microbiology and Biotechnology,* **2009**, *25*, 305.
12. Z. A. Siddiqui, "PGPR: Biocontrol and Biofertilization", Springer, Doordrecht, **2006**, chapter 2.
13. M. Sridevi, K.V. Mallaiah and N.C.S. Yadav, *Journal of Plant Sciences* **2007**, *2*, 635.
14. H. Chung, M. Park, M. Madhaiyan, S. Seshadari, J. Song, Hyunsuk C. and T. Sa, *Soil Biology and Biochemistry*, **2005**, *10*, 1970.
15. I.T. Jolliffe, *Springer Series in Statistics*, **2002**, *2*, 1.
16. H.H. Zahran, *Journal of Biotechnology*, **2001**, *3*, 143.
17. F. Ahmad, I. Ahmad, M.S. Khan, *Microbiological Research*, **2008**, *2*, 173.
18. G. Laguerre, P. Mavingui, M. Allard, M-P. Charnay, P. Louvrier, S-I. Mazurier, L.R. Gois, N. Amarger*, Applied and Environmental Microbiology*, **1996**, *6*, 2029.