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ISOLATION AND IDENTIFICATION OF NITROGEN-FIXING BACTERIA IN ORDER TO OBTAIN BACTERIAL INOCULANTS

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Abstract: The aim of this research was to isolate strains of *Rhisobium* specific for bean crops in the western part of Romania, adapted to local environmental conditions. Seven strains were identified and characterized morphologically and molecularly, which were found to belong to the species *Rhizobium leguminosarum faseoli*. For each of them the growth rate was evaluated in order to be able to select a strain that could be the basis for the production of bacterial inoculants.

Nitrogen is essential for plant growth and development. Since the annual nitrogen consumption from the soil reserve exceeds the rate of recovery through pedo-genetic processes, chemical fertilizers are required to be supplemented. But this source of nitrogen is expensive and also polluting soil and water. One of the cost-effective sources of soil enrichment with nitrogen in forms directly assimilated by plants is the biological fixation of molecular nitrogen.

The process is carried out within a symbiotic system, established between microorganisms called "nitrogen fixers" and leguminous plants, called host plants. Following the nitrogen fixation process, plants provide their nitrogen needs in reduced form (NH^{4+}) and the excess is releases it into the soil. There are species of leguminous plants with a very high degree of specificity, which may have symbiotes from a small number of species of nitrogen-fixing bacteria, but also legumes with a low degree of specificity, which can be infected by a greater number of such species.

Therefore, to the establishment of a leguminous culture it is necessary to ensure that the soil contains the specific symbiotic bacteria for the species of interest or to use bacterial inoculants. For the production of bacterial inoculants it is necessary to identify and characterize bacterial strains specific for the interest plant species, adapted to the environmental conditions of the region where the culture will be established. In general, after identification of bacterial strains they are morphologically characterized by microbiological methods but these methods are not always very accurate. Therefore these analyses can be complemented by molecular evaluations. In the case of taxonomy and phylogeny studies of bacteria, the 16s rRNA gene is most often used. This gene was chosen because it is present in all bacterial species, its sequence has remained constant in evolution and has a length of 1500bp, making it suitable for bioinformatics analysis. This gene encodes the small subunit of procariot ribosomal RNA and is part of a family of multigenes or may be part of an operon. Starting from this premise, one of the purposes of this paper was the isolation and characterization of some nitrogen-fixing bacteria native to the Romanian soils specific to the bean plants.

Material and method

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Biological material

To isolate nitrogen-fixing bacteria of the genus Rhizobium specific to beans, plants were harvested after flowering from several locations in the western part of Romania: F1, F2– Timis county, 3- Hunedoara county, F4, F5, F6 – Caras Severin county and F7- Arad county.

Working methods

To prevent damage of the nodules formed on the plant roots, in which nitrogen-fixing bacteria are found, the plants were harvested together with a quantity of soil in which they grew, and the isolation process began less than 24 hours after harvesting. The isolation procedure followed the Vincent method, 1970 .

DNA was extracted with the ISOLATE II Genomic DNA kit (Bioline) from a 1.5 ml volume of liquid bacterial culture. For cell lysis, in addition to the specific solutions, proteinase K is added to break down the samples proteins, followed by a heating step at 70ºC. ISOLATE II Genomic DNA Spin Column was used for DNA purification. DNA elution was performed with 100 µl of elution buffer.

The DNA concentration and its quality were evaluated based on spectrophotometric method with Nanodrop 8000 (Thermo Fisher Scientific, USA).

Amplification of the 16 S rRNA gene from DNA samples extracted from nitrogen-fixing bacteria was carried out using two primers as follows: F27 AGA GTT TGA TCM TGG CT and F485 CAG CAG CCG GGG TAA.

The amplification conditions were as follows:
A. Denaturation 90°C - 3 minutes B. 35 cycles 90°C - 30 seconds 53°C - 1 minute 72°C - 1 minute C. DNA synthesis 72°C - 3 minutes

Composition of the amplification mixture

Component	Amount
DNA	200 ng
Primer *nif*H reverse	1 pmol/reaction
Primer *nif*H forward	1 pmol/reaction
dNTP	200 µM
Taq polymerase	0,5 U
MgCl2	2,4 mM
Buffer solution	1x
Sterile distilled water	up to 20 µl

DNA amplified products run in a 1.6 agarose gel electrophoresis, in ethidium bromide presence for approximately 40 min under a voltage of 100 V. After completion of electrophoresis, the gels were examined using a UV light transilluminator. Finally the gels were examined using a UV light transilluminator.

After amplification of the DNA and analysis by agarose gel electrophoresis, it was necessary to purify it in order to send it for sequencing. In this context, purification of amplified DNA refers to its separation from the agarose gel in which electrophoresis took place, and this process was performed using the Monarch DNA Gel Extraction Kit (New England BioLabs).

The purified DNA fragments were sequenced at Macrogen (Holand).

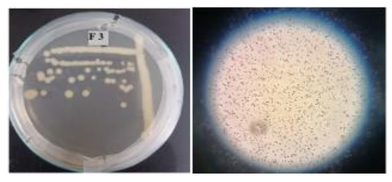
Results and discussions

Plants collected from different locations were processed in the laboratory. In the first step, the number of nodules per plant was determined. The highest number of nodules was observed in variant F2, with plants from Timisoara. The lowest number was observed in a variant from Caras-Severin County (F4).

The isolated bacterial strains were inoculated on solid culture medium and were maintained at 27°C for growth and then stored at 4°C. In order to purify the isolated bacterial strains, 3 - 4 subcultures were carried out, successively on solid and liquid media respectively. Finally, a single pure culture was isolated for each experimental variant and further analyzed.

Identification of bacterial strains by morphological methods

Morphological characterisation of the seven strains (**F1**, **F2**, **F3**, **F4**, **F5**, **F6** and **F7**) revealed round, regular, convex, pink-white, mucilaginous, rhizobial bacterial colonies. Microscopic observations also allowed the identification of the bacterial genus but did not make it possible to identify specific species.

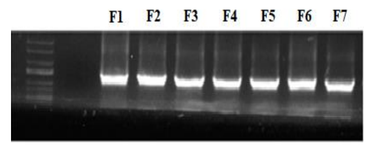


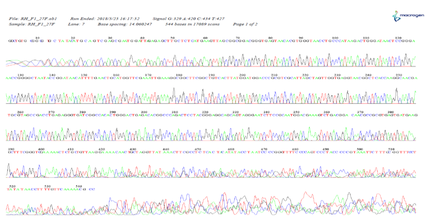
Identification of bacterial specie based on molecular analysis

Since the characterization of bacterial strains was performed by evaluating the sequence of a gene, it was necessary to use a method for bacterial DNA extraction that would lead to the best results in terms of both concentration and quality. It was previous identified that the ISOLATE II Genomic DNA kit (Bioline) leads to obtaining appropriate DNA samples.

The rRNA 16S gene occurs in most prokaryotes and is most commonly used in taxonomic identification and classification as it has specific and easily recognizable variable regions. For isolated bacterial strains, amplification of the 16S rRNA gene was carried out.

The amplifying products were analyzed using agarose gel electrophoresis and bands matching the 16S rRNA gene which were clearly distinguished for the seven samples were extracted from the gel and purified.

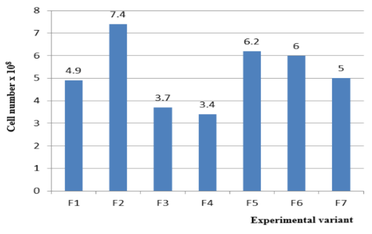




DNA fragments corresponding to the 16S rRNA gene corresponding to each bacterial strain analysed were sequenced at Macrogen, The Netherlands. The sequence obtained for strain F1 following sequencing with primer F 27 is shown for example.

The sequences obtained were compared with existing information in the NCBI (National Center for Biotechnology Information) Standard Nucleotide Blast.
All seven bacterial strains isolated had a 98-99% correspondence to *Rhizobium leguminosarum phaseol*i, a species known to be specific to beans. Therefore, all single bacterial strains may be used for the production of inoculants for bean crops. Although all the isolated strains were part of the species *Rhizobium leguminosarum phaseoli* it was of interest to determine their developmental rate, because it is an important factor in the production of inoculants.

The development capacity was evaluated by spectrophotometric method. The optical density was measured at 600 nm. The number of cells corresponding to the optical density was determined with the formula: 1 unit optical density ($1OD_{600}$) = $8x10^8$cells/ml (http://www.labtools.us/bacterial-cell-number-od600/).



It was observed that the highest growth rate was recorded for strain F2, followed by F5, F6 and F7. A lower rate was observed for strains F3 and F4. Therefore, strains with a high growth rate can be recommended for inoculant production**.** It was observed that strain F2, which was isolated from the plants with the highest number of nodules, had the highest growth rate.

Conclusions

In the present work, a methodology was developed to isolate 7 bacterial strains from plants collected from different locations in the western part of the country, which were identified on the basis of both morphological characteristics and 16S rRNA gene sequences as *Rhizobium leguminosarum faseoli*.
Following the growth assessment, the strains with the highest development rate were identified as fost F2, F5 and F6. The strain F2, originated from plants with a high nodulation capacity could be recommended for inoculants production specific for beans.