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HALOPHILIC MICROORGANISMS FROM SALINE WASTES OF STAROBIN POTASH DEPOSIT

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Abstract. 3 cultures of halophilic phosphate-solubilizing bacteria isolated from solid saliferous wastes of Starobin potash mines run by Belaruskali concern are capable to grow in the presence of 10-15 % sodium chloride and to promote germination and growth of alfalfa (*Medicago sativa*).

Key words: halophilic microorganisms, phosphate solubilization, salinization, biotechnology, phytoremediation.

The stressful environmental factors like salinization, drought, xenobiotics, heavy metals, radiation etc. are extremely unfavorable for agricultural practice. In most cases contamination of environmental media in industrial regions is distinguished by a complex pattern. Being added to increased levels of pollutants represented by mono- and polyaromatic hydrocarbons, the soil is subjected to extreme action of other factors, e.g. enhanced salt background [1].

Belaruskali company exploiting the mines since the 1960s has dumped on formerly fertile lands over 730 mln tons of solid clay-saline wastes across the overall area of about 2 thousand hectares [8].

Salt stress negatively affects growth and productivity of farm crops. Despite adverse salinization impact, soils with high technogenic load generate microbiota capable to withstand hypersaline exposure [2]. A promising strategy

for remediation of salinized soils envisages application of legume varieties, like alfalfa to upgrade soil fertility via atmospheric nitrogen fixation. Alfalfa salt tolerance is promoted by inoculation of selected rhizobia strains and PGPR bacteria.

The aim of the study consists in the isolation of microbial cultures resistant to soil salinization and stimulating germination and growth of alfalfa (*Medicago sativa*).

Objects and Methods

Salt-resistant phosphate-solubilizing microorganisms were recovered from saline wastes of Starobin deposit on solid nutrient medium in compliance with the Muromtsev guidelines [6]. Tolerance of microbial isolates to sodium-, potassium- and calcium chlorides in concentrations 3, 5, 10, 15, 20 % was tested on TY medium [4]. Nitrogen-fixing *Ensifer meliloti* strains able to grow on TY medium containing 300 mM NaCl were isolated from nodules of alfalfa, *nifH* gene encoding a small subunit of nitrogenase complex was used as nitrogen fixation marker. A couple of primers *nifH*-1F and *nifH*-1R proved suitable for all examined samples of alfalfa-specific nodulating bacteria [7].

Growth-stimulating action of bacterial isolates under salinization conditions was evaluated using alfalfa seeds. Tested plant seeds were steeped in 2 % aqueous bacterial suspension, control plant seeds were dipped into sterile tap water.

The seeds placed into vials were incubated at 28 °C during 2 hours and transferred into sterile Petri plates on damp filter paper impregnated with sodium chloride in 100 mM concentration. The treated seeds were germinated in incubation chamber for 2-3 days at 28 °C.

Results and Discussion

Unique microflora capable to survive under extreme environmental conditions is generated in soil with high salinization and technogenic load. In this respect special emphasis is focused on microorganisms adapted to elevated mineralization background [5]. Such bacteria appear attractive due to increased biotechnological potential. They may be applied to design biopreparations remediating saline

soils and effluents with complex multichemical composition of pollutants [3].

Isolation of halophilic microorganisms showing resistance to chlorides of sodium, potassium, calcium was performed from solidsaliferous wastes of Starobin potash deposit. The enrichment culture was carried out by inoculating homogenated solid saliferous wastes into selective liquid nutrient media and subsequent fermentation during 1 week. Submerged culture was conducted in 250 ml flasks containing 100 ml of selective medium on laboratory shaker (agitation rate – 200-220 rpm) at temperature +28 °C. Glucose-asparagine medium proposed by Muromtsev served as selective medium for enrichment culture of phosphate-solubilizing microorganisms. Isolates of phosphate-solubilizing bacteria were recovered by plating samples of enrichment culture on agar selective medium of the same composition.

At the next research stage isolates were screened according to the criteria of phosphate-solubilizing activity and resistance to enhanced salt concentrations. 4 cultures were sorted out of phosphate-solubilizing bacterial colonies dominating on glucose-asparagine medium with calcium phosphate. All isolates were Gram-positive rods showing catalase activity, while variant 4F displayed oxidase activity. Based on physiological and biochemical characterization using automatic system VITEK-2 (bioMerieux) and taxonomic nomenclature of Bergey's Manual of Determinative Bacteriology (1994) all four phosphate-solubilizing strains were affiliated to genus *Bacillus*.

Survival of selected bacilli exposed to the action of sodium-, potassium- and calcium chloride in different concentrations was assessed in a series of model experiments (see Table).

In accordance with the aim of our investigation strains capable to grow on media containing elevated chloride levels should be chosen as promising objects for further studies. It may be deduced from our data that 3 strains - *Bacillus sp.* 2F, *Bacillus sp.* 4F and *Bacillus sp.* 8F suit perfectly to meet the set research objectives. They may grow on media with 15 % sodium-, potassium- and calcium chloride and form halo zones on glucose-asparagine medium with calcium orthophosphate, indicating conversion of added salt into water-soluble form.

Growth of halophilic phosphate-solubilizing bacteria on TY medium in the presence of sodium-, potassium- and calcium chloride

Isolate N	Control (salt free)	Concentration, %														
		NaCl					KCl					CaCl ₂				
		3,5	5	10	15	20	3,5	5	10	15	20	3,5	5	10	15	20
<i>Bacillus sp.</i> 2F	++	+++	+++	++	+	-	+++	+++	++	+	-	+++	+++	+	+	-
<i>Bacillus sp.</i> 4F	++	+++	+++	++	+	-	+++	+++	++	+	-	+++	+++	+	+	-
<i>Bacillus sp.</i> 5F	++	+++	+++	+	-	-	+++	+++	+	-	-	+++	+++	-	-	-
<i>Bacillus sp.</i> 8F	++	+++	+++	++	+	-	+++	+++	++	+	-	+++	+++	+	+	-

Note: +++ – abundant growth, ++ – good growth, + – satisfactory growth, - – no growth.

4 nitrogen-fixing strains of bacteria *E. meliloti* able to grow on TY medium comprising 300 mM NaCl were isolated from nodules of alfalfa (*M. sativa*).

Strain capacity to fix nitrogen mediated by nitrogenase enzyme complex was confirmed by presence of *nifH* gene. So far, 10 *nif* genes have been identified in Rhizobia, including *nifH* encoding a small subunit of enzyme complex. Amplification of *nifH* gene fragment with a couple of primers *nifH*-1F and *nifH*-1R revealed one specific zone sized approximately 430 bp (Fig. 1). Partial *nifH* gene amplification involving primers *nifH*-1F and *nifH*-1R demonstrated that specific PCR product available in all 4 strains of nodulating bacteria *E. meliloti* is identical to that of nitrogen-fixing reference strain *Rhizobium loti*.

A series of model experiments was conducted to evaluate growth-stimulating effect

of selected halophilic phosphate-solubilizing bacterial strains on seed germination and development of alfalfa sprouts in saline media (NaCl concentration 100 mM) (Fig. 2).

The obtained result points out the leading role of strain *Bacillus sp.* 8F promoting germination of alfalfa seeds by 50 % and elongation of seedlings by 16 %. The other halophilic phosphate-solubilizing bacteria *Bacillus sp.* 2F and *Bacillus sp.* 4F did not cause any favorable impact on germination and length of alfalfa seedlings.

Mixed influence of nitrogen-fixing strain *E. meliloti* S3 and phosphate-mobilizing culture *Bacillus sp.* 8F on growth and development of alfalfa crop (*M. sativa*) in salinized environment (NaCl concentration 100 mM) was tested in model experiment (Fig. 3).

The analysis of the results evidences stimulating action of strain *E. meliloti* S3 on alfalfa

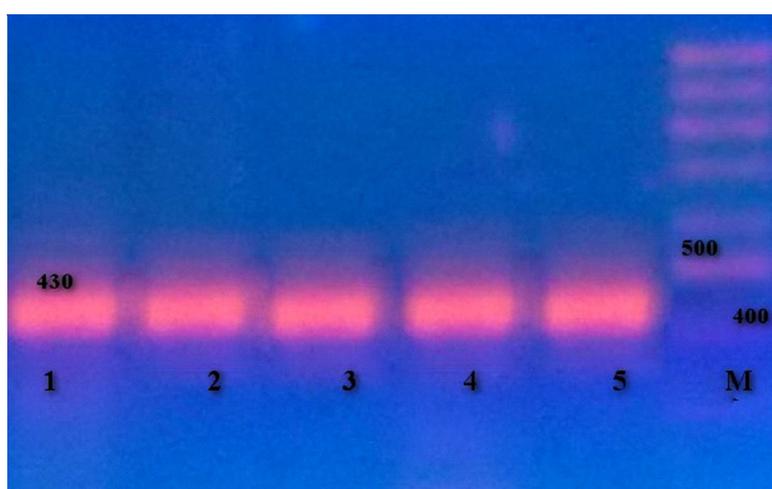


Fig. 1. Amplification of *nifH* gene fragment with primers *nifH*-1F and *nifH*-1R using DNA isolated from pure culture:

1 – *Rhizobium loti* L3 (positive control); 2 – *Ensifer meliloti* S3; 3 – *Ensifer meliloti* S5; 4 – *Ensifer meliloti* IS0; 5 – *Ensifer meliloti* 3583; M – DNA molecular weight marker (100 bp)

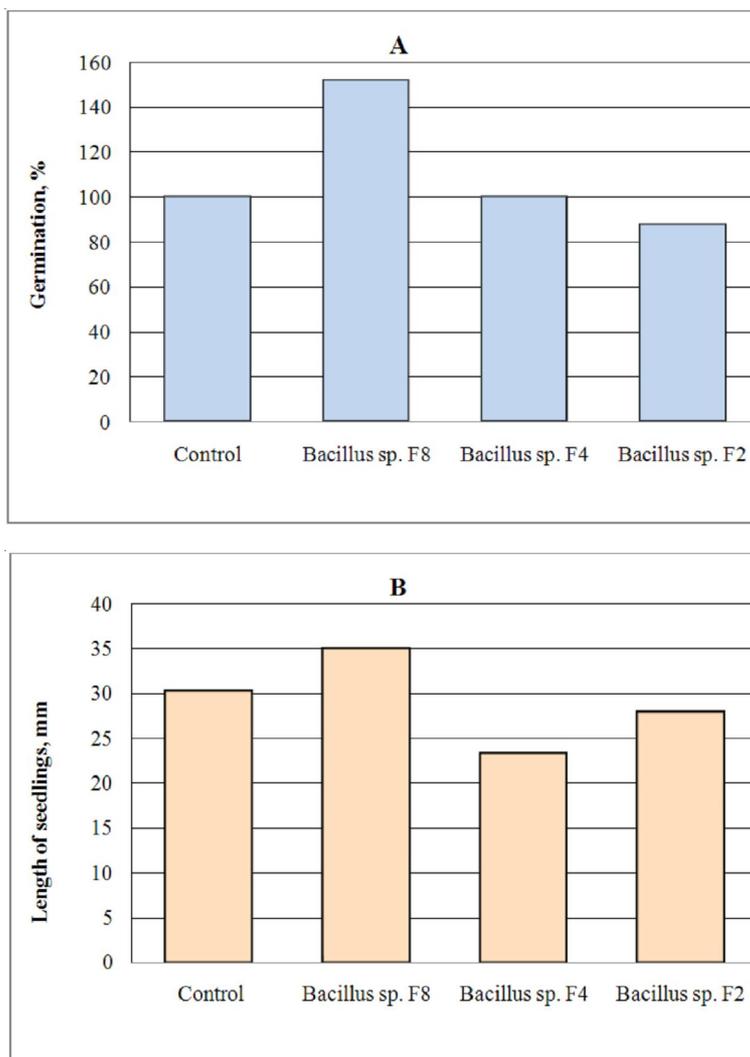


Fig. 2. Effect of halophilic strains of phosphate-solubilizing bacteria on seed germination (A) and length of alfalfa seedlings (*M. sativa*) (B) under saline conditions

seed germination and development of seedlings – 17 % and 34 %, respectively. Beneficial effect on both parameters was also demonstrated by phosphate-solubilizing strain *Bacillus sp.* 8F. Dual application of nitrogen-solubilizing strain *E. meliloti* S3 plus phosphate-solubilizing strain *Bacillus sp.* 8F exposed to excessive salinity ensured increase in seed germination rate by 9 % and length of seedlings by 21 %.

Conclusion

3 bacterial strains representing genus *Bacillus* and distinguished by the maximum tolerance to soil salinization and active ability to grow on media in the presence of 15 %

concentrations of sodium-, potassium- and calcium chloride were isolated from solid saliferous wastes of Starobin potash mines managed by Belaruskali concern.

Screening of 3 isolates for phosphate-solubilizing activity in pure culture demonstrated the ability of all selected variants to mobilize calcium orthophosphate. Strains *E. meliloti* S3 and *Bacillus sp.* 8F stimulated germination of alfalfa seeds and promoted development of *Medicago sativa* sprouts. The selected efficient cultures of nitrogen-fixing and phosphate-solubilizing bacteria arouse vivid interest as potential components of plant-microbial association applied in biotechnology of salinized soil phytoremediation.

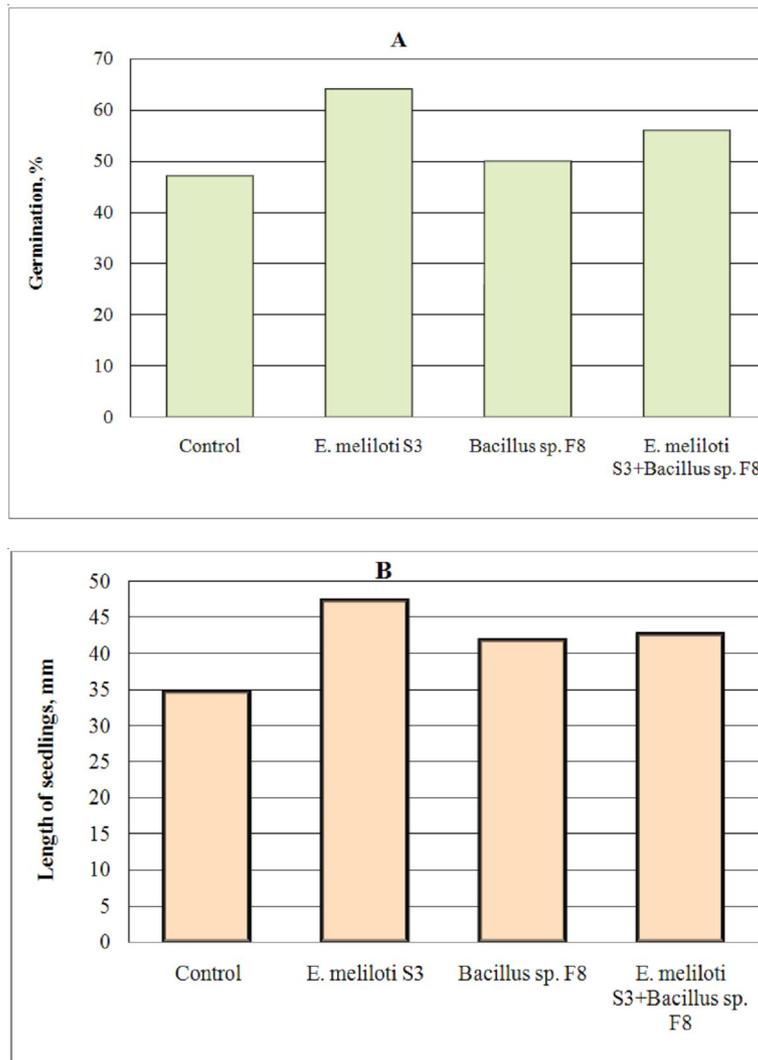


Fig. 3. Combined effect of strains *E. meliloti* S3 and *Bacillus sp.* 8F on seed germination (A) and length of alfalfa (*M. sativa*) seedlings (B) under the salinized conditions

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**АДАПТАЦИЯ ГАЛОФИЛЬНЫХ МИКРООРГАНИЗМОВ
К ЗАСОЛЕНИЮ ПОЧВЫ**

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Аннотация. В работе были исследованы 3 культуры галофильных фосфат-растворимых бактерий, выделенных из калийсодержащих отходов. Было показано, что они способны расти в присутствии 10–15 % натрия хлорида и способствовать прорастанию и росту люцерны (Люцерна посевная).

Ключевые слова: галофильные микроорганизмы, фосфаты, засоление, биотехнологии, фиторемедиация.