Introduction

All symbioses involve an association, permanent or not, between two or more species, association beneficial for all the involved organisms. Symbiotic associations are common in nature, from bacteria and fungi that form close alliances with terrestrial plants, to annelids and sulfur-fixing bacteria on the ocean floor (Paracer and Ahmadjian, 2000). Plants cover almost the entire surface of the earth and many types of microorganisms grow in close association with them. Although many microbial activities may be detrimental to plants, microorganisms can bring benefits to plants, especially nutritional benefits. Various interactions have been identified between plants and microorganisms (bacteria, fungi and algae), resulting in mycorrhizae, actinorhizae, root nodules involved in biological nitrogen fixation, the control of pests, plant tolerance to environmental factors, and plant growth-promoting rhizobacteria. Soil is one of the most obvious environments where these interactions occur. In the symbiosis between plants and bacteria, the enzyme system necessary for nitrogen fixation is found only in prokaryotes and this symbiotic association shows a high degree of specificity with benefits for both partners: the plant provides carbon and energy to the bacteria and the bacteria fix nitrogen required for amino acids synthesis in the plant (Barton and Northup, 2011).

Study objectives

One of the current methods used to avoid saturating the soil with synthetic fertilizers is the application of inoculants (solid or liquid media containing viable bacterial cells) to the plant root of leguminous crops. Rhizobia fix atmospheric nitrogen in root nodules of legume plants. After harvest the roots remain in the soil thus forming the organic nutrients used by future crops. Also, the ability of legumes to fix nitrogen in symbiosis with rhizobia, allows development on degraded or low nutrient intake soils.

One problem of the inoculants may be represented by the poor adaptation of commercial rhizobial strains to different pedo-climatic conditions and situations may arise where these strains may be ineffective. Native, indigenous strains present an advantage over commercial ones, because they are already adapted to local environmental conditions. A major objective of this study is to identify indigenous rhizobial strains efficient in nitrogen fixation and the phylogenetic relationships between them and some reference strains.

For this study, the following objectives were proposed:

1. The isolation of some rhizobial strains from white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) plant root nodules found in pastures and natural habitats for genetic characterization and taxonomy studies.

2. The evaluation of nitrogen fixing efficiency of some rhizobial isolates by biomass accumulation and protein content of red and white clover plants inoculated with rhizobial strains.

3. *Material and research methods*

Between June 1st and June 15th field trips were conducted in order to collect plant material in the following localities: Ciumârna, Izvorul Alb, Mestecăniș, Vârful Rarău, Pojorâta, Câmpulung Moldovenesc – Stulpicani, Vama, Palma (Suceava county), Oituz, Poiana Sărată, Hârja, Borzești, Jevreni (Bacău county). The plant material consisted of mature white clover (*Trifolium repens*) and red clover (*T. pratense*) plants.

For statistical analysis, the collection points were divided into two groups, called "Rarău" and "Oituz". Plants were harvested from cropland and grassland and from different soil types. Root nodules were isolated, washed and transferred to Eppendorf tubes containing glycerol. Each nodule was assigned a code that contains the geographical origin of the plant, the plant number and nodule number. In the lab, each nodule was crushed in order to free its content and that content was inoculated on YMA solid culture medium. Each rhizobial strain used for this study was isolated only from a single nodule. After confirming that the strains were indeed rhizobial, they were taken with a sterile loop and inoculated into liquid YEM medium, in order to subsequently carry out total bacterial DNA isolation. After inoculation of the medium, the rhizobial cultures were incubated for 3-5 days at 28°C. For long-term keeping (up to 1-2 weeks), the bacterial cultures were maintained at 4°C on solid culture medium. For culturing in a liquid medium to obtain the inoculum required for nodulation tests and isolation of bacterial DNA, we used the same recipe for the medium with no agar.

For molecular genetic analysis (RAPD, ARDRA, gene sequencing of the 16S gene and 16S-23S intergenic spacer) of the isolated rhizobial strains, total bacterial DNA was isolated by the CTAB-NaCl method.

4. Results and discussion

RAPD analysis

Following the DNA isolation by the CTAB-NaCl protocol, 32 rhizobial isolates were chosen from different geographical locations and 3 reference strains: *Bradyrhizobium japonicum* LMG 4252, *Ensifer meliloti* LMG 6133, *Rhizobium leguminosarum* LMG 8820 for RAPD analysis. Three decamer primers were selected: OPA-18, OPC-02, S-17. For the statistical analysis of the data generated by RAPD, the rhizobial isolates were divided into two groups, according to their geographic origin: Rarău and Oituz.

The generated RAPD pattern was used for the construction of the cladogram.

The genetic distances were estimated by the degree of variance among and within the two groups, using AMOVA. The genetic diversity among groups was 7.64% and within the groups was 92.35%. The value of Φ_{ST} among the two groups is lower than 0.1, which means that there are no differences between the two groups ($p<0.0001$, from 1000 permutations).

The genetic relationships between the rhizobial isolates were also investigated by PCoA (Principal Coordinate Analysis or Classical Multidimensional Scaling - CMDS). According to the PCoA analysis, the rhizobial isolates were distributed in a 3D environment on 3

principal coordinates responsible for 10.98%, 9.12% and 8.93% of variation.

From the analysis of the RAPD-generated cladogram, it's obvious that the high degree of variability of the rhizobial isolates does not allow their grouping on well-defined clusters and there are differences even between rhizobial strains isolated form the same sampling location. Among the factors responsible for this population heterogeneity could be the primers' lack of specificity, the disappearance of appearance of new RAPD loci, horizontal gene transfer and the native variability of the rhizobial strains.

ARDRA analysis

Just like in the RAPD analysis, reference rhizobial strains were included in the analysis: *Bradyrhizobium japonicum* LMG 4252, *Ensifer meliloti* LMG 6133, *Rhizobium leguminosarum* LMG 8820, *Bradyrhizobium japonicum* USDA 110, *Sinorhizobium (Ensifer) meliloti* 1021 and *Mesorhizobium loti* MAFF 303099.

16S gene amplification was done with specific primers. PCR products were digested with the restriction enzymes *Hae*III, *Alu*I and *Mbo*I. *Hae*III generated the most restriction fragments: 464 for the rhizobial isolates from red clover nodules and 339 for the rhizobial isolates from white clover nodules. There are no signifficant differences between the averages of the restriction fragments generated by the three restriction enzymes for the two groups of rhizobial isolates.

The genetic distances between the rhizobial isolates were estimated by the AMOVA analysis. The genetic diversity among groups was 1.93% and 98.06% within groups. The value of Φ_{ST} among groups was lower than 0.1, which means there is no statistical significant difference between the rhizobial isolates form red and white clover ($p = 0$, from 1000 permutations). AMOVA was based on Jaccard coefficients and the results show a low level of variability

between the rhizobial isolates. ARDRA revealed a homogenous bacterial community.

16S gene sequence analysis

The 16S sequences showed a length of 1382 nucleotides with a content of 20.04% thymine, 23.64% cytosine, 24.38% adenine and 31.94% guanine. The estimated value of transitions/transversions is 0.54. The substitutions models and rates were estimated by the Kimura 2-parameter and a discrete Gamma distribution was used to model the differences in evolutionary rates among sites. For the Maximum Likelihood (ML) estimated values, a dendrogram topology was automatically calculated.

Two main clusters are visible on the dendrogram, noted **A** and **B**. The rhizobial isolates from cluster **A** grouped together with *R. leguminosarum* SVD FK0, *R. leguminosarum* CIAM 685, *R. leguminosarum* bv. *viciae* 3841 and *R. leguminosarum* bv. *trifolii* ATCC 14480.

Like in the case of RAPD and ARDRA analysis, the rhizobial isolates did not cluster together according to their geographic origin, having a uniform distribution on the cladogram.

16S-23S intergenic spacer sequence analysis

The sequences of the 16S-23S IGS varied in length from 1056 to 1255 nucleotides, with a content of 25.16% thymine, 22.97% cytosine, 20.47% adenine and 31.39% guanine. The estimated value of transitions/transversions is 1.74. The substitutions models and rates were estimated by the Kimura 2-parameter and a discrete Gamma distribution was used to model the differences in evolutionary rates among sites. For the Maximum Likelihood (ML) estimated values, a dendrogram topology was automatically calculated.

Similar sequences were downloaded from GenBank and included in the analysis. Five main clusters are visible on the dendrogram: **A**, **B**, **C**, **D** and **E**.

Strains R31, R87 and R1, isolated from the "Rarău" area did not cluster in any of the five groups mentioned earlier, being very divergent from the strains included in the analysis. Strain R31, isolated from a mountain grassland shows a similarity of 94% with the reference strain *Rhizobium sp.* CCBAU 83530. Strain R87 shows a similarity of 99% with the reference strain *Rhizobium pisi* DSM 30132, isolated from *Pisum sativum* nodules in Peru.

Bacterial population structure and gene flow

The genetic structure and gene flow were assessed on the basis of 16S gene and 16S-23S IGS sequences with a Bayesian clustering approach using a Markov Chain Monte Carlo (MCMC) assignment method as implemented in the Structure software. The results showed that the value of K (number of genetic units) was 4 for both the 16S gene and the 16S-23S. Accordingly, all the sequenced rhizobial isolates were distributed into four genetic units and their presence and distribution were overlaid on the sampling locations map. For the 16S gene, there is a relatively homogenous distribution of the rhizobial isolates and the percentage of the isolates assigned to the four genetic units differs in the two large groups, Rarău and Oituz. In the case of the 16S-23S IGS, the distribution is more heterogeneous and the percentage of the four genetic units fluctuate.

Quantitative evaluation of plant material dry weight

After the genetic analysis of the rhizobial isolates, they were used for plant inoculations in order to determine the plant dry weight. The solution that was used for watering the plants lacked nitrogen to favor symbiosis. The most efficient rhizobial isolates in nitrogen fixation were considered the ones that lead to the highest plant biomass accumulation. After 45 days of growth, the inoculated plants (135 red clover plants and 107 white clover plants) were analyzed. The roots were detached from the stems and weighed separately.

The high positive values of the Pearson coefficient between the dry weight of the roots and stems for the white clover plants (r = 0.946) and red clover plants (r = 0.950), indicate a strong correlation between the biomass of these two plant components. As the dry weight of the root is higher, so is the dry weight of the stems and leaves and vice-versa.

Fresh weight and protein content of plants inoculated with rhizobial isolates

For this analysis, 13 rhizobial isolates were used for white clover plants inoculation and 20 rhizobial isolates were used for red clover plants inoculation. After 45 days, an average of 15 plants for each rhizobial isolate were weighed and their total protein content were determined by the Bradford assay.

The inoculated red clover plants were generally more vigorous, with an average weight of 592.7 mg and the white clover plants had an average weight of 179.6 mg. Among the inoculated white clover plants, the most developed were the ones inoculated with isolates A40 (340 mg), A29 (273 mg) and A19 (257 mg). The least developed plants were the ones inoculated with isolates A51 (70 mg) and A64 (62 mg). Among the inoculated red clover plants, the most developed were the ones inoculated with isolates R82 (976 mg), R108 (910 mg) and R41 (752 mg). The least developed plants were the ones inoculated with isolates R130 (420 mg), R77 (370 mg) and R127 (68 mg).

For protein content analysis, the fresh plants were ground in lysis buffer and part of the extract was mixed with Bradford reagent for the Bradford assay. Serial dilutions of 1, 2, 7, 10, 20, 50 µg bovine serum albumin (BSA) were used for the standard curve.

The plant protein content was calculated by using the equation y = 0,0208x + 0.0226:

- µg of protein = (extinction – 0.0226) / 0.0208

The amount of protein was calculated relative to the fresh biomass of the plants and was noted as milligrams of protein / gram of fresh plant material.

In the case of white clover plants, the highest amount of protein was found in the plants inoculated with isolates A64 (16.49 mg/g), A100 (11.49 mg/g) and A54 (10.7 mg/g) and the lowest amount was found in the plants inoculated with isolates A40 (5.6 mg/g) and A51 (4.14 mg/g). The plants inoculated with the commercial strain *R. leguminosarum* LMG8820 and the plants watered with nutrient solution had a protein content close to the average: 8.01 mg/g and 7.73 mg/g.

In the case of red clover plants, the highest amount of protein was found in the plants inoculated with isolates R127 (6.09 mg/g), R130 (4.07 mg/g) and R30 (3.95 mg/g) and the lowest amount was found in the plants inoculated with isolates R117 (3.06 mg/g), R36 (2.65 mg/g) and *R. leguminosarum* LMG8820 (2.51 mg/g). The plants watered with nutrient solution had a protein content of 3.76 mg/g, a little above the average.

Regarding the correlation between fresh weight and protein content, the Pearson coefficient had a value of -0.35 for the white clover plants and -0.59 for the red clover plants. In both cases, the Pearson coefficient shows a weak to moderate negative correlation, which could indicate and inverse proportionality between fresh weight and protein content of the inoculated plants.

Rhizobial cells abundance in the root nodules

Rhizobial cells abundance was done by qPCR. Total DNA was isolated from root nodules, all the samples were diluted to the same DNA concentration (2.5 ng/µl) and were used as template for the qPCR assay, using specific bacterial primers.

Two reference genes were used: *ACT* and *UBC2*. The number of copies of the two reference genes show an interdependent growth in

relation to the 16S gene, regardless of the rhizobial isolate used to inoculate the plants. There is a strong positive correlation between the copy numbers of the two genes, with a coefficient value of 0.9376.

In relation to the *ACT* reference gene, the 16S, *nifH* and *nodA* genes show a higher level of accumulation (a bigger number of copies) for all the rhizobial isolates. There is a big number of copies of the *nifH* (involved in nitrogen fixation) and *nodA* (involved in the establishment and maintenance of the symbiosis) genes for the rhizobial isolates R19 and R112.

Just as in the case of reference gene *ACT*, the 16S, *nifH* and *nodA* genes show a high level of accumulation in relation to the *UBC2* reference gene.

There is a strong positive correlation ($r > 0,98$) between the *nifH* and *nodA* gene copy number, when reported to the *ACT* ($r = 0,993$), *UBC2* ($r = 0,982$) or 16S ($r = 0,988$) genes, which means that the copy number of the *nifH* gene rises proportionally to the copy number of the *nodA* genes.

Conclusions

The studies undertaken in order to fulfill the proposed objectives (the taxonomical studies of the rhizobial isolates and their ability to fix atmospheric nitrogen) have led to the following conclusions:

1. The cladogram constructed from the RAPD analysis and on the basis of the similarity matrix there is an apparent clustering of the rhizobial isolates, depending on their geographic origin. The

AMOVA analysis showed a genetic diversity of 7.64% among the groups and 92.35% within the groups. The value of Φ_{ST} among the two groups is lower than 0.1 which means that there is no difference between the Rarau and Oituz groups.

2. The ARDRA analysis and the subsequent AMOVA analysis showed a genetic diversity of 1.93% among the groups and 98.06% within the groups. The value of Φ_{ST} among the two groups is lower than 0.1, which means that there is no difference between the isolates form white clover and red clover plants. No correlation has been identified between the geographical origin of the rhizobial isolates and their genetic diversity.
3. The dendrogram constructed from the 16S gene sequences shows two apparent clusters and the 16S-23S IGS dendrogram shows five apparent clusters. There is no correlation between the geographic origin of the rhizobial isolates and their clustering options. By comparing the 16S and 16S-23S sequences to sequences from GenBank, there was a similarity of over 95% to species of the genus *Rhizobium*.
4. The genetic structure of the rhizobial population and gene flow were estimated with a Bayesian clustering approach on the 16S and 16S-23S IGS sequences. This analysis showed a relative admixture between isolates from different populations. The haplotype network resembles the clustering pattern of the 16S and 16S-23S sequences cladogram.
5. To test their nitrogen fixing efficiency, 49 rhizobial isolates from red clover and 44 rhizobial isolates from white clover plants were used to inoculate clover plants. The dry weight of the roots and aerial parts showed a high correlation coefficient for both white clover (r = 0.946) and red clover plants(r = 0.950). The most efficient rhizobial strains were considered R121, R9, R37 and R73 for red clover plants and A19 and A2 for white clover plants.

6. There is a moderate positive correlation (r = 0.87 – for white clover) and a weak positive correlation (r = 0.27 – for red clover) between the number of active nodules and the fresh weight of the inoculated plants.
7. The total protein content (mg of protein / g of fresh plant material) from inoculated plants was determined. These values were higher for the white clover plants (8.5 mg/g) than for red clover plants (3.59 mg/g). There was a negative correlation between the protein content and fresh weight for both the white clover plants (r = -0.35) and red clover plants (r = -0.59). The highest protein content was recorded for the plants inoculated with the A64, A100, A54, R127, R130 and R30 rhizobial isolates.
8. Regarding the rhizobial cells' abundance in the nodules, there is a strong positive correlation (r > 0.98) between the number of *nifH* and *nodA* genes in relation to the *ACT* (r = 0.993), *UBC2* (r = 0.982) or 16S (r = 0.988) control genes. The highest abundance was recorded in the R19 and R112 rhizobial isolates

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