

## MICROBIAL PRODUCTS AS NATURAL ALTERNATIVE TO FERTILISERS: ISOLATION AND CHARACTERISATION OF NITROGEN FIXING BACTERIA

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*Bacteriile fixatoare de azot au o importanță mare în ciclul biologic al azotului, ei sunt capabile să fixeze azotul atmosferic cu ajutorul sistemelor de enzime – nitrogenaze, și așa fel să imobilizeze azotul pentru plante<sup>14</sup>. Izolarea se întâmplă cu inoculare repetată pe medii selective. Caracterizarea culturilor pure a fost realizat prin determinarea proprietăților morfologice al celulelor și tulpinilor (formă, pigmentare, suprafață, margină, consistență). Pentru caracterizarea genetică am folosit digestia cu enzime de restricție (RFLP - restriction fragment length polymorphism)<sup>9,10</sup>. Diferența între ADN ribosomal a fost realizat folosind enzimele de restricție *TaqI*, *HaeIII*, *Apal*<sup>17</sup>.*

*The nitrogen fixing bacteria have stimulating effect on the plant, they are able to fix the nitrogen in symbiosis with leguminous plants using the nitrogenase enzyme complex<sup>14</sup>. The isolation of the strains was made from the soil on selective media, and the strains were characterised through colony morphology analysis (form, elevation, margin, appearance, optical property, pigmentation, texture) and cellular morphology. For the genetically characterizations we have used restriction fragment length polymorphism (RFLP) of the 16S rRNA gene<sup>9,10</sup>. The differences in the rRNA region were mapped using *TaqI*, *HaeIII* and *Apal* restriction enzymes<sup>17</sup>.*

**Keywords:** nitrogen fixing rhizobacteria, rhizosphere, isolation, RFLP.

### 1. Introduction

The interactions between plants and the rhizosphere microorganisms are diverse. Carbon compounds released from plant roots into the soil drives to a great microbial development in the rhizosphere. Rhizobacteria living on the root surface

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have many positive effects: they can promote the plant growth, the crop production, and play a role in providing resistance to microbial diseases. Most plant species interacts with the rhizobacteria to obtain the essential mineral elements such as N or P. Some groups of microorganisms are capable to solubilise or to transform these elements, making them available to the plants [3]. Symbiotic N<sub>2</sub>-fixation is a well-known process, consisting in the interaction of the bacteria with legume roots which leads to the formation of N<sub>2</sub> fixing nodules. The reduction of the atmospheric N<sub>2</sub> to ammonia in the symbiotic root nodules is driven by the nitrogenase enzyme complex.

The most important symbiotic associations in the agriculture are the relations between leguminous plants and bacteria belonging to the genera *Rhizobium spp.* and *Bradyrhizobium spp.* These nitrogen fixing bacteria are used in many countries as inoculants.

The nitrogen fixing bacteria can be isolated directly from the root nodules of the host plant or from the soil [6, 7], using yeast extract mannitol selective culture media (YEM) [4, 9, 13]. Bromthimol blue (BTB) is used as indicator in order to detect the multiplication of the nitrogen fixing bacteria. The indicative of the acid production were yellow halos around the colonies on blue. Alberton et al. [1] determined the morpho – physiologic characteristics of the soybean and bean rhizobia. The isolates were grown on yeast extract – mannitol agar (YMA), and the morphologic characterization was realised by Gram stain. Ribosomal DNA genes have been studied for the genetic analysis of the *Rhizobium* strains, , using the restriction fragment length polymorphism (RFLP) method [10]. The most often used restriction enzymes for the digestion of rhizobial rRNA genes were: *AluI*, *CfoI*, *DdeI*, *HaeIII*, *HinfI*, *MspI*, *NdeII*, *RsaI*, *ApaI* and *TaqI*. The PCR – RFLP method was used successfully for the characterization and determination of the nitrogen fixing bacterial strains [5, 10, 11, 12, 16].

The main aim of our study was to isolate and characterize new nitrogen fixing bacterial strains from Harghita County using microbiological and molecular methods, in order to obtain microbial products as alternative to chemical fertilisers.

## 2. Materials and methods

### 2.1. Isolation of the bacteria

Brown forest soil samples from the Ciuc Basin (sample I) and cambisoil samples from Gheorgheni Basin (sample II) were tested for the isolation of the nitrogen fixing bacteria. The sampling was made from agricultural sites in which beans had been cultivated the previous year. The brown forest soil (sample I) can be characterized as being rich in organic matter and it is also agriculturally important in this region, due to the good crumb structure and mild acidity. Cambisols

(sample II) are characterized by an incipient soil formation having a weak horizon differentiation, being used as agricultural land. Samples were taken from 10 – 15 cm depth and were preserved in sterile plastic bags.

### **2.2. Multiplication of the bacteria and preparation of the pure cultures**

Colonies characteristic for rhizobia developed on the selective medium were picked and then purified by single-colony streaking on solid media. Pure isolations were kept on YMA medium (YMA: 10 g mannitol, 0,2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0,1 g NaCl, 0,5 g K<sub>2</sub>HPO<sub>4</sub>, 0,2 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0,01 g FeCl<sub>2</sub>·6H<sub>2</sub>O, 1 g yeast extract, 20 g agar, 1 L distilled water, 25 µg/ml BTB, pH = 6,7 – 7). The inoculated media were incubated 7 days at 28°C.

### **2.3. Morphological characterization**

Characterisation of pure cultures obtained by isolation was realized using methods of determination of the cells (Gram stain) colonies morphological properties (form, margins, appearance, pigmentation, texture).

### **2.4. Polymerase chain reaction (PCR) – and restriction fragment length polymorphism (RFLP)**

#### **2.4.1. Isolation of the DNA**

The isolation of the DNA was realized using Wizard Genomic DNA Purification Kit (Promega).

#### **2.4.2. Polymerase chain reactin (PCR)**

The amplification of the 16S rRNA was realized with the universal 27f and 1492r primer pairs. The sequences of the used primers were:  
27F - 5' AGAGTTTGATCMTGGCTCAG 3' and  
1492R - 5'TACGGYTACCTTGTACGACTT3'.

The PCR reaction was set up to a 50 µL final volume and contained the following reaction mixture: 5 µL 10XPCR buffer, 5 µL MgCl<sub>2</sub> (2.5 mM), 4 µL dNTP (0.2mM per nucleotide), 1-1 µL each primer (10 pmole), 0.25 µL Taq Polymerase (1 U), 1-3 µL DNA and ultrapure water.

Amplifications were carried out in an ESCO thermocycler with the following temperature profile: 5 min at 94°C, 30 cycles of denaturation (30 s at 94°C), annealing (25 s at 50°C) and extension (2 min at 72°C) and a final extension for 7 min at 72°C. Negative controls were included to check for the presence of false positives due to reagent contamination. Amplified products were separated on 1.5% agarose gels in 1XTBE buffer at 10 V cm<sup>-1</sup> for 30 minutes. Amplification products were stained with ethidium bromide, observed with a BioRad UV transilluminator and certified with GelDocXR program.

#### 2.4.3. *Restriction fragment length polymorphism – RFLP*

The final volume of the digestion was 20  $\mu$ L, containing the following: 5 U of restriction enzyme, 2  $\mu$ L of 10X reaction buffer (according to each enzyme requirements) and 10  $\mu$ L of the PCR products. After the preparation, the reaction media were incubated for 3 h at 37°C for *Hae III*, *Apa I*, and the *TaqI* reactions were incubated at 65°C for the same time. The restriction fragments obtained were separated in 1.5% agarose gel. Gels were run with 1X TBE at 10 V  $\text{cm}^{-1}$  for 60 minutes, and visualized using BioRad UV transilluminator. The dendrogram was constructed using the PAST program.

### 3. Results

#### 3.1. *The morphology of the nitrogen fixing bacteria*

12 pure isolates were obtained from the first sample (Ciuc basin) and 14 pure isolates were obtained from the second sample (Gheorgheni basin) during isolations on selective medium. Different separated colonies have been obtained after 2 days of incubation on 28°C on a selective media, as shown in figure 1. The pigmentation of the colonies vary among transparent, cream and yellow (Fig.1). The observed shape of the colonies was round with smooth margins and surface, and a convex shape.

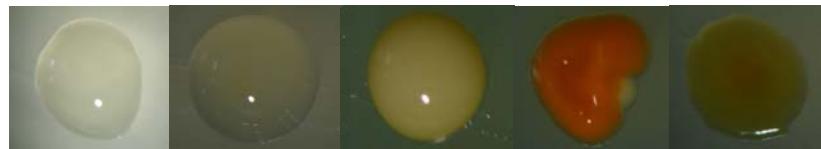


Fig. 1. Morphology of *Rhizobium* colonies obtained from pure cultures

The texture of the colonies was mucous, given to their extracellular polysaccharide (EPS) production.

The observed morphological traits of our isolates correspond to the culture properties of the rhizobia presented in the literature [2]. Regarding the cell morphological analysis, our isolates proved to be Gram negative bacteria.

#### 3.2. *Genetic characterization of the nitrogen fixing bacteria*

The polymerase chain reaction was realized with the *f27* și *r1492* universal primers. PCR amplification of the bacterial 16S rDNA gave a 1.4 kb fragment, which was subject to RFLP analysis.

The three enzymes used had different discriminating abilities; *TaqI* distinguished 4 types of restriction profiles, *ApaI* 2 types and *HaeIII* 7 types. *HaeIII* and *TaqI* restriction enzymes were the most discriminatory.

The four types of the restriction profile given by the *TaqI* enzyme are presented on Fig.2. The length of the obtained restriction fragments varies between 1000 and 200 base pairs (bp).

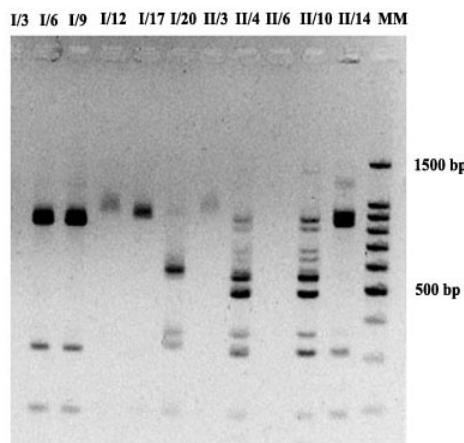


Fig. 2. Separation of the restriction fragments obtained after digestion with the *TaqI* enzyme

The digestion with the *Apal* enzyme resulted in two different restriction profiles, as it can be observed in figure 3. The length of the restriction fragment varied between 900 and 600 bp. The PCR product remained intact for the two samples I/4 and II/7, because the enzyme did not find a recognition site. These two isolates have been considered as different from others.

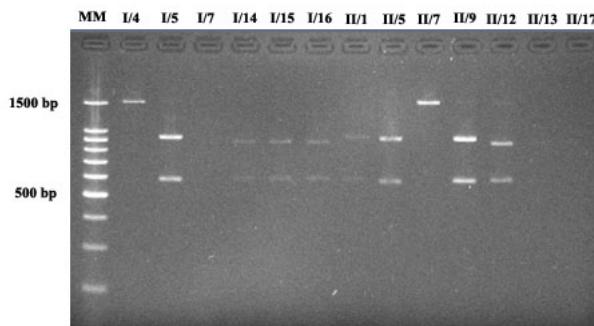


Fig. 3. Separation of the restriction fragments obtained after digestion with the *Apal* enzyme

7 different restriction profiles have been obtained after the digestion with the *HaeIII* enzyme, as it can be observed in figure 4. The length of the restriction fragments varies between 800 and 140 base pairs.

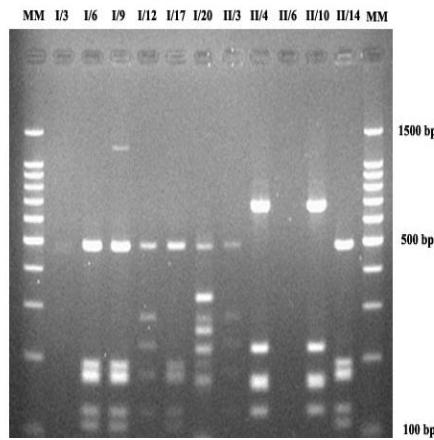


Fig. 4. Separation of the restriction fragment obtained after digestion with the *HaeIII* enzyme

By combining the results obtained from the three enzymes, it was possible to distinguish 8 different strains. The dendrogram shows 5 groups of divergent lineages (Fig. 5).

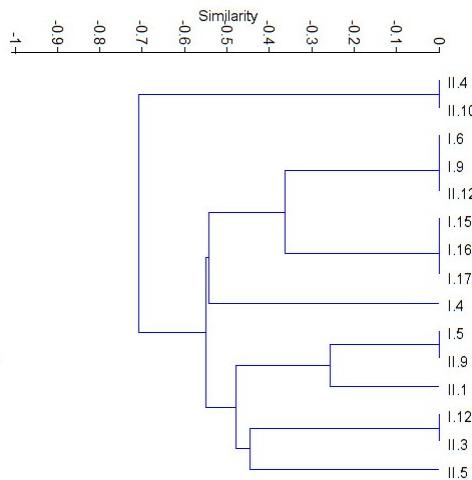


Fig.5. Genetic divergence of the isolated bacterial strains based on 16S ribosomal DNA sequence

A further sequence analysis of the 16S ribosomal DNA would be necessary in order to identify the distinguished 8 strains with the RFLP analysis.

#### 4. Conclusions

In this study the first and the most important step in the development of a new biopreparate, as the isolation of the potential beneficial bacterial strains, was achieved. On selective medium, pure culture of nitrogen fixing bacteria was obtained out of soil samples prelevated from Ciuc and Gheorgheni Basins in which beans had been previously cultivated. We obtained 20 respectively 24 pure bacterial cultures, showing the characteristic culture properties of rhizobia. 15 of the isolates were analysed for the differences in the ribosomal DNA with *HaeIII*, *ApalI*, *TaqI* restriction enzymes, being grouped in 8 strains. The isolated strains after a characterization of the beneficial traits can be used to obtain microbial products as alternative to chemical fertilisers. The next step will be the development of a kinetic model for the microbial biomass production, in order to determine the optimal culture conditions [18].

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#### R E F E R E N C E S

- [1]. *O. Alberton, G. Kaschuk, M. Hungria*, Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean, *Soil Biology & Biochemistry*, **vol. 38**, 2005, pp. 1298-1307
- [2]. *A. Bala, P. Murphy, K.E. Giller*, Genetic diversity of rhizobia from natural populations varies with the soil dilution sampled, *Soil Biology & Biochemistry*, **vol. 33**, 2000, pp. 841-843.
- [3]. *J.M. Barea, M. Pozv, R. Azcón, C. Azcón-Aquilar*, Microbial co-operation in the rhizosphere, *Journal of Experimental Botany*, **vol. 56**, 2005, pp. 1761-1778
- [4]. *I.V. Castro, E.M. Ferreira, S.P. McGrath*, Survival and plasmid stability of rhizobia introduced into a contaminated soil, *Soil Biology & Biochemistry*, **vol. 35**, 2003, pp. 49-54.
- [5]. *H.L.C. Coutinho, B.A. Handley, H.E. Kay, L. Stevenson, J.E. Beringer*, The effect of colony age on PCR fingerprinting, *Lett. Appl. Microbiol.*, **vol. 17**, 1993, pp. 282-284
- [6]. *E. Geniaux, G. Laguerre, N. Amarger*, Comparison of geographically distant populations of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris*, *Mol. Ecol.*, **vol. 2**, 1993, pp. 295-302
- [7]. *B.A. Handley, A.J. Hedges, J.E. Beringer*, Importance of host plants for detecting the population diversity of *Rhizobium leguminosarum* biovar *viciae* in soil, *Soil Biology & Biochemistry*, **vol. 30**, 1998, pp. 241-249
- [8]. *S.P. Harrison, L.R. Mytton, L. Skot, M. Dye, A. Cresswell*, Characterization of *Rhizobium* isolates by amplification of DNA polymorphisms using random primers, *Can. J. Microbiol.*, **vol. 38**, 1992, pp. 1009-1015
- [9]. *Küçük Ç., M. Kivanç, E. Kinaci*, Characterization of *Rhizobium* Sp. Isolated from Bean, *Turk J. Biol.*, **vol. 30**, 2006, pp. 127-132

- [10]. *G. Laguerre, E. Geniaux, S.I. Mazurier, R. Rodriguez-Casartelli, N. Amarger*, Conformity and diversity among field isolates of *R. leguminosarum* bv. *viciae*, bv. *trifoli* and bv. *phaseoli* revealed by DNA hybridization using chromosome and plasmid probes, *Can. J. Microbiol.* **vol. 39**, 1994, pp. 412–419
- [11]. *G. Laguerre, S.I. Mazurier, N. Amarger*, Plasmid profiles and restriction fragment length polymorphism of *Rhizobium leguminosarum* bv. *viciae* in field populations, *FEMS Microbiol. Ecol.* **vol. 101**, 1992, pp. 17–26
- [12]. *G. Laguerre, P. Mavingui, M.-R. Allard, M.-P. Charnay, P. Louvrier, S.-I. Mazurier, L. Rigottier-Gois, N. Amarger*, Typing of Rhizobia by PCR DNA Fingerprinting and PCR-Restriction Fragment Length Polymorphism Analysis of Chromosomal and Symbiotic Gene Regions: Application to *Rhizobium leguminosarum* and Its Different Biovars, *Applied and Environmental Microbiology*, **vol. 62**, 1996, pp. 2029–2036
- [13]. *A. Lakzian, P. Murphy, A. Turner, J.L. Beynon, K. E. Giller*, *Rhizobium leguminosarum* bv. *viciae* populations in soils with increasing heavy metal contamination: abundance, plasmid profiles, diversity and metal tolerance, *Soil Biology & Biochemistry*, **vol. 34**, 2002, pp. 519-529
- [14]. *J.A.W. Morgan, G.D. Bending, P.J. White*, Biological costs and benefits to plant-microbe interactions in the rhizosphere, *Journal of Experimental Botany*, **vol. 56**, 2005, pp. 1729-1739
- [15]. *K.G. Nandasena, G.W. O'Hara, R.P. Tiwari, R.J. Yates, J.G. Howieson*, Phylogenetic relationships of three bacterial strains isolated from the pasture legume *Biserrula pelecinus* L., *International Journal of Systematic and Evolutionary Microbiology*, **vol. 51**, 2001, pp. 1983-1986
- [16]. *A.E. Richardson, L.A. Viccars, J.M. Watson, A.H. Gibson*, Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers, *Soil Biology & Biochemistry*, **vol. 27**, 1995, pp. 515-524
- [17]. *J.É. Zilli, R.R. Valisheski, F.R.F. Filho, M.C.P. Neves, N.G. Rumjanek*, Assessment of cowpea Rhizobium diversity in Cerrado areas of Northeastern Brazil, *Brazilian Journal of Microbiology*, **vol. 35**, 2004, pp. 281-287
- [18]. *C. Ungureanu, O. Muntean, A.A. Chirvase, I. Nagy, A. Onu, A. Sălăgeanu*, Technological study of an aerobic bioprocess for a bacterial pharmaceutical preparation, *UPB Sci. Bull., Series B*, **vol. 69**, no. 3, 2007, pp. 19-26.