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PHOSPHORUS MOBILIZATION FROM DIFFERENT INORGANIC PHOSPHATES BY BACTERIA PROPOSED FOR BIOFERTILIZER

LASLO ÉVA[a,*], GYÖRGY ÉVA[b], MARA GYÖNGYVÉR[b], SZENTES SAROLTA[a], ANDRÁS CSABA[b], LÁNYI SZABOLCS[b]

ABSTRACT. Phosphorus is absorbed by the plants as orthophosphate anions (HPO_4^{2-} and $H_2PO_4^-$). The concentration of soluble phosphate in soil is low and it must be supplemented from other sources. Some of the bacteria with phosphate solubilising capacity are able to transform the accumulated insoluble phosphates in the soil into soluble forms, making them available for plants.

Our aim was to assay phosphorus mobilization from different inorganic phosphates (calcium and iron phosphate, hydroxyapatite) by bacteria isolated from rhizospheric soil proposed for biofertilizer production.

Keywords: *calcium phosphate, iron phosphate, hydroxyapatite, phosphorus solubilisation*

INTRODUCTION

Plant growth promoting rhizobacteria use different mechanisms to improve the growth of plants. One of these plant growth promoting traits is mineral phosphate solubilisation. Due to this process, the bacteria increase the bioavailability of the phosphorus and improve soil fertility.

Phosphorus is the second most important plant growth limiting macronutrient. This element plays a central role in different metabolic pathways, photosynthesis, respiration, cell division, energy transport, as well as signal transduction. With these, it contributes to the development and growth of the plant biomass [2].

The insoluble mineral phosphate content of soils is high, while the soluble orthophosphate content, which is available both for bacterial and plant use, is relatively low [2]. Phosphorus used as fertiliser rapidly enters the immobilized complexes, reacts with metal ions such as Fe^{2+}, Al^{3+} in acidic soils and Ca^{2+} in alkaline soils. The insoluble forms are $CaHPO_4$, $Ca_3(PO_4)_2$,$FePO_4$ and $AlPO_4$ [3,4].

[a] *Universitatea POLITEHNICA din Bucureşti, Facultatea de Chimie Aplicată şi Ştiința Materialelor; Spl. Independenței, 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*

Phoshate-solubilising bacteria mobilize the insoluble mineral phosphate through two mechanisms: production of low molecular weight organic acids and H^+ extrusion [5]. In the first mechanism the transformation of the accumulated insoluble phosphates of soil into a soluble form is related to the direct oxidation pathway in the periplasmatic space of the bacteria. This is the primary mechanism for aldose utilization in many bacteria. One of these mechanisms is the enzymatic conversion of glucose to gluconic acid and 2-keto gluconic acid by quinoprotein glucose dehydrogenase [6,7,8]. Carboxylic anion groups of the secreted acids chelate the metal ions of the mineral phosphate (Ca^{2+} from tricalcium phospahate and hydroxyapatite) with ligand exchange reaction. The result of this reaction is the release of the soluble orthophosphate [9].

This fact was demonstrated in the case of many bacteria with phosphate solubilisation capacity. *Azospirillum* spp. produced different organic acids that take part in the mobilization of insoluble phosphates [10]. In *Rhizobium* species the phosphorus solubilisation is the result of the production of 2-glukonic acid [3]. *Burkholderia cepacia* DA23 released gluconic acid in medium during the solubilisation of approximately 800 mg/l orthophosphate. It was shown that the expression of this pathway in solubilising calcium phosphates may be induced by phosphate starvation conditions in the medium [11].

The other proposed mechanism of the solubilisation is through proton release from the cytoplasm of the bacteria to the outer surface. In this way the negative charge of the surface facilitate the sorption of negatively charged phosphorus ions [6].

For the genetics of this mechanism it was identified some genes and plasmids that play a role in the mineral phosphate solubilisation, such as *mps* in *Erwinia herbicola*, *gabY* in *Pseudomonas cepacia* [12].

The aim of this paper was to assay phosphorus mobilization from different inorganic phosphates (calcium and iron phosphate, hydroxyapatite) by bacteria, isolated from leguminous plants nodules and rhizospheric soil, proposed for biofertilizer production.

RESULTS AND DISCUSSION

In this study, using the spectrophotometric method, we measured the solubilised orthophosphate content by eleven isolated bacteria in the presence of three insoluble phosphates. The isolated bacteria originated from the root nodule and rhizospheric soil of different leguminous plants. The orthophosphate concentration was measured during a period of ten days (72 h, 96h, 144 h, 172h, 240h).

From the assayed isolates the bacterial culture originated from the rhizospheric soil of *Cytisus hirsutu*s L. (T1310-2/1) had the maximum phosphate mobilization capacity (Fig.1). In the case of the calcium phosphate (CaP) the

measured orthophosphate concentration varied between 347.59 mg/l and 421.73 mg/l. The maximum value was detected on the eighth day. In the presence of hydroxyapatite (CaPOH), the obtained values were also similar. We measured also the maximum value in the case of iron phosphate (FeP). The maximum orthophosphate concentration was 89 mg/l after 240 h.

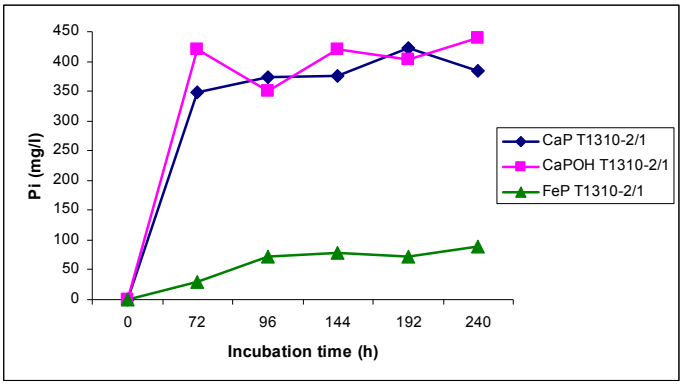

Figure 1. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate originated from the rizosphere of *Cytisus hirsutus* L.

Bacterial isolate (T1110-2/3) originated from the rhizosphere of *Vicia sepium* L. had the lowest phosphate solubilisation capacity. The orthophosphate content (Fig.2) of the liquid culture medium, which contained calcium phosphate, varied between 1.75 mg/l and 114.82 mg/l.

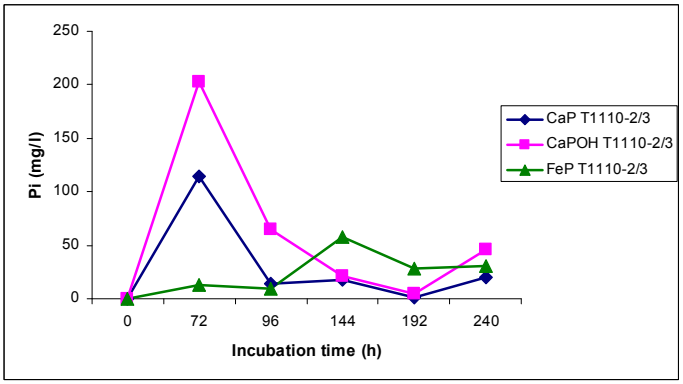

Figure 2. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate originated from the rizosphere of *Vicia sepium L.*

In the presence of hydroxyapatite the measured values were between 4.3 mg/l and 202 mg/l. In both cases the highest concentration was measured on the third day of the incubation time. In the case of iron phosphate the measured values were between 9.4 mg/l and 57 mg/l.

The phosphate concentration mobilized by the bacterial isolate (1G/2) originated from the root nodule of the *Anthyllis vulneraria L.* changed between 148.76 mg/l (96 h) and 92.06 mg/l (Fig.3) (at 192 h).

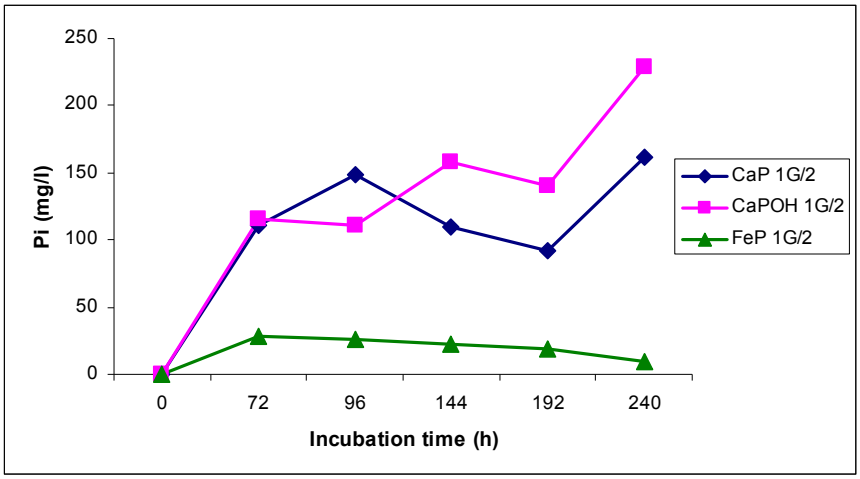

Figure 3. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate originated from the root nodule of *Anthyllis vulneraria L.*

The free phosphate concentration varied between 110.68 mg/l and 228.97 mg/l in the presence of hydroxyapatite and 21 mg/l and 28 mg/l in the case of iron phosphate.

The free phosphate concentration (Fig.4.) solubilised by the bacteria isolated (T510-1/2) from the rhizosphere of *Trifolium montanum L.* changed between 80.68 mg/l and 181.43 mg/l in the presence of the calcium phosphate. In the case of hydroxyapatite we measured the highest concentration (231.64 mg/l) after 240 h. The highest solubilised phosphate content was 27.97 mg/l in the culture medium which contained the insoluble iron phosphate.

The maximum solubilised phosphate content (Fig.5) in the presence of calcium phosphate was 186.80 mg/l on the tenth day of the incubation. Again, the highest concentration was detected after 240 h in the presence of hydroxyapatite. The solubilised phosphate content was between 12.44 mg/l and 43 mg/l in the culture medium with iron phosphate content.

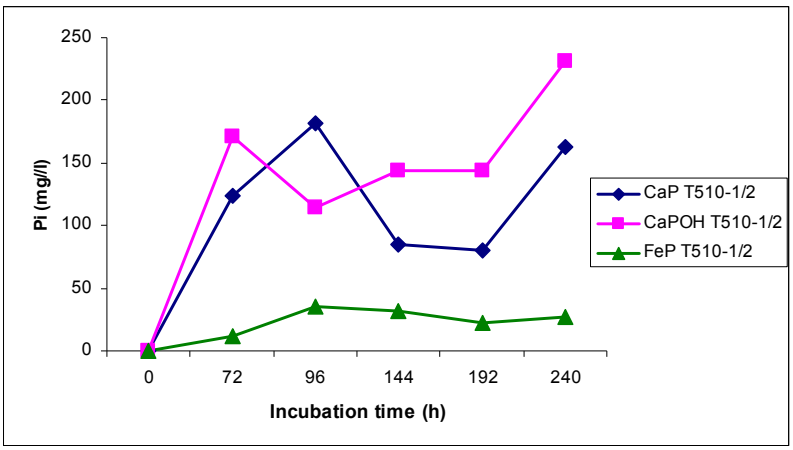

Figure 4. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate originated from the rizosphere of *Trifolium montanum L.*

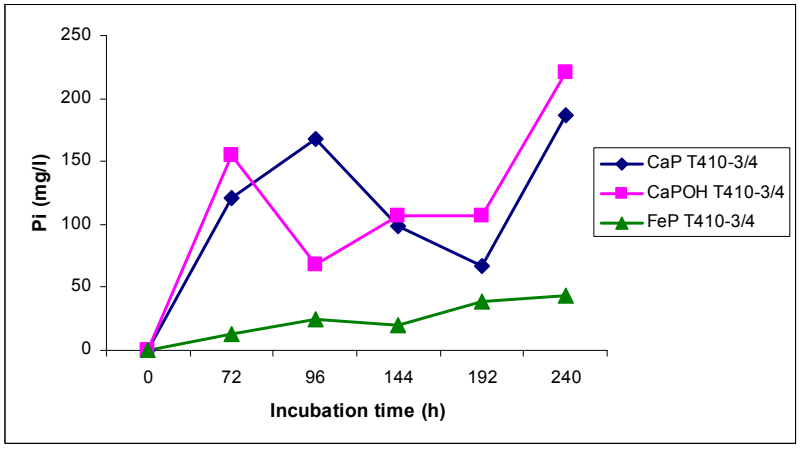

Figure 5. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate (T410-3/4) originated from the rizosphere of *Trifolium repens L.*

The phosphate mobilization capacity differed in the case of two bacterial isolates (T810-2/1,T810-1/3) that originate from the rhizosphere of the same plant (Fig.6 and Fig.7).

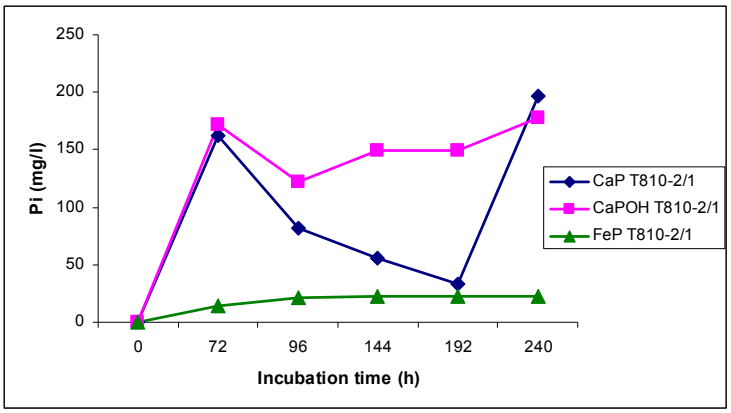

Figure 6. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate originated from the rizosphere of *Trifolium alpestre L.*

The highest phosphate concentration was measured at both of the isolates on the tenth day of the incubation in the presence of the calcium phosphate.

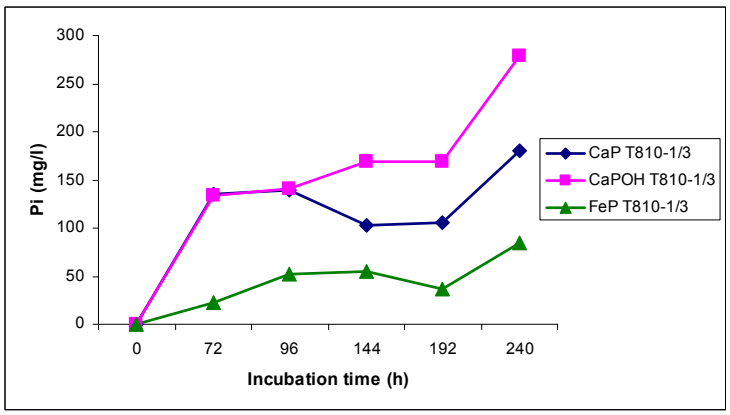

Figure 7. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacteria isolate originated from the rizosphere of *Trifolium alpestre L.*

The highest free phosphate concentration (Fig.8) (mobilized by the isolated bacteria originated from *Lupinus polyphyllus Lindl.*(KLG16) in the case of the three phosphates (calcium phosphate, hydroxyapatite and iron phosphate) were 203.14 mg/l, 209.14 mg/l and 59.83 mg/l.

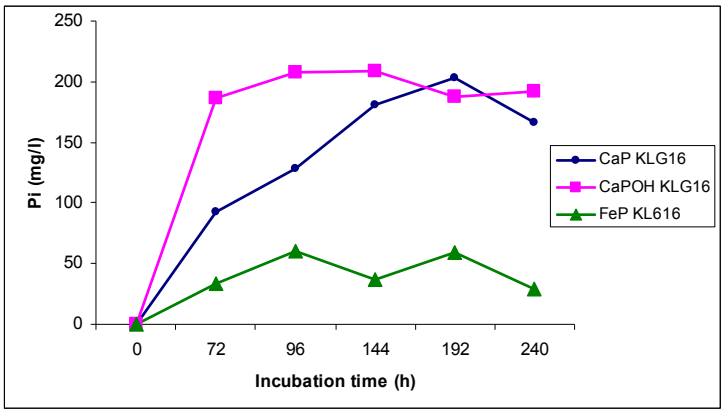

Figure 8. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate originated from the root nodule of *Lupinus polyphyllus*

The detected phosphate concentration (Fig.9) solubilised by the isolate (KLT20) originated from *Lupinus polyphyllus* Lindl. in the presence of the calcium phosphate were between 65.9 mg/l and 170.33 mg/l. In the culture medium with hydroxyapatite content the measured free phosphate concentration was 86.23 mg/l and 220.04 mg/l. In the case of iron phosphate the highest orthophosphate concentration was measured on the tenth day.

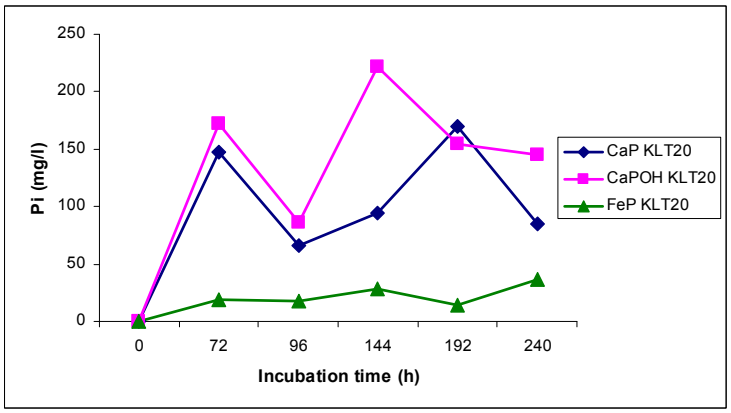

Figure 9. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial isolate originated from the the rizosphere of *Lupinus polyphyllus*

The detected phosphate concentration (Fig.10 and Fig.11) was between 92.7 mg/l and 203.4 mg/l, 81.05 mg/l and 148.58 mg/l in the presence of calcium phosphate in the case of the two bacteria cultures (H39, H31) isolated from the rhizosphere of *Trifolium hybridum* L. In the culture medium with iron phosphate the free phosphate concentration varied between 7.84 mg/l and 65.99 mg/l, 10.79 mg/l and 54.09 mg/l. The highest values were measured in these two isolates in the presence of hydroxyapatite 276.01 mg/l (Fig.11) and 335.55 mg/l (Fig.10).

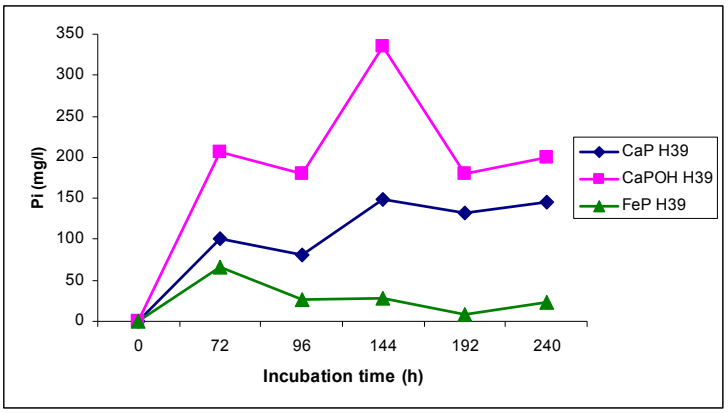

Figure 10. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial i solate originated from the rizosphere of *Trifolium hybridum L.*

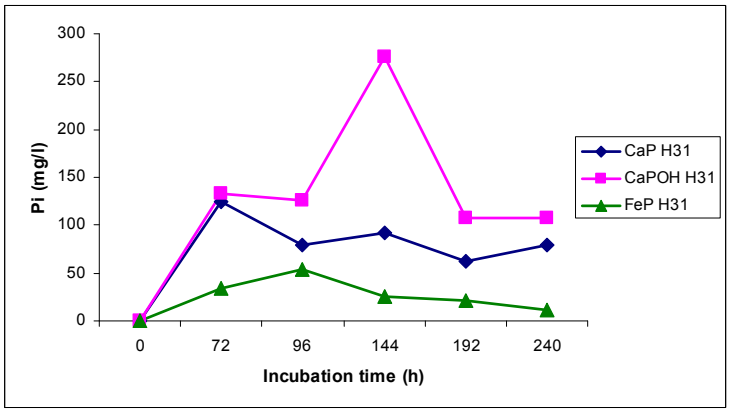

Figure 11. The measured phosphate concentration (in the liquid medium containing the three different phosphates) during the cultivation of the bacterial solate originated from the rizosphere of *Trifolium hybridum L.*

The measured solubilised phosphate concentration is very variable during the incubation period in all of the isolates. This variation is not linear, and it can be explained by the fact that many bacteria accumulate the inorganic phosphates in poly polyphosphate form if it is in excess in their environment. The phosphorus acquisition in bacteria takes place via inorganic transport system [13].

According to the principal component analysis, based on colony morphology properties, the bacteria isolates from the nodules and the soil samples are grouped in three main classes. The morphological properties, with the exception of a few strains, were the same or very similar (Fig 12).

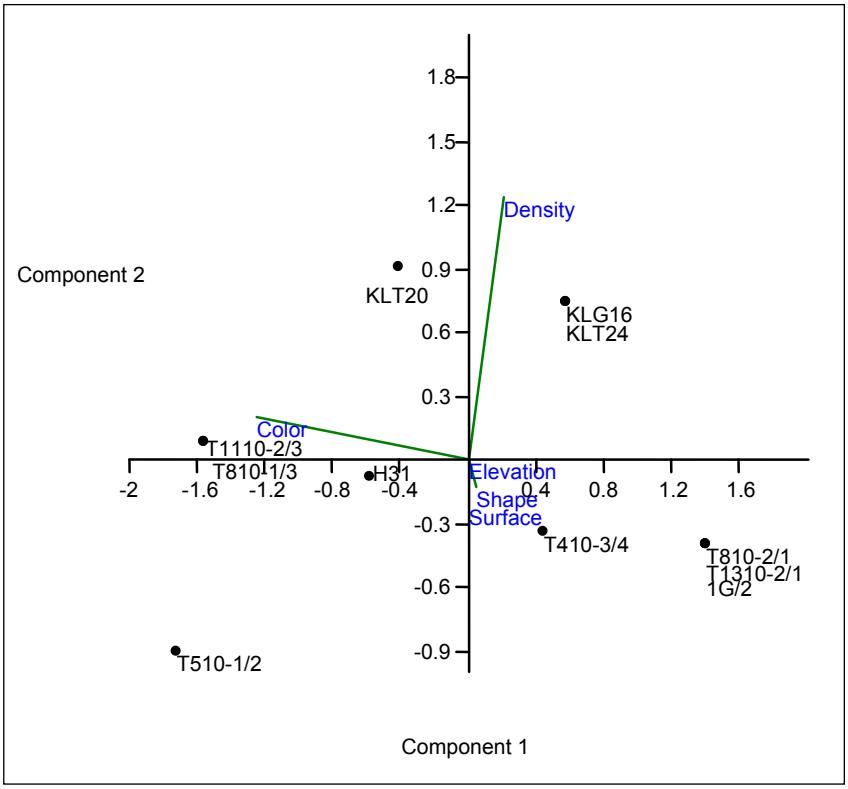

Figure 12. Principal component analysis of bacterial isolates based on morphological characteristics

In most of the cases, the shape of the colonies were round, only one of the colonies presented irregular shaped colony (T410-3/4). The elevation of the colonies was raised in all cases and the margins were entire. At three isolates (1G/2, T1310-2/1, T810-2/1) the middle of the colonies were yellow and the margins were creamy. In the case of six bacterial strains, the colonies were

yellow and one colony had a creamy color. The surfaces of the colonies of the isolates were glistening. The density of colonies in the middle were opaque with transparent margins at seven isolates, while in the case of three bacteria was transparent (KLT24, KLG16, KLT20). Only one bacterial colony was found to be opaque (T510-1/2).

CONCLUSIONS

The eleven bacterial isolates are able to mobilize a relatively high amount of free phosphate, thus increasing the bioavailability of orthophosphates for plants.

The solubilised phosphate concentration from the different three highly soluble phosphate compounds in the case of some isolates is similar with the values described in the literature [15].

On the basis of the results these bacteria can be used for the development of phosphorus solubilisation biofertilizers.

EXPERIMENTAL SECTION

Bacterial Strains

The assayed bacterial cultures were isolated from the rhizosphere and root nodule of different leguminous plants: *Trifolium hybridum L, Lupinus polyphyllus, Trifolium alpestre L., Cytisus hirsutus L., Anthyllis vulneraria L., Trifolium montanum L., Trifolium repens L.*

The qualitative phosphate solubilisation capacity of these isolates was detected on Pikovskaya agar medium [14].

Determination of solubilised phosphate content

The quantitative determination of phosphate solubilisation was carried out in modified Sperber medium (glucose 10 g/l, yeast extract 0.5 g/l, $CaCl_2$ 0.1 g/l, $MgSO_4 \cdot 7H_2O$ 0.25 g/l, $(NH_4)_2SO_4$ 0.5 g/l) with three different phosphates: calcium phosphate 5g/l, hydroxyapatite 5g/l, iron phosphate 3 g/l.

The 24 h cultures were inoculated in sterile physiology solution and cell density was adjusted to 55% transmittance on a Biolog turbidimeter for each. 90 ml of modified Sperber medium was inoculated with 250 μl bacterial suspension and incubated on rotary shaker at 28°C with 150 rpm for ten days. The mobilized free phosphate concentration was measured five times after 72 h, 96 h, 144 h, 192 h and 240 h.

Before the measurement of the phosphate content of the culture medium, the cultures were centrifuged at 6000 rpm for 15 min. 500 μl of the supernatant was mixed with 500 μl 10% trichloroacetic acid for the protein precipitation.

Then 4 ml of mixed reagent (ammonium paramolybdate, sulfuric acid, ascorbic acid, potassium-sodium tartarate) was added, and was incubated on room temperature for 15 min. The absorbance of the solution was measured at 880 nm. The amount of mobilized phosphate was detected from the standard curve of KH_2PO_4 [15, 16].

Determination of the morphological characteristics

The colony morphology properties of the isolates with phosphate solubilisation capacity were determined on YEM medium (contained per liter of distilled water: 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 1 g yeast extract, 0.2 $CaCl_2 \cdot 2H_2O$, 0.01 g $FeCl_3 \cdot 6H_2O$, 20 g agar, 25 μg/ ml bromthimol blue pH 6.7–7.0.).

The determined morphological characteristics included the colony size, elevation, density, color, shape and margin.

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