PRELIMINARY DATA ON BORSÁROS RAISED BOG NATURAL RESERVE BRYOPHYTE ASSOCIATED BACTERIA

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Turbăria Borsáros este un ecosistem oligotrof, sărac în elemente nutritive, unde materialele organice sunt acumulate şi depozitate. Microorganismele au un rol important în procesele de degradare, contribuind astfel în circulația locală a elementelor nutritive.

Scopul cercetării noastre este analizarea speciilor bacteriene asociate Briofitelor, izolate din turbăria Borsáros.

Rhizobacteriile favorizante ale creşterii plantelor (PGPR) au efect benefic prin diferite mecanisme (producerea unor metaboliți secundari, competiție pentru nutrienți).

Speciile bacteriene izolate au fost caracterizate prin proprietățile lor biochimice şi benefice. Pentru caracterizarea genetică am folosit metoda polimorfismul fragmentelor de restricție (RFLP). Speciile bacteriene au fost comparate cu alte rhizobacterii identificate taxonomic.

Borsáros is a nutrient poor oligotrophic raised Bog, where organic materials accumulate and are stored. Microorganisms play important role in the degradation processes, thus contributing to the local circulation of nutrients[1].

The aim of our study is to analyze Bryophyte associated bacterial strains isolated from Borsáros raised bog.

Plant growth promoting rhizobacteria (PGPR) are beneficial trough different mechanisms (production of secondary metabolic substances, competing with the pathogens for nutrients[2]).

The isolated bacterial strains were characterized trough their biochemical and beneficial properties. For the genetically characterization restriction fragment length polymorphism (RFLP) was used. Bacterial strains were compared with other taxonomically identified strains, studied with similar methods.

Keywords: beneficial bacteria, Bryophytes, RFLP

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1. Introduction

Borsáros raised-bog is a nutrient poor oligotrophic ecosystem, but many plants were able to adapt and found the minimal necessary living conditions. Bryophytes and their representatives are one of the many plants found in Borsáros raised bog. Bryophytes mostly are known for their antimicrobial effects, but are important host plants for microorganisms because of their small size and limited availability of substratum, and also because these plants could adapt to extreme environmental factors such as too much or too little water. Those bacteria which are able to adapt to these circumstances are successful [1].

Decomposition processes of organic materials in raised-bogs are very slow because of the low pH and temperature of the soil. Cellulose and lignin are the two most important organic compounds found in peatlands. Bacteria play an important role in the degradation of these components. Both cellulose and lignin compounds come from plant residues and are degraded to mono- and disaccharides respective to phenol [3, 4, 5]

Plant growth promoting bacteria have stimulating effects on plants using a lot of mechanisms: nitrogen fixation, phosphorous and iron solubilization, production of phytohormones, facilitating the uptake of nutrients or biocontrol of the plant pathogens [6, 7]. One of the biocontrol methods is the production of low molecular mass compounds as siderophores, which dissolve ferric ion from soil. Siderophores also stimulate the plant defense capacity [8].

The biochemical and beneficial properties studied in this article give us information about the metabolism and enzyme system of the isolated bacterial strains. These properties are important in the degradation processes and also in the plant growth promotion. The genetic characterization helped us to identify taxonomically the isolated and selected bacterial strains.

2. Materials and Methods

2.1. Bacterial strains

Bacterial strains (selected as *Bacillus sp.*, *Pseudomonas sp.* and E*nterobacteriaceae*) were isolated from the surface, the rhizosphere and from the tissues of Bryophytes. Pure bacterial cultures were kept on Nutrient agar medium (5 g NaCl, 5 g peptone, 2 g yeast extract, 1 g meat extract and 1 L distilled water).

2.2. Biochemical characterization

The studied biochemical characteristics permit the classification of the studied bacterial strains. During the analysis the metabolism and enzyme system of bacterial strains is studied.

Utilization of lactose and glucose

First we studied the ability of bacterial strains to metabolize carbohydrates (glucose and lactose). Bacterial strains were grown in peptone water (peptone 10 g, NaCl 5 g, distilled water 1 L) supplemented with glucose/lactose (10% final concentration). After incubation at 28°C for 24 hours an indicator solution (bromothymol-blue) was added to the samples. Disappearance of the blue colour of the bromothymol-blue solution indicated the utilization of carbohydrates.

Nitrate reduction

To demonstrate the nitrate reduction, bacterial strains were grown in peptone water supplemented with KNO_3 (10 g in 1 L) and incubated at 28°C for 24 hours. The reduction of nitrates was checked with Griess-Ilosvay solution. In case of a positive reaction the growth medium changed its colour into red.

Gelatinase test

The gelatinase test gives us information about hydrolyzing the protein macromolecules by bacteria. Bacterial strains were grown on solid medium supplemented with gelatine for 5 days on 28°C. Liquefaction of the medium indicated a positive reaction (gelatinase activity).

2.3. Characterization of bacterial strains by their beneficial properties

Siderophore production

Siderophore production was detected by using agar plates containing chrom azurol S (CAS) dye. An orange halo around the bacterial colonies indicated the siderophore production.

Cellulose degradation

Cellulose degradation analysis was conducted using Congo red dye. Bacterial strains were grown on agar plates, containing carboximethyl-cellulose (CMC) (1 g $K_2H_2PO_4$, 0,5 g $MgSO_4*7H_2O$, 0,5 g NaCl, 0,01 g $MnSO_4*H_2O$, 0,3 g NH_4NO_3, 10 g CMC, 12 g agar-agar, 1 L distilled water). After incubation for 24 hours at 28 °C, agar plates were stained with Congo red solution (1%) for 15 minutes, and then treated with NaCl solution (1 M) for 15 minutes. The clear halo around the bacterial strains indicated cellulose production and cellulose degradation.

2.4. Genetic characterization

Isolation of the DNA

Genomic DNA was isolated from the bacterial strains with PROMEGA Wizard Genomic DNA Purification Kit using the protocol provided by the firm.

Polymerase chain reaction (PCR)

The amplification of the 16S ribosomal DNA was realized with the universal primer pair: 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (**5'** TACGGYTACCTTGTTACGACTT 3'). The reaction mixture had 50 μL final volume and contained: 5 μL 10X PCR buffer, 5 μL $MgCl_2$ (2,5 mM), 5 μL dNTP

(2 mM), 0,5 μL each primer, 0,25 μL Taq Polymerase (1 Ui), 1 μL genomic DNA and distilled water. Amplification was carried out in a Corbett Plam-Cycler thermocycler with the following programme: 3 min. at 94 °C initial denaturation, 32 cycles of amplification (30 sec. at 94 °C – denaturation, 30 sec at 52 °C – annealing, 1 min. at 72 °C - extension) and final extension 7 min. at 72 °C.

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) was realized with *MspI* (from *Moraxella sp.*) and *HaeIII* (from *Haemophylus aegypticus*) restriction endonucleases. The reaction mixture had 20 μL final volume and contained: 2 μL 10X RE buffer, 0,2 μL bovine serum albumin (BSA), 0,6 μL restriction enzyme, 5-10 μL of PCR product and distilled water. The reaction mixture was incubated for 3 hours on 37 °C according to the manufacturer's suggestion.

The amplicons resulted from the PCR reaction and restriction fragments obtained were separated in agarose gel, and visualized using BioRad transilluminator. DNA fragments were compared with molecular weight marker (Fermentas GeneRuler 100bp DNA ladder), containing standard fragments between 1500 and 100 base pairs. Results were processed and dendogram was constructed using PAST program.

3. Results

3.1. Biochemical properties of bacterial strains

A number of 64 pure isolates were obtained from Borsáros Raised Bog's Bryophytes (21 isolated as *Bacillus sp.*, 23 as *Pseudomonas sp.* and 20 isolated as *Enterobacteriaceae*).

According to the scientific literature bacterial strains belonging to *Bacillus sp.* are able to degrade glucose. Our results correspond to these data. From the bacterial strains isolated as *Bacillus sp.* 85,7 % (18 strains) were capable to utilize glucose and 9% (2 strains) were lactose positive (Fig. 1).

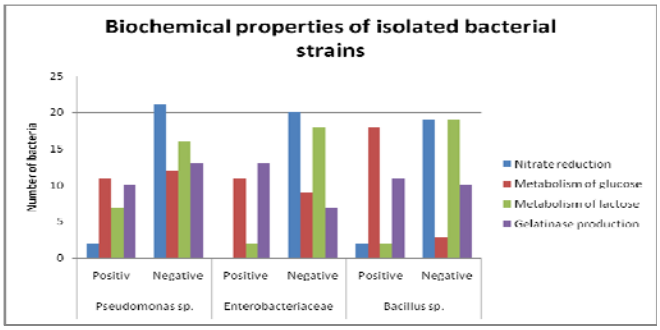

Fig. 1. Results of the biochemical characterization of the isolated bacterial strains

Utilization of oxygen from nitrates is one of the abilities that represent *Bacillus sp.* strains according to the literature, also the capability of degrading gelatine. 11 from the 21 isolates gave positive results of gelatinase activity, but only 2 were able to reduce the nitrate.

Pseudomonas sp. is described as a group of bacteria unable to utilize glucose or lactose, but capable using the oxygen from nitrates. Our result indicated that 11 strains from 23 were able to metabolize glucose and 7 to utilize lactose. A number of 10 strains had the ability of hydrolyzing the protein macromolecules by gelatinase enzyme and 21 gave positive results of nitrate reduction.

Although bacterial strains belonging to the family of *Enterobacteriaceae* are described as good glucose and lactose utilizers, our results show that 11 strains from the 20 gave positive results of glucose reduction and 2 strains of lactose utilization. 13 strains had gelatinase activity and none of the strains showed the ability to utilize oxygen from nitrates as electron acceptor.

3.2. Beneficial properties

In case of the bacterial strains isolated as *Pseudomonas sp.* 4 out of 23 gave positive result to siderophore production and 6 strains to cellulose degradation. These are contradictory to data found in the scientific literature, where bacterial strains belonging to the *Pseudomonaceae* family are described as good siderophore producers (ex.: pyoverdin or pseudobactin). The production of siderophores was detected in 25% (5 strains) of *Enterobacteriaceae* isolates and 19% (4 strains) of *Bacillus sp.* isolates. 4 from each group of bacteria isolated as *Enterobacteriacea*e and *Bacillus sp.* had the property to decompose the CMC, contributing thus to the local circulation of carbon (Fig. 2).

From all 64 isolates 20% were able to fix ferric ions from the growth medium by producing siderophores. 23% of the bacterial isolates were able to degrade the carboximethyl-cellulose present in the growth medium due to the presence of cellulase enzyme.

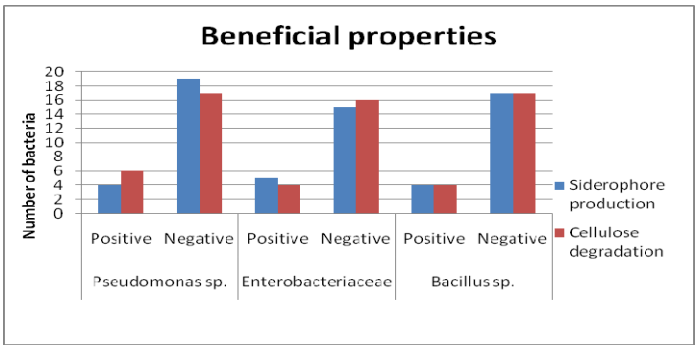

Fig. 2. Siderophore production and cellulose degradation ability

3.3. Genetic characterization

Restriction fragment length polymorphism is a technique that can be used to find out the variations in homologue DNA sequences. Digestion was realized using *MspI* and *HaeIII* restriction endonucleases. In Fig. 3 is presented the restriction profile of the bacterial strains isolated as *Pseudomonas sp.* given by the *MspI* enzyme (obtained restriction fragment varies between 700 and 120 base pairs).

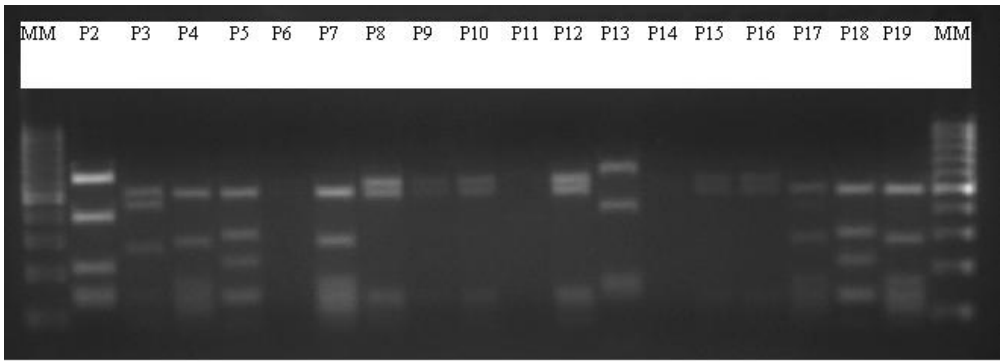

Fig. 3. Separation of DNA fragment, obtained after digestion with *MspI* endonuclease

Fig. 4 presents the restriction profile of *Pseudomonas sp.* bacterial strains, DNA fragments being digested with *HaeIII* enzyme (the obtained fragment varies between 700 and 120 base pairs). The two restriction profile (Fig 3, 4) suggests that P8, P9, P10 bacterial strains are identical due to the similar DNA fragments resulted after digestion with the two enzymes.

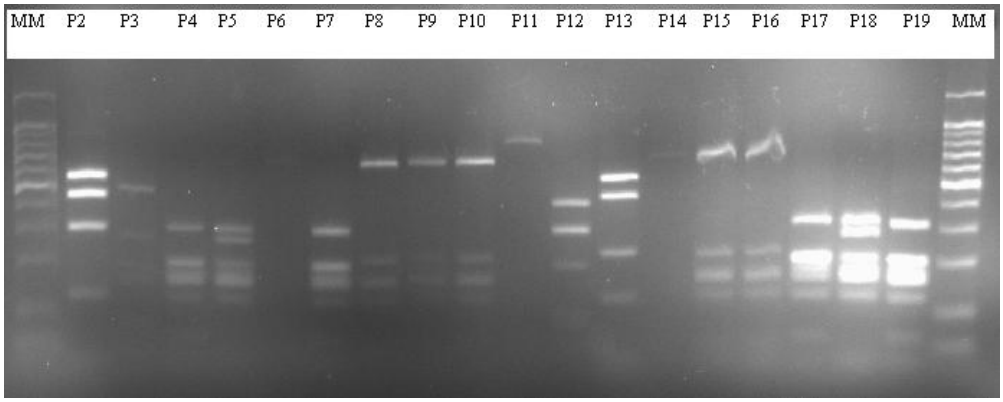

Fig. 4. Separation of DNA fragment, obtained after digestion with *HaeIII* endonuclease

Similar restriction profiles resulted for the bacterial strains isolated as *Bacillus sp.* and *Enterobacteriaceae* using the same enzymes. The obtained fragments were compared with digestion profiles of known bacterial strains as:

Delftia lacustris, *Bacillus cereus*, *Bacillus fordii*, *Serratia plymuthica*, *Pseudomonas sp.* isolated from soil samples and studied with similar genetic methods. The dendogram below (Fig. 5.) shows the similarity rate between the isolated bacterial strains and the taxonomically identified ones. For ex. bacterial strains E8, E9, E14, E16, E19 are identical, their similarity is 100%. At the same time the similarity rate between the group of bacterial isolates E8, E9, E14, E16, E19 and *Delftia lacustris* is approx. 55%.

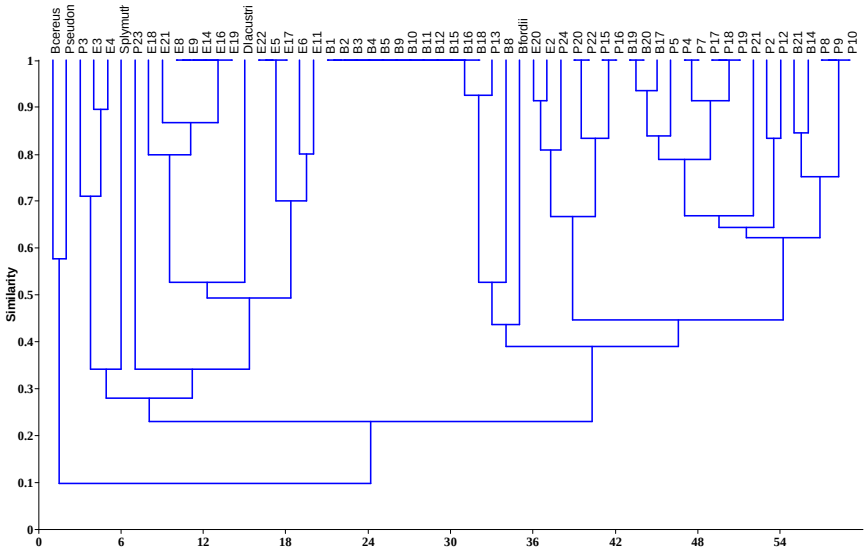

Fig. 5. Genetic variability of the isolated bacterial strains

4. Conclusions

In this study we analyzed a number of 64 bacterial strains isolated from the rhizosphere of different Bryophytes. Biochemical, beneficial and genetic characteristics were studied. From the analysis of the enzyme system the results show that 13 isolated bacterial strains were able to uptake ferric ion from soil. A number of 14 bacterial strains were demonstrated to produce cellulose enzyme. Only 5 strains have both abilities: to produce siderophores and to degrade CMC. Our future plan is to analyze other beneficial properties (phosphorus mobilization, production of secondary metabolic substances) of the isolated bacterial strains.

The results of the genetic analyses showed, that none of the isolated bacterial strains is identical with the used and taxonomically identified ones. Further genetic studies and 16S rDNA sequence analyses are needed to identify taxonomically the bacterial strains isolated from Borsáros raised bog.

Those strains which prove to be the best will be selected for use in biotechnological applications such as production of bacterial based biopreparates.

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