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THE STUDY OF *Agrobacterium radiobacter* 10 AND *Pseudomonas fluorescens* PG7 PHOSPHATE-MOBILIZING ABILITIES in vitro

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Abstract

There is a need to improve the phosphorus nutrition of agricultural plants due to the mobilization of phosphorus from hard-to-reach soil compounds and fertilizers by useful rhizosphere microorganisms (PGPB). For this purpose, phosphate-mobilizing bacteria are being selected to create biologicals with a fertilizing action. Here, our research data for the first time show that the strain *Agrobacterium radiobacter* 10 can metabolize phytate to utilize it as a source of carbon and energy in the absence of other sources, and the strain *Pseudomonas chlororaphis* PG7 can solubilize inorganic phosphates (tricalcium phosphate, hydroxyapatite) and organic phosphates (calcium phytate). The aim of the work is to investigate the potential of phosphate-mobilizing ability of two strains, *A. radiobacter* 10 and *P. chlororaphis* PG7. The stock cultures were propagated on pea agar (according to Khotyanovich). The phosphate mobilizing ability of the strains was assessed in vitro on selective nutrient media at 28 °C. Dephosphorylation of sodium phytate was examined in two liquid media. Medium II had the following composition (g/l distilled water): $(NH_4)_2SO_4$ — 1.0, K_2SO_4 — 0.2, Na phytate (Sigma-Aldrich, USA) — 10, corn extract — 0.2, pH 6.8. PSM (phytase screening medium) composition was as follows (g/l distilled water): D-glucose — 15.0, $(NH_4)_2SO_4$ — 5.0, KCl — 0.5, $MgSO_4 \cdot 7H_2O$ — 0.1, NaCl — 0.1, $CaCl_2 \cdot 2H_2O$ — 0.1; $FeSO_4 \cdot 7H_2O$ — 0.01, $MnSO_4 \cdot 7H_2O$ — 0.01; Na phytate (Sigma-Aldrich, USA) — 5, pH 6.5. The content of total phosphorus added to media with Na phytate was determined by the method of E. Truog and A.H. Meyer modified by J.B. Rodriguez et al. (1994) after ashing as per N.E. Ginsburg and G.M. Shcheglova (1960). The growth of strains in liquid media was estimated by the bacteria abundance (CFU/ml of suspension) during incubation. The ability of the strains to solubilize inorganic phosphates (tricalcium phosphate, hydroxyapatite) and organic phosphate (calcium phytate) was carried out on three solid nutrient media, the NBRIP, glucose-aspartic medium (according to G.S. Muromtsev) and PSM. NBRIP (National Botanical Research Institute's phosphate growth medium) composition was as followed (g/l of distilled water): D-glucose — 10, $Ca_3(PO_4)_2$ — 5.0, $MgCl_2 \cdot 6H_2O$ — 5.0, $MgSO_4 \cdot 7H_2O$ — 0.25, KCl — 2.0, $(NH_4)_2SO_4$ — 0.1, agar-agar — 20, pH 6.8. The glucose-aspartic medium with hydroxyapatite (according to G.S. Muromtsev) (43) contained (g/l of distilled water) D-glucose — 10, asparagine — 1, K_2SO_4 — 0.2, $MgSO_4 \cdot 7H_2O$ — 0.2, corn extract — 0.2, $Ca_5(PO_4)_3O_5$ — 4, agar-agar — 20, pH 6.8. The PSM composition is as hereinabove, added with agar-agar 20 g/l, pH 6.5 adjusted to by adding a 1 0% aqueous solution of $Ca(OH)_2$ to convert soluble sodium phytate into insoluble calcium phytate. The formation of halos around the colonies was recorded. The research revealed that *A. radiobacter* 10 cultured in the liquid medium uses phytate as a source of carbon and phosphorus for growth and enzymatically dephosphorylates phytate. This was evidenced by a significant increase in abundance of the bacteria during 4-day growth, a relatively small decrease in pH of the liquid broth compared to the control without inoculation, and the accumulation of immobilized phosphorus in the bacterial cell sediment and free orthophosphate in the liquid medium. *P. chlororaphis* PG7 could not mobilize phytate in the medium II. In particular, despite an increase in the *P. chlororaphis* PG7 aundance, there was no noticeable accumulation

of bacterial cell sediment and free orthophosphate in the liquid medium. It was shown that when cultured in the liquid PSM, both strains actively grew and multiplied, obviously using glucose as a source of carbon and energy. Under these conditions, a significant amount of immobilized phosphorus accumulated in the bacterial cell sediment, while the content of free orthophosphate in the medium remained at the control level. In addition, bacterial growth led to significant acidification of the medium, which contributed to the non-enzymatic hydrolysis of sodium phytate. Therefore, the research data could not drive to an unambiguous conclusion about the ability of strains to enzymatic hydrolysis of sodium phytate when cultured in the liquid PSM with two carbon sources. The halos around the colonies of *P. chlororaphis* PG7 on solid media indicated its ability to dissolve inorganic phosphates and phytin by solubilization. Unlike the *Pseudomonas* strain, the *A. radiobacter* 10 showed no solubilizing ability. This indicates its individual physiological features, since, as follows from special publication, many representatives of the genus *Rhizobium* are potential solubilizers. Thus, the ability of strains to solubilize mineral phosphates should be tested on solid nutrient media, where the formation of halos around colonies is a criterion for evaluating phosphate dephosphorization. The ability of strains to mobilize phosphorus from phytates should be assessed in liquid media in order to avoid false positive or false negative results. The main indicators of the enzymatic hydrolysis of phytates are the accumulation of immobilized phosphorus in the sediment of bacterial cells and free orthophosphate in the medium.

Keywords: phosphate-mobilizing ability, *Agrobacterium radiobacter*, *Pseudomonas fluorescens*, phytate, tricalcium phosphate, hydroxyapatite, selective nutrient media, immobilized phosphorus, orthophosphate

Phosphorus is one of the key elements in plant life. It is part of a number of organic compounds, in particular nucleic acids, nucleotides, nucleoproteins, vitamins, phospholipids, phytin, etc., which play a central role in metabolism. Phosphorus deficiency affects almost all life processes of plants. Phosphorus enters the root system and functions in the plant in the form of oxidized compounds, mainly orthophosphoric acid residues ($H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}) [1, 2]. Mycorrhizal fungi involved in the transport of phosphorus from the soil solution to the roots of the host plant also absorb phosphorus mainly in the form of the $H_2PO_4^-$ ion [3-5].

In soils, phosphorus occurs almost exclusively in the form of orthophosphates and is part of mineral and organic compounds. The gross content of phosphorus in the arable layer of soils is 0.03-0.2%, or 1-6 t/ha. Despite the significant reserves of phosphorus in soils, its availability for plants is largely hindered due to the low content of orthophosphoric acid ions in the soil solution, which is due to the intense retrogradation of phosphates (transition of easily digestible forms into hardly digestible ones) [3, 6]. In the mineral compounds of soils, phosphorus is mainly represented by inactive forms, such as primary minerals of soil-forming rocks, various compounds of secondary origin in the form of salts with alkaline and alkaline earth bases and sesquioxides.

The solubility of mineral phosphates depends on the reaction of the soil. Thus, calcium and magnesium phosphates become insoluble in an alkaline environment, and aluminum and iron phosphates become insoluble in an acidic one. The greatest amount of phosphorus compounds available for plants is present in soils with a slightly acidic and neutral reaction, the pH of which is in the range of 6.5-7.0 [6, 7].

It is known that soil microorganisms (bacteria and fungi) play a key role in the cyclic cycle of soil phosphorus and its availability for plant nutrition [8]. Rhizosphere bacteria involved in the release (solubilization) of phosphates from insoluble inorganic raw materials use various strategies to convert forms inaccessible to plants into available ones. Many bacteria release carbon dioxide and acidify the environment or, utilizing sugars, release low molecular weight organic acids (acetic, malic, gluconic, etc.) which have chelating properties and form organomineral complexes with cations associated with phosphorus, thereby converting it into soluble forms. Mobilization of phosphorus bound in the rhizosphere may result from activation of proton transport from root cells and acidification of

the medium in response to bacterial inoculation. In addition, bacterial siderophores that chelate iron and other metals with the formation of stable complexes can play an important role in increasing the availability of phosphorus [9]. Representatives of the genera *Arthrobacter*, *Bacillus*, *Burkholderia*, *Beijerinckia*, *Mesorhizobium*, *Flavobacterium* [10], *Rhizobium* [11, 12], *Pseudomonas* [13], *Enterobacter*, *Klebsiella*, *Stenotrophomonas* [14], *Streptomyces*, *Leifsonia* [15] and *Lisinobacillus* (16) associated with mycorrhizal fungi participate in the solubilization of inorganic phosphates in the soil.

Soil organic phosphorus makes up from 30 to 50% of its total content and is represented by two groups of compounds that are different in nature, i.e., products of biological synthesis and humus formation. The first group includes nucleoproteins, phytin, phospholipids, phosphoproteins and other organic compounds that are part of living organisms. The largest part (30-60%) among soil organic phosphates is occupied by phytates, the salts of phytic acid (D-myo-inositol-1,2,3,4,5,6-hexakisdihydrophosphoric acid) which is an ester of the cyclic hexahydric alcohol myo-inositol and six residues of orthophosphoric acid. The distribution of phytates depends on the acidity of the soil. Calcium and magnesium phytates are common in neutral soils while iron and aluminum phytates are found in acid soils [17]. Phytates are found in large quantities in plants, especially in seeds, where they represent the main form of phosphorus storage. Phytates enter the soil with plant residues and manure [18].

The most common isomer of inositol phosphate in soil, myo-inositol hexakisphosphate (InsP6), is a strong polyanion chelating agent. It can form insoluble complexes with vital divalent metal cations, as well as with proteins, carbohydrates, amino acids, turning them into insoluble conglomerates [19]. In acidic solutions, protonation (addition of protons to the molecule) of the phosphate groups of phytate promotes the formation of a free form of the molecule. In neutral and alkaline solutions, deprotonation of phosphate groups increases the affinity for divalent metal cations, which significantly reduces the solubility of phytate [20].

Hydrolysis of InsP6 is carried out by phytase enzymes. Phytases are a special group of phosphatases capable of stepwise phytate dephosphorylation with the formation of less phosphorylated derivatives of myo-inositol phosphate, inorganic phosphate, and free metal ions [21]. Based on the pH optimum, phytases are divided into two classes — acidic and alkaline. At present, the biochemical properties and mechanism of catalysis of individual enzymes, representatives of these classes, as well as their structural features, substrate specificity, and temperature dependence have been studied [19].

The main producers of phytase in the soil are microorganisms that make the phosphorus of organic compounds available for plant nutrition [22-24]. Weak phytase activity was also found in plant roots. However, the enzyme is not secreted into the rhizosphere, so plants cannot independently absorb the phosphorus bound in phytate [25]. Among soil microorganisms, the most active producers of extracellular phytase are micromycetes from the genera *Aspergillus*, *Penicillium*, and *Mucor* [26]. Phytases have been found in yeast [27] and bacteria of various taxa, including *Pseudomonas*, *Bacillus*, *Klebsiella*, *Enterobacter* [22]. Bacterial phytases are mainly intracellular enzymes, but bacteria of the genera *Bacillus* [28] and *Enterobacter* [29] are also able to produce extracellular phytases, and in *Escherichia coli* phytase is a periplasmic protein that most likely has access to phytate substrates in vivo (30). The ability to hydrolyze phytates was also found in some representatives of *Arthrobacter* [31], *Flavobacterium* [32], *Burkholderia* [33], and *Pantoea* [34].

According to the G.S. Muromtsev's concept [32], the microbiological mobilization of phosphorus from phytic acid salts proceeds in two phases. These are

the "non-specific" phase (dissolution of Ca, Mg, Fe and Al phytates) is carried out by various acid-forming microorganisms, the "specific" (enzymatic dephosphorylation of phytic acid) is carried out by specific microorganisms, among which there those using phytin or the product of phytin hydrolysis myo-inositol as a carbon source, e,g,, *Bacillus subtilis*, *Enterobacter aerogenes*, *Rhizobium leguminosarum* bv. *viciae*, *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Corynebacterium glutamicum* and *Lactobacillus casei* [32]. In general, the catabolic pathway of myo-inositol after cellular uptake has been studied in *Bacillus subtilis*. It includes multiple and stepwise reactions involving dehydrogenase, dehydratase and other enzymes. The end result of the myo-inositol catabolic pathway is an equimolar mixture of dihydroxyacetone phosphate, acetyl-CoA, and CO_2 [35].

The ability of rhizosphere bacteria to mobilize hard-to-reach soil phosphates has long been considered by scientists as an important mechanism for the positive effect on plant phosphorus nutrition [36, 37]. It is also known that most phosphate-mobilizing bacteria have a beneficial effect on the growth and development of the plant as a whole. This is due to an increase in the availability of other mineral elements (N, Fe, Zn, etc.), the release of vitamins and phytohormones, the production of antibiotics that inhibit the development of pathogens, the induction of mechanisms of systemic resistance to abiotic and biotic stresses [9, 38], and, finally, due to the formation of intracellular signaling molecules (second messengers), such as InsP3, a positive regulator of many signaling pathways [39] which is important for plant-microbial interaction and specific interaction between plants and nitrogen-fixing bacteria [40).

However, for the effective use of biological preparations, more complete knowledge of the physiological characteristics of the microorganisms included in their composition is needed. Among the most important characteristics of strains of rhizosphere bacteria selected for the development of biological preparations of fertilizer, stimulating or protective action is their ability to mobilize soil phosphates and fertilizers. In the absence of such information, the results of the action of biological preparations on the phosphorus nutrition of plants remain little predictable [7, 13]. Research in this direction will contribute to a better understanding of the potential interactions between PGPB (plant growth-promoting bacteria) and plants, as well as between PGPB and mycorrhizal fungi.

In the present work, the phosphate mobilizing ability of two bacterial strains, *A. radiobacter* 10 and *P. chlororaphis* PG7, of scientific and practical importance, was studied for the first time. The ability of *A. radiobacter* 10 to metabolize phytate, using it as a source of carbon and energy in the absence of other sources, and the ability of *P. chlororaphis* PH7 to solubilize inorganic (tricalcium phosphate, hydroxyapatite) and organic (calcium phytate) phosphates have been established.

Our goal was to study the potential ability of the Agrobacterium radiobacter 10 and Pseudomonas chlororaphis PG7 strains to mobilize phosphorus from mineral and organic compounds that are difficult for plant nutrition.

Materials and methods. The strains of *Agrobacterium radiobacte*r 10 and *Pseudomonas chlororaphis* PG7 stored in the collection of the All-Russian Institute of Agricultural Microbiology (ARRIAM) were used. It is known that the former is capable of fixing atmospheric nitrogen when grown on a nitrogen-free Vinogradsky medium, has a growth-stimulating effect for many types of agricultural plants, and serves as the basis for the commercial biopreparation Agrofil (EKOS BIOPRODUCTS, Russia) [41, 42]. The second, as a potential agent in the biocontrol of phytopathogenic microorganisms, is being tested in the geographical network [42]. The strains were identified based on the analysis of the 16S rRNA gene sequence at the Department of Genomic Technologies of the ARRIAM Center

for Collective Use.

To obtain an enrichment culture, the strains were propagated on bean agar (according to the prescription of A.V. Khotyanovich) with the following composition (g/l of pea broth): sucrose — 10; KH_2PO_4 — 0.5; $MgSO4 \cdot 7H2O$ — 0.3, chalk — 1, agar-agar — 20 (pH 6.8-7.0)

The study of the phosphate-mobilizing ability of the strains was carried out in vitro on selective nutrient media at 28 °C according to the methodological recommendations of G.S. Muromtsev [43], V.F. Pavlova et al. [43], B. Sasirekha et al. [44] and C.S. Nautiyal [45].

The ability of the strains to dephosphorylate sodium phytate was evaluated in two liquid media. Medium II [43] had the following composition (g/l distilled water): $(NH_4)_2SO_4$ — 1.0, K_2SO_4 — 0.2, Na phytate (Sigma-Aldrich, USA) — 10, corn extract — 0.2 (pH 6.8). PSM (phytase screening medium) [44] contained (g/l distilled water) D-glucose — 15.0, $(NH_4)_2SO_4$ — 5.0, KCl — 0.5, $MgSO_4 \cdot 7H_2O$ — 0.1, NaCl — 0.1, $CaCl_2 \cdot 2H_2O$ — 0.1; $FeSO_4 \cdot 7H_2O$ — 0.01, $MnSO_4 \cdot 7H_2O$ — 0.01, phytate-Na (Sigma-Aldrich, USA) — 5 (pH 6.5). For this purpose, 25 ml of media were poured under sterile conditions into 200 cm^3 flat-bottomed flasks; 100 ml of initial suspensions of bacteria were preliminarily prepared in 0.9% aqueous NaCl solution (washing from the surface of one Petri dish from bean agar). Then, 400 μl of initial suspensions of bacteria were added to flasks with media (bacteria were not added in the control) and cultured on a GFL 3015 orbital shaker (LAUDA-GFL, Germany) at 220 rpm for 4 days. The content of total phosphorus introduced into media with Na phytate was determined by the method of E. Truog and A.H. Meyer [46] with modification by J.B. Rodriguez et al. [47] after the preparation was ashed according to the method of N.E. Ginsburg and G.M. Shcheglova [48]. In terms of 1 ml of medium II, it amounted to 3.98 mg P_2O_5, in terms of 1 ml of PSM medium 1.99 mg P_2O_5.

The ability of strains to grow on liquid media was evaluated by the change in titers (CFU/ml) during the incubation period. The titers were determined by the serial dilutions of bacteria suspensions in saline followed by inoculation of 100 μl portions on Petri dishes with bean agar. The growth of colonies was assessed after 2 days of culture at 28 °C. The pH of suspensions was measured using a H1230B combined electrode on a portable pH meter HI 83141 (Hanna Instruments, Inc., USA) and optical density on a KFK-2 photoelectrocolorimeter (OAO Zagorsk Optical and Mechanical Plant, Russia) at λ = 590 nm. Bacterial cells (4 ml suspension) were sedimented by centrifugation in Eppendorf tubes for 5 min at 12000 g in a PE-6926 centrifuge (OOO EKROSKHIM, Russia). The resulting precipitates were washed twice from the culture medium with saline, after removing the supernatant, 1 ml of concentrated H_2SO_4 was poured into the test tubes, and the precipitates were charred for 2 days at 24 °C. The intensity of acid staining in brown color directly depended on the amount of sediment in the test tube. The accumulation of immobilized phosphorus (total phosphorus in the sediment of bacterial cells) was determined by the methods described above, recalculating it in mg P_2O_5 per 1 ml of suspension.

The accumulation of free orthophosphate in the medium during the incubation period was assessed by centrifuging bacterial suspensions for 5 min at 12000 g, then filtering the supernatant through a Whatman FP 30/0.2 CA-S membrane filter (Cytiva, UK). The phosphorus content was determined as per the method of E. Truog and A.H. Meyer [46] modified by J.B. Rodriguez et al. [47].

The ability of strains to dissolve inorganic and organic phosphates was studied on solid nutrient media of the following composition (g/l of distilled water): NBRIP (National Botanical Research Institute's phosphate growth medium) [45] — D-glucose 10, $Ca_3(PO_4)_2$ 5.0, $MgCl_2 \cdot 6H_2O$ 5.0, $MgSO_4 \cdot 7H_2O$ 0.25, KCl

2.0, $(NH_4)_2SO_4$ 0.1, agar-agar 20 (pH 6.8). Glucose-aspartic acid with hydroxyapatite medium (according to G.S. Muromtsev) [43] was as follows (g/l of distilled water): D-glucose 10, asparagine 1, K_2SO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, corn extract 0.2, $Ca_5(PO_4)_3O_5$ 4, agar-agar 20 (pH 6.8). The PSM medium composition is presented above [44], an additional 20 g/l of agar-agar was added and the pH was adjusted to 6.5 by 10% aqueous solution of $Ca(OH)_2$ to convert soluble sodium phytate to insoluble calcium phytate. The formation of zones of enlightenment of the nutrient medium (halo) around the colonies were recorded.

Statistical processing (calculation of mean values, standard deviations, and errors of sample means) was performed using Microsoft Excel 2010. To test the null hypothesis when compared sample means, we used an interval estimate of the distribution parameters, for which we calculated confidence intervals for the general means. Student's *t*-test were used for 5% significance level according to B.A. Dospekhov (the number of degrees of freedom is 3, Table 1 of the appendix) [49].

Results. Analysis of the phosphate mobilizing ability of when cultivated in liquid medium II showed that the *A. radiobacter* 10 strain can enzymatically mobilize phosphorus from sodium phytate. The content of free orthophosphate in the medium increased vs. control without inoculation, and the sediment of bacterial cells significantly accumulated immobilized phosphorus (Table).

The growth of bacteria utilizing phytate as a carbon source was evidenced by an increase in cell titers (from 3.59×10^6 to 9.71×10^8 CFU/ml), turbidity of the suspension, a decrease in the pH of the medium (see Table), and accumulation of bacterial cell biomass (Fig. 1, A, b). To verify the biological nature of phytate dephosphorization, an analysis was carried out for the content of free orthophosphate in a 1% aqueous solution of sodium phytate with a change in pH in the operating range from 6.83 to 6.11. The results did not reveal a significant effect of increasing the acidity of the medium on the hydrolysis of phytate. The obtained results are consistent with the literature data on the presence of extracellular phytase in some members of the genus *Agrobacterium* which can hydrolyze phytic acid with the formation of substrates containing less than six phosphoric acid residues, i.e., the inositol phosphates, inositol and inorganic phosphate [32]. Probably, the ability of the *A. radiobacter* 10 strain to enzymatically hydrolyze phytate under conditions when phytate serves as the only source of carbon in the environment is directly related to the ability to assimilate myo-inositol as a carbon and energy source using the mechanisms described for *Bacillus subtilis* [35].

Dephosphorylation capability of the *Pseudomonas chlororaphis* PG7 and *Agrobacterium radiobacter* 10 strains when cultured in liquid nutrient media with sodium phytate at 28 °C ($n = 4$, day 4)

Treatment	pH	OD_{590}	Free phosphorus concentration in the medium, mg P_2O_5/ml	Total phosphorus in bacterial sediment, mg P_2O_5/ml suspension
		Medium II		
P. chlororaphis PG7	6.47±0.15	0.10±0.01	1.34±0.16	0.0005±0.0002
A. Radiobacter 10	6.12±0.13	0.53±0.09	3.04±0.47	0.0440±0.0100
Without inoculation (control)	6.47±0.09	0.08±0.01	1.34±0.11	0.0001±0.0000[c]
		Medium PSM		
P. chlororaphis PG7	4.25±0.13	1.50±0.22	0.02±0.01	0.0720±0.0180
A. Radiobacter 10	4.52±0.13	1.50±0.18	0.04±0.02	0.0380±0.0070
Without inoculation (control)	6.13±0.12	0.01±0.00	0.04±0.01	0.0001±0.0000[к]

Note. For the composition of the media, see the "Materials and methods" section. The Table shows the confidence intervals for the general means at the 5% significance level; c indicates the control for reagents.

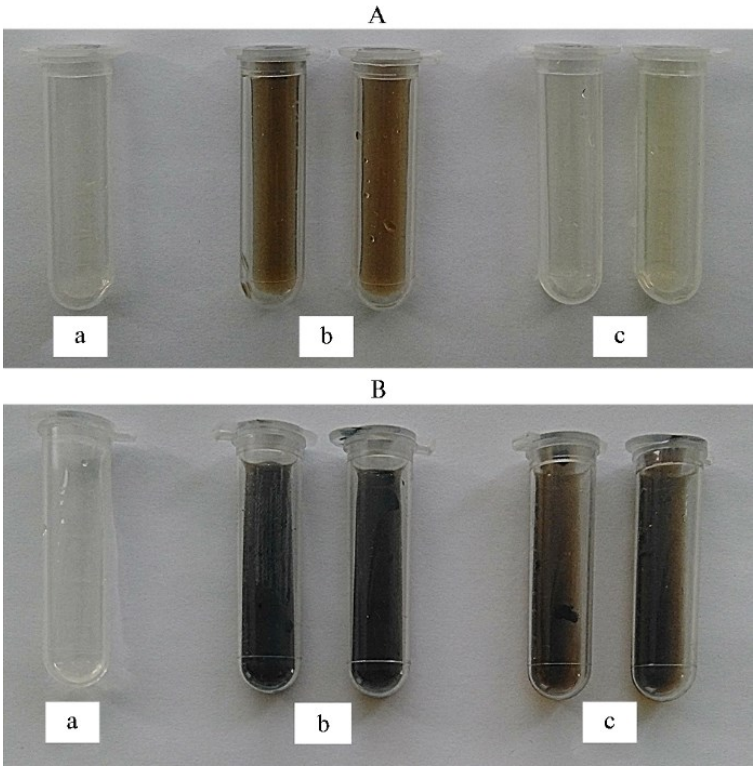

Fig. 1. Bacterial sediments charred with concentrated H_2SO_4 depending on growth medium: A — medium II, B — medium PSM; a — control (without inoculation), b — *Agrobacterium radiobacter* 10, c — *Pseudomonas chlororaphis* PG7. For the composition of the media, see the "Materials and methods" section.

In turn, the strain *P. chlororaphis* PG7 did not show the ability to enzymatic hydrolysis of sodium phytate when cultured in liquid medium II. Despite the increase in titers from 1.03×10^6 to 3.34×10^8 CFU/ml, there was no accumulation of free orthophosphate in the medium, no change in pH and turbidity of the medium, and the formation of a sediment of bacterial cells and the accumulation of immobilized phosphorus in it was insignificant (see Table, Fig. 1, A, c). It can be assumed that bacteria could not use phytate as a source of carbon and energy in the absence of other sources and activated other metabolic pathways.

In liquid PSM medium with two carbon sources, active growth of both strains occurred. Thus, during the incubation period, the titers of *A. radiobacter* 10 and *P. chlororaphis* PG7 increased from 3.59×10^6 to 1.33×10^9 and from 1.03×10^6 to 1.41×10^8 CFU/ml, respectively. The bacterial cultures became significantly turbid and the pH of the medium decreased vs. control; the biomass of cell sediments and the amount of immobilized phosphorus in the sediments increased, while the content of free orthophosphate in the solution remained comparable to the control (see Table, Fig. 1, B, b, c).

In experiments with the *Bacillus subtilis* 60015 strain which is able to metabolize inositol, it was shown that the presence of D-glucose and other easily metabolized carbohydrates in the medium suppresses the production of inositol-2-dehydrogenase (the first enzyme in the myo-inositol catabolic pathway) [30]. Based on these data, we preliminaryly conclude that the strains *A. radiobacter* 10 and *P. chlororaphis* PG7, when cultured in the liquid PSM medium, use glucose rather than phytate as a source of carbon and energy.

The experiments with the PSM liquid medium did not allow us unambiguously draw a conclusion about the ability of the strains to enzymatic hydrolysis

of sodium phytate, since the test of the effect of acidification of the medium on the dephosphorization of phytate in this case gave positive results. With a decrease in the pH of a 0.5% aqueous solution of sodium phytate from 6.50 to 4.19, a significant (by 16.7%) increase in the content of free orthophosphate in the solution occurred, from 0.048±0.001 to 0.056±0.006 mg P_2O_5/ml for 1 h at room temperature. The presence of sodium phytate and glucose in the medium, as well as aeration, can have a significant positive effect on the production of extracellular phytase in microorganisms, as evidenced by the data obtained for *P. aeruginos*a [50], *Mucor racemosus* NRRL (51), and *Bacillus subtilis* DR6 [52]. On this basis, we made a preliminary conclusion that the phosphorus nutrition of the strains in the liquid PSM medium was carried out at the expense of free orthophosphate, the reserves of which were replenished due to both nonenzymatic hydrolysis of phytate upon acidification of the medium and enzymatic hydrolysis of phytate.

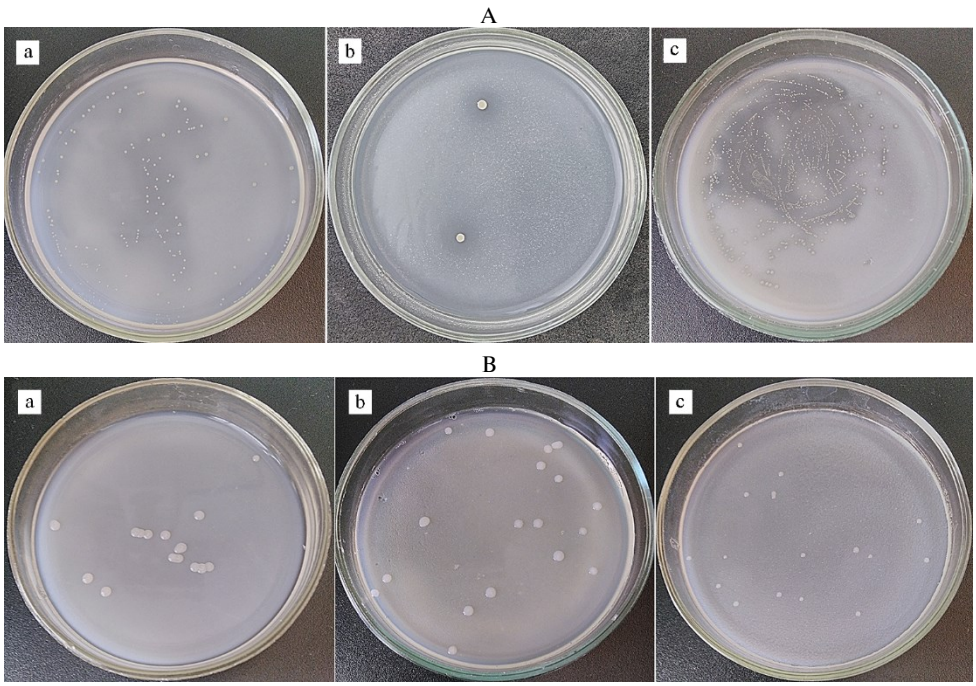

Fig. 2. Growth of the strains *Pseudomonas chlororaphis* PG7 (A) **and *Agrobacterium radiobacter* 10** (B) **on NBRIP medium with tricalcium phosphate** (a)**, glucose-aspartic medium with hydroxyapatite (as per G.S. Muromtsev)** (b) **and PSM medium with calcium phytate** (c)**.** For the composition of the media, see the "Materials and methods" section.

In experiments on solid media, *P. chlororaphis* PG7 is able to dissolve inorganic phosphates (tricalcium phosphate, hydroxyapatite) and organic phosphate (calcium phytate). This was evidenced by the formation of halo zones around the colonies when the strain was cultured on NBRIP, glucose-aspartic (according to G.S. Muromtsev), and PSM media (Fig. 2, A). The results obtained are consistent with data on the ability of some species of Gram-negative bacteria from the genus *Pseudomonas* to dissolve calcium phosphates according to the solubilization scheme [13]. Data on the solubilizing capacity of *P. chlororaphis* in the special literature are very scarce [53]. The data we obtained allow us to consider the strain *P. chlororaphis* PG7 not only as an agent in the biological defense of plants against pathogens, but also as a growth stimulator that improves phosphorus nutrition of plants.

The absence of halo zones around the colonies of the *A. radiobacter* 10 strain on the same solid media indicated that it did not have the ability to solubilize

mineral phosphates and calcium phytate (see Fig. 2, B). As follows from the literature sources, many representatives of the genus *Rhizobium* are such potential solubilizers. They secrete low molecular weight organic acids and dissolve inorganic phosphates [11], which is facilitated by the presence of glucose in the medium [12]. The absence of a halo on the PSM agar medium remains inexplicable, since, as noted earlier, the liquid PSM medium was strongly acidified during culturing the strain (see Table). It can be assumed that this is due to the lower metabolic activity of the *A. radiobacter* 10 strain on a solid medium compared to a liquid one.

Discussing phytase activity in the strains, given the physicochemical properties of phytic acid and its complexes with metals [20, 54], we can conclude that the clear zones around bacterial colonies on a solid medium cannot indicate calcium phytate dephosphorization by phytase, because acid-forming bacteria can dissolve calcium phytate by the solubilization scheme. It is seen in the example of *P. chlororaphis* PG7. False positive results obtained on solid media when testing acid-forming bacteria for the ability to enzymatic hydrolysis of calcium phytate are also known from the literature [55]. Thereof, the ability of bacteria to mobilize phosphorus from phytates should be assessed using liquid rather than solid media or using a technique that allows one to neutralize the halo zones formed as a result of acidification of the PSM medium and preserve those that appeared as a result of the enzymatic hydrolysis of calcium phytate [55].

Thus, the *Agrobacterium radiobacter* 10 strain can use sodium phytate as a source of carbon and phosphorus in the absence of other more available sources, while the *Pseudomonas chlororaphis* PG7 strain does not have this ability. *A. radiobacter* 10 enzymatically hydrolyzes phytate, as a result, free orthophosphate is released into the medium, and immobilized phosphorus accumulates in the sediment of bacterial cells. It was shown that when cultured in a liquid medium with two carbon sources, glucose and sodium phytate, both strains actively multiply using glucose as a carbon source. In this case, a strong acidification of the medium occurs which contributes to the non-enzymatic hydrolysis of phytate. This does not allow us to make an unambiguous conclusion about the ability of strains to enzymatic hydrolysis of phytate. It was shown that the strain *P. chlororaphis* PG7 can dissolve inorganic phosphates (tricalcium phosphate, hydroxyapatite) and organic phosphate (calcium phytate) according to the solubilization scheme when cultured on solid nutrient media, while *A. radiobacter* 10 does not have this property. It is advisable to analyze the ability of bacterial strains to dissolve mineral and organic phosphates according to the solubilization scheme on solid nutrient media with the identification of clear zones around the colonies, while the enzymatic hydrolysis of phytates must be evaluated in liquid media to avoid false positive or false negative results. The main indicator of the ability of strains to perform the enzymatic hydrolysis of phytates is the accumulation of immobilized phosphorus in the sediment of bacterial cells and free orthophosphate in the culture medium.

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