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Original article

Bacillus Strains as an Effective Treatment of Mobile Forms of Phosphorus in Bulgarian Soils

Tsvetana Licheva [a, *], Dilyana Nikolova [a] & Evstatieva Yana [a]

[a] Department of Biotechnology, Faculty of Biology, Sofia University St. Kliment Ohridski, 8, Dragan Tsankov Blvd., 1164, Sofia, Bulgaria

Abstract

Strains of genus *Bacillus* have the potential to increase the availability of phosphorus to plants by the dissolution of inorganic phosphate, which favours the growth of plant species and that has an important economic and agricultural impact. Soils with low phosphate intensity (Leached chernozem) were enriched with poorly soluble phosphorus compounds and inoculated with the tested newly isolated strains from genus *Bacillus* (*Bacillus subtilis* T 2, *Bacillus amyloliquefaciens* T 3, *Bacillus subtilis* T 4, *Bacillus subtilis* T 10, *Bacillus thuringiensis* T 17 and *Bacillus cereus* T 18). Six newly isolated strains from different regions in Bulgaria were identified by classical phenotypic techniques and 16S rDNA sequence analysis. Tested strains were inoculated (2 ml and 15 ml) in the soil and incubated for 25 days at 28°C. After the incubation period, the degradation of phosphorite flour to available phosphorus was examined by the classical method of Egner-Riehm and by extraction with $CaCl_2$.

A vegetation experiment was conducted with a test plant *Pelargonium zonale*, characterized by its ability to absorb large amounts of phosphorus. A peat substrate enriched with all macro-and microelements was used, and phosphorus was added to the medium in the form of phosphorite flour. The amounts of phosphorus absorbed by one plant *Pelargonium zonale* treated with newly isolated strains *Bacillus subtilis* T 10 and *Bacillus cereus* T 18 absorbed 23% more phosphorus than the control variant.

As a result of the experiments, it was found that the studied strains have a positive impact on the increase of phosphorus mobility in soils with low phosphate intensity treated with hardly degradable phosphors.

Keywords: *Bacillus*, Leached chernozem, Phosphorus, Pelargonium zonale.

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* **Corresponding author:**

Licheva Ts. is an assistent professor in the Department of Biotechnology at Sofia University St. Kliment Ohridski, Bulgaria. Her research interests include the Agriculture, Biology, Microbiology, Biotechnology.
Email: cvlicheva@uni-sofia.bg

INTRODUCTION

The need for phosphorus for the plants results from the multiple physiological and biochemical functions of this element in plant biology. Phosphorus participates in several processes that determine the optimum growth and development of plants (Shen *et.al*., 2011).

Phosphorus compounds have important functions in the metabolism of plants. Phosphorous level in the tissues and organs of crops is crucial for their development, cellular processes, cell division, enzyme activation/inactivation and carbohydrate metabolism (Holford, 1997; Zandi, 2018, Tairo *et.al.,* 2013; Razaq *et.al.,* 2017).

At the plant level, phosphorus stimulates seed germination; development of roots, stalk and stem strength; flower and seed formation; crop yield; and quality. In addition, the availability of phosphorus increases the N-fixing capacity of leguminous plants. Phosphorus is essential at all developmental stages, right from germination to maturity (Hina *et.al.,* 2018).

Several groups of factors - physical, physico-chemical, and biological - effect the phosphorous mobility in soil. Mineralogy of soil and organic matter content are among the main factors determining the mobility of phosphorus. As a rule, free and water-soluble forms of phosphorus are rarely found in the soil (Chen *et.al.,* 2006). Phosphorus compounds are associated with soil minerals and organic compounds in virtually insoluble forms (Zaidi *et.al.,* 2009). To make them available, the plant organism typically produces substances that dissolve phosphorus compounds to phosphate anions. The “life" of these free phosphate anions is very short, and they almost immediately associate with soil colloids (Rodriguez *et.al*, 2009). Especially important are aluminium and ferrous ions, which almost immediately fix phosphorus ions thus making them inaccessible to living organisms.

Biological activity in the soil is another very important factor in determining the mobility of phosphorus for the plant organism. Certain groups of microorganisms have a strong influence on the transformation of phosphorus compounds. The ability of microorganisms to transform phosphorus compounds is considered one of the most important characteristics associated with the phosphorus nutrition of plants (Chen *et.al*., 2009). Several bacterial species are able to increase the availability of phosphorus to plants by the dissolution of inorganic phosphate by producing acids (Rodriguez, 1999). These bacteria are called phosphate solubilizing bacteria (PSB) and have potential applications as a fertilizer to improve plant growth and yield (Zaidi *et.al.,* 2009; Rodriguez, 1999; Vessey, 2003; Adhya *et.al.,* 2015). Many authors study intensively PSB isolated from different soil types and the ability of these bacteria to increase the availability of phosphorus to plants (Peix *et.al.,* 2001a; Peix *et.al.,* 2001b). Currently, representatives of the genus *Bacillus* and the genus *Pseudomonas* are the most studied phosphate degrading bacteria (Rodriguez *et.al.,* 2009).

MATERIALS and METHODS

Bacterial strains

The bacterial strains used in this study were isolated from different soils from Bulgaria by classical methods. Samples for analysis were taken from soils from different regions of Bulgaria, giving preference to areas/soil types with specific microflora. The sampling and storage of the samples until their analyzes were performed according to standard procedures for conducting microbiological analysis, as the samples were not preserved and were stored at 10 ° C for 24 hours. A classical procedure was used to obtain pure cultures, and the purity of the isolated cultures was checked microscopically. The isolates were maintained in a Nutrient agar (Diffco) medium. The newly isolated strains from genus *Bacillus* after identification were indicated as *Bacillus subtilis* T 2, *Bacillus amyloliquefaciens* T 3, *Bacillus subtilis* T 4, *Bacillus subtilis* T 10, *Bacillus thuringiensis* T 17 and *Bacillus cereus* T 18.

Extraction and amplification of bacterial genomic DNA

Genomic DNA was isolated by Macherey-Nagel™ Bacterial Genomic DNA Kit in accordance with the manufacturer's instructions. Ready To GoTM PCR beads (GE Healthcare) and the primer set 27F and 1492R for 16S rDNA PCR were applied. Obtained 16S rDNA PCR products were used as a template for a standard sequencing procedure (Macrogen Inc.). The sequences were compared with the available nucleotide database from the NCBI GenBank. A similarity of >96% to the 16S rDNA sequences of the reference strains was used as a criterion for the identification.

Cultivation and storage of studied microorganisms

To the experiment, the tested strains were cultivated in Nutrient broth (Diffco) at 28^{o}C for 24 hours. All tested strains were preselected from a single colony and were cultivated on Nutrient agar (Diffco) to avoid contamination. The strains are stored in the form of stock culture on Nutrient agar (Diffco) with 20% glycerol at -20 ^{o}C.

Study on mobile forms of phosphorus in treated soils with studied strains

The soil used in this experiment was with low phosphate intensity and was taken from Knezha town, Bulgaria. For the purpose of the experiment, the soil (with and without phosphorite flour) was autoclaved for 30 min. at 121^{o}C to eliminate the allochthonous microflora. Phosphorite flour that is difficult to degrade and unusable for plants was added to the soil. Batch cultivated cultures of the tested strains at a concentration of 10^8 colony forming units (cfu/ml) were inoculated (2 ml and 15 ml) in the soil and incubated for 25 days at 28 ^{o}C. The breakdown of phosphorite flour was examined after the incubation period by the classical method of Egner-Riehm (Egnér et al, 1960) and extraction with $CaCl_2$. Treatment options are presented in Table 1.

Table 1. Variants of treatment

Control 1- soil without phosphorite
Control 2- soil with phosphorite
T 2 – 4 ml/L – 2 ml
T 2 – 4 ml/L – 15 ml
T 3 – 4 ml/L – 2 ml
T 3 – 4 ml/L – 15 ml
T 4 – 4 ml/L – 2 ml
T 4 – 4 ml/L – 15 ml
T 10 – 4 ml/L – 2 ml
T 10 – 4 ml/L – 15 ml
T 17 – 4 ml/L – 2 ml
T 17 – 4 ml/L – 15 ml
T 18 – 4 ml/L – 2 ml
T 18 – 4 ml/L – 15 ml

Determination of macronutrients in the plant biomass of *Pelargonium zonale*

Agrochemical analyzes to determine the influence of the studied strains on the mineral nutrition of *Pelargonium zonale* were performed during the flowering phase. The total content of P, K, Ca, Mg in the tissues was measured as follows: K - flame-gauge, Ca and Mg - complexometric, P - photocolorimetric. Total nitrogen was determined after decomposition of the sample with phenolic sulfuric acid in the presence of Se powder (modified Keldahl method) and distillation of the Parnas – Wagner apparatus.

RESULTS and DISCUSSION

Molecular characterization based on 16S rDNA sequence analysis

Six newly isolated strains from different regions in Bulgaria were identified by classical phenotypic techniques and 16S rDNA sequence analysis. The classical phenotypic characteristics were done according to established phenotypic criteria.

The sequences of the standard nucleotide were BLAST analysis and compared with the NCBI database. After analyzing the newly obtained sequence in the GenBank DNA database, the BLAST algorithm found that strain T 2 showed a higher percentage of similarity with the type culture of *Bacillus subtilis subsp. subtilis* p. NCIB 3610 (96%), strain T 3- 99% similarity to *Bacillus amyloliquefaciens* FZB42 and strain T4 shows 98% similarity to the type culture of *Bacillus subtilis subsp. subtilis* p. NCIB 3610. In strain T 10 was observed 97% (27F) similarity with the type culture *Bacillus subtilis subsp. subtilis* p. NCIB 3610 and 98% (1492R) similarity to the *Bacillus subtilis subsp. subtilis* str168. Upon identification of the strains T 17 and T 18 for BLAST analysis in GenBank DNA database were used

1009 and 1027bp in primer 27F and 1036 and 1031bp in primer 1492R, obtained after sequencing and processing. In the performed analyzes it was found that in strain T17 97% (27F - refseq_genomic BLAST) similarity with *Bacillus thuringiensis str. Al Hakam* and in strain T 18 95% (1492R) similarity to *Bacillus cereus EB16.*

All strains were identified from genus *Bacillus* and indicated as *Bacillus subtilis* T 2, *Bacillus amyloliquefaciens* T 3, *Bacillus subtilis* T 4, *Bacillus subtilis* T 10, *Bacillus thuringiensis* T 17 *and Bacillus cereus* T 18.

Study on mobile forms of phosphorus in treated soils with studied strains.

To the experiment soils with low phosphate intensity (Leached chernozem, FAO) of the region of Knezha, were treated with poorly soluble phosphorus compounds and inoculated with the tested bacterial strains from genus *Bacillus*. In the conditions of a model experiment an attempt was made to identify the individual effect of the different strains of the genus *Bacillus* on the poorly soluble phosphate compounds in one of the soils characteristics for Bulgaria – leached chernozem. For this purpose, soil with and without phosphorite was sterilized to eliminate the effect of all other organisms. In this way, the physicochemical nature of mobility and the action of microorganisms were observed. The data from the experiment are shown in Table 2.

Table 2. Mobility of phosphorus compounds in soil inoculated with the tested strains

Variants	**pH**	**Total P**	**Phosphorus by Egnér- Riehm**	**% to Control 2**	**Phosphorus by extraction with $CaCl_2$**	**% to Control 2**
Control 1- soil without phosphorite	6.67	180.03	6.88	no data	0.1036	no data
Control 2- soil with phosphorite	6.63	270.65	11.82	100	0.1071	100
T 2 – 4 ml/L – 2 ml	6.69	270.65	12.29	109.34	0.0786	-714.29
T 2 – 4 ml/L – 15 ml	6.80	270.65	13.49	133.61	0.1143	305.71
T 3 – 4 ml/L – 2 ml	6.75	270.65	13.79	139.66	0.075	-817.14
T 3 – 4 ml/L – 15 ml	6.82	270.65	11.64	96.27	0.0786	-714.29
T 4 – 4 ml/L – 2 ml	6.87	270.65	12.89	121.51	0.075	-817.14
T 4 – 4 ml/L – 15 ml	6.68	270.65	9.75	58.02	0.1107	202.86
T 10 – 4 ml/L – 2 ml	6.74	270.65	13.12	126.14	0.964	24582.86
T 10 – 4 ml/L –15 ml	6.71	270.65	11.43	92.08	0.857	21525.71
T 17 – 4 ml/L –2 ml	6.83	270.65	12.43	112.17	0.0714	-920.00
T 17 – 4 ml/L – 15 ml	6.81	270.65	12.75	118.67	0.0786	-714.29
T 18 – 4 ml/L – 2 ml	6.82	270.65	13.53	134.58	0.0721	-900.00
T 18 – 4 ml/L – 15 ml	6.81	270.65	12.96	122.93	0.0821	-614.29

It was found that the sterile soil without phosphorite (Control 1) allows 6.88% mobile forms of phosphorus from the total phosphorus content to be liberated physicochemically in the soil. The sterile soil enriched with phosphorite (Control 2) was able to mobilize physicochemically 11,82% mobile forms - almost twice as many as Control 1 (Table 2). Strains *Bacillus amyloliquefaciens* T 3 and *Bacillus cereus* T 18 had the greatest effect on the phosphorus mobility when inoculated at a dose of 2 ml, followed by strains *Bacillus subtilis* T 10 and *Bacillus subtilis* T 4. The treatment with high doses of the microorganisms (15 ml) had the strongest positive influence when the inoculation was with strain *Bacillus subtilis* T 2. When the treatment was done with *Bacillus thuringiensis* T 17 and *Bacillus cereus* T 18, the mobile phosphorus was increased by about 20%. After inoculation with strains *Bacillus amyloliquefaciens* T 3, *Bacillus subtilis* T 4 and *Bacillus subtilis* T 10 (15 ml), a decrease of the phosphorus mobility was observed. Probably the higher number of living microbial cells of strains *Bacillus amyloliquefaciens* T 3, *Bacillus subtilis* T 4 and *Bacillus subtilis* T 10 at a dose of 15 ml has some indirect influence – the released mobile phosphorus is decreased because they use it for their own metabolism. The results from the study of mobile forms of phosphorus extracted with $CaCl_2$ („mild“ extraction) showed that strains *Bacillus subtilis* T 2 and *Bacillus subtilis* T 4 at a dose of 15 ml lead to an actual increase of the mobile phosphorus, and the lower dose of 2 ml leads to about 8 times decrease. Strain *Bacillus amyloliquefaciens* T 3 was not able to increase the mobile forms of phosphorus in either dose. Similarly, strains *Bacillus thuringiensis* T 17 and *Bacillus cereus* T 18 decreased from 6 to 9 times the mobilisation of phosphorus in the phosphorite-enriched soil. The method of mild extraction showed that the inoculation with strain *Bacillus subtilis* T 10 leads to an increase of the mobile forms of phosphorus – 24,5 times (2 ml) and 21,5 times (15 ml).

Study of mobile forms of phosphorus in a test plant *Pelargonium zonale* by inoculation with the studied strains

A vegetation experiment was conducted with a test plant *Pelargonium zonale*, characterized by its ability to absorb large amounts of phosphorus. A peat substrate enriched with all macro- and microelements was used, and phosphorus was added to the medium in the form of phosphorite flour.

Table 3. Assimilated macronutrients from one plant

Variants	N	P	K	Ca	Mg
	g/plant				
Control	0.51	0.12	0.55	1.22	0.10
T 2	0.59	0.13	0.62	1.13	0.11
T 3	0.76	0.13	0.59	1.25	0.13
T 4	0.60	0.12	0.52	1.22	0.13
T 10	0.84	0.55	0.67	1.45	0.12
T 17	0.61	0.12	0.62	1.26	0.10
T 18	0.62	0.44	0.72	1.35	0.13

In the case of macronutrients assimilated by plants (Table 3), it was found that strains of *Bacillus amyloliquefaciens* T 3 and *Bacillus subtilis* T 10 assisted the assimilation of the highest amounts of nitrogen. The amounts of phosphorus absorbed by one plant were close to or equal to those of the control, but the plants treated with *Bacillus subtilis* T 10 and *Bacillus cereus* T 18 absorbed 23% more phosphorus than the control. The same strains have assisted the uptake of potassium by plants and there is almost no difference in the uptake of calcium and magnesium as a total. These changes in the mineral nutrition with basic macronutrients of the experimental plant have some influence on its development and decorative appearance (Figure1.)

Figure 1. Decorative type of the studied variants (A - control, strain T 2; B - control, strain T 3; C - control, strain T 4; D - control, strain T 10; E - control, strain T 17; F - control, strain T 18)

Conclusion

The studied strains have a positive impact on the increase of phosphorus mobility in soils with low phosphate intensity treated with hardly degradable phosphors. The method of mild extraction with $CaCl_2$ showed that the inoculation with strain *Bacillus subtilis* T 10 leads to an increase of the mobile forms of phosphorus – 24,5 times (dose of 2 ml) and 21,5 times (dose of 15 ml) compare with the control. The method of Egnér- Riehm showed that the strains *Bacillus amyloliquefaciens* T 3 and *Bacillus cereus* T 18 demonstrated the highest activity on the phosphorus mobility (inoculated at a dose of 2 ml). The treatment with high doses of the microorganisms- *Bacillus subtilis* T 2, *Bacillus thuringiensis* T 17 and *Bacillus cereus* T 18 (15 ml) had the strongest positive effect, the mobile phosphorus was increased by approx. 20%.

The amounts of phosphorus absorbed by one plant *Pelargonium zonale* treated with newly isolated strains *Bacillus subtilis* T 10 and *Bacillus cereus* T 18 absorbed 23% more phosphorus than the control variant.

Based on the obtained data it could be concluded, that tested *Bacillus* strains demonstrated a positive impact on the increase of phosphorus mobility in soils with low phosphate intensity treated with hardly degradable phosphors.

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