NITROGEN FIXING EFFICIENCY OF SOME INDIGENOUS *RHIZOBIUM LEGUMINOSARUM* ISOLATES FROM RED CLOVER (*TRIFOLIUM PRATENSE* L.) AND WHITE CLOVER (*TRIFOLIUM REPENS* L.) NODULES

ANDREI ȘTEFAN[1*], CĂTĂLINA STEDEL[2], GOGU GHIORGHIȚĂ[3], DUMITRU COJOCARU[1], RODICA CĂTĂLINA EFROSE[2]

Keywords: *Rhizobium leguminosarum*, clover, biological nitrogen fixation, symbiotic efficiency
Abstract: The increasing demand for food leads to intensive agricultural practices which require a high input of synthetic fertilizer. The excess fertilizer ends up in aquatic ecosystems, leading to eutrophication and hypoxia. An alternative would be the use of microorganisms capable of incorporating atmospheric nitrogen into organic compounds. Nitrogen fixing bacteria, collectively known as rhizobia, establish symbiosis with leguminous plants in exchange for a carbon source, this relationship being beneficial for both parties. Commercial rhizobial strains have been used as crop inoculants to improve biomass production but the commercial strains are not as adapted to pedo-climatic conditions as the local, indigenous strains. We tested the nitrogen fixing efficiency of some native *Rhizobium leguminosarum* isolates from Romania, on biomass production of two clover species. Plants inoculated with the A2, R37 and R73 rhizobial isolates showed the highest amount of biomass accumulation.

INTRODUCTION

Nitrogen is the most abundant gas in Earth’s atmosphere and is a vital element, being included in biological molecules such as nucleic acids, proteins, vitamins. It is a tasteless, odorless, and inert gas and is found as a molecule comprised of two atoms (N_2). Most organisms use ammonia as a primary source of nitrogen (Lindemann and Glover, 2003). Biological nitrogen fixation (BNF) is the process by which non-reactive atmospheric nitrogen is included into organic compounds and it’s restricted to only a few prokaryotic organisms (Franche et al., 2009). BNF is an energy-consuming process and is accomplished by the nitrogenase enzymatic complex (Kim and Rees, 1994). The energy is required to break the triple bonds between the two nitrogen atoms, 16 ATP molecules being consumed for each reduced nitrogen molecule (Kneip et al., 2007).

Earth’s growing population requires an increased amount of food which, in turn, requires high amounts of synthetic fertilizer. As a result of intensive agricultural practices, the excess fertilizer is washed by rain water as surface runoff or is leaching into groundwater, leading to eutrophication and hypoxia of aquatic environments, among other environmentally damaging side-effects (Vance, 2001).

The symbiotic associations between rhizobia and forage/fodder legumes are considered the most important nitrogen fixing systems in agriculture (Herridge et al., 2008). BNF is an alternative to extensive synthetic fertilizer use and the application of commercial inoculants has become common practice, millions of hectares of crops being inoculated with nitrogen fixing organisms in South America (Castro-Sowinski et al., 2007). One setback of this is that the commercial rhizobial strains are not adapted to local environment conditions, unlike highly competitive indigenous rhizobial strains (Catroux et al., 2001; Streeter, 1994; Fabiano and Arias, 1991; Meade et al., 1985; Laguerre at al., 2003). Indigenous rhizobial strains are an important resource and their isolation and characterization could lead to the improvement of BNF in crops (Lindstrom et al., 2010).

Clovers are primarily used as fodder, being the second largest leguminous crop in Romania, as cultivated surface, after alfalfa (The Romanian Statistical Yearbook, 2012). Clovers establish symbiotic relationships only with *Rhizobium leguminosarum* bv. *trifolii* (Denarie et al., 1992), a nitrogen fixing alphaproteobacteria.

We tested the nitrogen fixation efficiency of some wild, indigenous rhizobial strains isolated from red and white clover, by biomass accumulation and, to a lesser extent, by number of nitrogen fixing nodules.

MATERIALS AND METHODS

Cell cultures preparation and plant inoculation

The rhizobial isolates used in this study are included in the collection of the Institute of Biological Research Iași – Department of Experimental and Applied Biology. The rhizobial isolates are stored in 20% (v/v) glycerol, kept at -80^{o}C and were isolated from nodules of red clover (*Trifolium pratense*, L.) and white clover (*Trifolium repens*, L.) plants. For making the inoculum, the rhizobial cultures were transferred from the stock collection to liquid Yeast Mannitol Agar –

YMA medium (Vincent, 1970) and incubated at 28°C for 3-5 days. All the rhizobial isolates used in this study belong to *Rhizobium leguminosarum* bv. *trifolii*.

Two species of clover were used for this study: red clover (*T. pratense*, L.) cultivar Livada Sara and white clover (*T. repens*, L.) cultivar Carmencita. The clover seeds were provided by the University of Timișoara and the Agricultural Research-Development Station Livada, Satu Mare. The seeds were immerged in sulfuric acid for 5 minutes and rinsed three times with de-ionized water. Surface sterilization was carried out in 2% sodium hypochlorite and 0,02% Tween 20 for 20 minutes. The seeds were then rinsed with double distilled water and placed in Petri dishes on 3 mm Whatmann wet filter paper. The Petri dishes were kept in the dark for 72 hours at room temperature. After seed germination, 1ml of liquid culture containing rhizobial isolates (with an OD_{600}: 1) was applied to the roots and the plants were placed in sterilized perlite in open pots. Five plants were planted in each pot, all of them inoculated with the same isolate. The plants were kept in a plant growth chamber in adequate conditions (Handberg and Stougaard, 1992): 18 hours of light/6 hours of darkness, temperature of 26°C and humidity of 65% in a Nüve TK600 test cabinet. The plants received Hogland nutritive solution (Broughton and Dilworh, 1971) alternating with distilled water, once every two days. The nutritive solution lacks nitrogen, thus favoring symbiosis with the rhizobial isolates.

The red clover plants were inoculated with 49 rhizobial isolates from red clover nodules which are denoted by the letter 'R' followed by a number. The white clover plants were inoculated with 44 rhizobial isolates from white clover nodules which are denoted by the letter 'A' followed by a number. The plants were removed from the growth chamber after 45 days, air-dried, the roots were detached from the stems and weighed separately. There was an uninoculated control group but at the end of the growth period, the uninoculated plants were not developed and were discarded. A Kern 770 scale (Kern & Sohn GmbH, Balingen, Germany) was used for weighing the plants.

After this initial screening, eighteen rhizobial isolates were chosen for nodulation tests: A8, A15, A22, A26, A33, A40, A59, A76, A107, R19, R47, R55, R58, R71, R77, R103, R108 and R112. Five plants were inoculated with these isolates each, and after 45 days the nodules were counted and based on their color it was established if they were nitrogen fixing or non-fixing nodules. Nitrogen fixation occurs in nearly anoxic conditions and leghemoglobin (a red pigment similar to animal hemoglobin) acts as a buffer for oxygen diffusion, keeping a low oxygen level in the cell. Based on this, all pink nodules were considered capable of nitrogen-fixation. The white nodules were also counted but they were considered to be non-fixing nodules.

DNA extraction and amplification

To test the presence of bacteria in the pink nodules, some of the nodules were isolated from the roots and total DNA extraction was carried out by the Promega DNA IQ™ System (Promega, Madison WI, USA), according to the manufacturer's specifications. DNA was extracted only from the nodules of plants inoculated with the isolates present in Figure 6. The DNA extraction from white clover nodules was unsuccessful. DNA amplification was carried out by PCR in a 25µl reaction volume, using the GoTaq® Green Master Mix (Promega) kit, which contains the reaction buffer, $MgCl_2$, dNTP mix and DNA polymerase. The primers used (nifH For: 5'- TACGGNAARGGSGGNATCGGCAA -3' and nifH Rev: 5'- AGCATGTCYTCSAGYTCNTCCA -3') are designed (Laguerre et al., 2001) to amplify the *nifH* gene. This gene was used to test the presence of nitrogen-fixing bacteria in the nodules. The PCR was performed in a Corbett Palm-Cycler (Qiagen, Venlo, The Netherlands): an initial DNA denaturation step at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds and extension at 72°C for 1 minute. An additional extension step at 72°C for 10 minutes was added after the 35 cycles.

After DNA amplification, the resulting products were subjected to a 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light (Gorgan, 2008).

Data analysis

All the calculations were done in Microsoft Excel and XLSTAT. Descriptive statistics was also applied to the set of plant weight values, to present quantitative descriptions in a manageable form. This kind of analysis describes and summarizes data in a meaningful way but does not allow us to draw conclusions beyond the analyzed data; this is simply a way to describe and visualize the large amount of data from this study and its overall distribution. The boxplot is a graphical way to express data resulting from descriptive statistics. The data is divided into four parts and the three values that divide it are the lower quartile (Q1) – the middle value between the lowest value and the median, the median (or Q2) and the upper quartile (Q3) – the middle value between the median and the highest value. Fifty percent of these values are between Q1 and Q3 – the rectangle on the graph. The boxplots for our data can be seen in Figures 3 and 4.

RESULTS AND DISCUSSION

On average, three out of five plants had developed, for each inoculated rhizobial isolate. After weighing the roots and stems with leaves, their values were used to create a scatter plot in

which the average weight of the roots from the plants inoculated with the same rhizobial isolate was distributed along the Y axis and the average weight of the stems and leaves was distributed along the X axis. This scatter plot for the red clover plants can be seen in Figure 1 and the scatter plot for the white clover plants can be seen in Figure 2. As can be observed from these two graphs, the plants inoculated with the rhizobial isolates in the upper-right corner show the highest biomass accumulation, probably because of a higher rate of nitrogen fixation. Accordingly, the plants inoculated with the rhizobial isolates in the lower-left corner show the least biomass accumulation, probably because of the rhizobial isolates' inefficiency at fixing atmospheric nitrogen. There is no correlation between the geographical location of the isolates and their ability to fix nitrogen. Also, there is no correlation between the nitrogen fixing efficiency and the type or pH of soil.

The root weights and the stems and leaves weights for the red clover plants are distributed as can be seen in Figure 3. The maximum weight for the roots was 28,17 mg and the minimum weight was 2,06 mg with a mean of 11,87 mg. The maximum weight for the stem and leaves was 70,02 mg and 5,11 mg with a mean of 28,95 mg.

The distribution of the root weights and the stem and leaves weights from the white clover plants can be seen in Figure 4. The maximum weight for the roots was 13,77 mg and the minimum weight was 0,87 mg with a mean of 4,19 mg. The maximum weight for the stem and leaves were 39,04 mg and the minimum weight was 2,46 mg with a mean value of 12,28 mg.

We wanted to know if there is any correlation between the weight of the roots and the weight of the stems and leaves and how one might influence the other. There was a strong positive correlation between the weight of roots and the weight of stems and leaves, with a correlation coefficient r=0,9467 for white clover plants and r=0,9521 for red clover plants, meaning that if there is an increase in root mass there will also be an increase in stem and leaves mass and vice-versa.

The highest number of non-fixing nodules was on the plants inoculated with the A22 isolate for white clover, with a total of 37 nodules and isolate R77 for red clover, with a total of 22 nodules. The highest number of nitrogen fixing nodules was found on the white clover plants inoculated with the A22 isolate, with a total of 11 nodules and on the red clover plants inoculated with the R103 isolate. The plants inoculated with the A76 isolate lacked nitrogen fixing nodules. These values can be seen in Figure 5.

DNA amplification of the *nifH* gene – a gene encoding a subunit of the nitrogenase, showed the presence of rhizobial cells in the nodules (Figure 6).

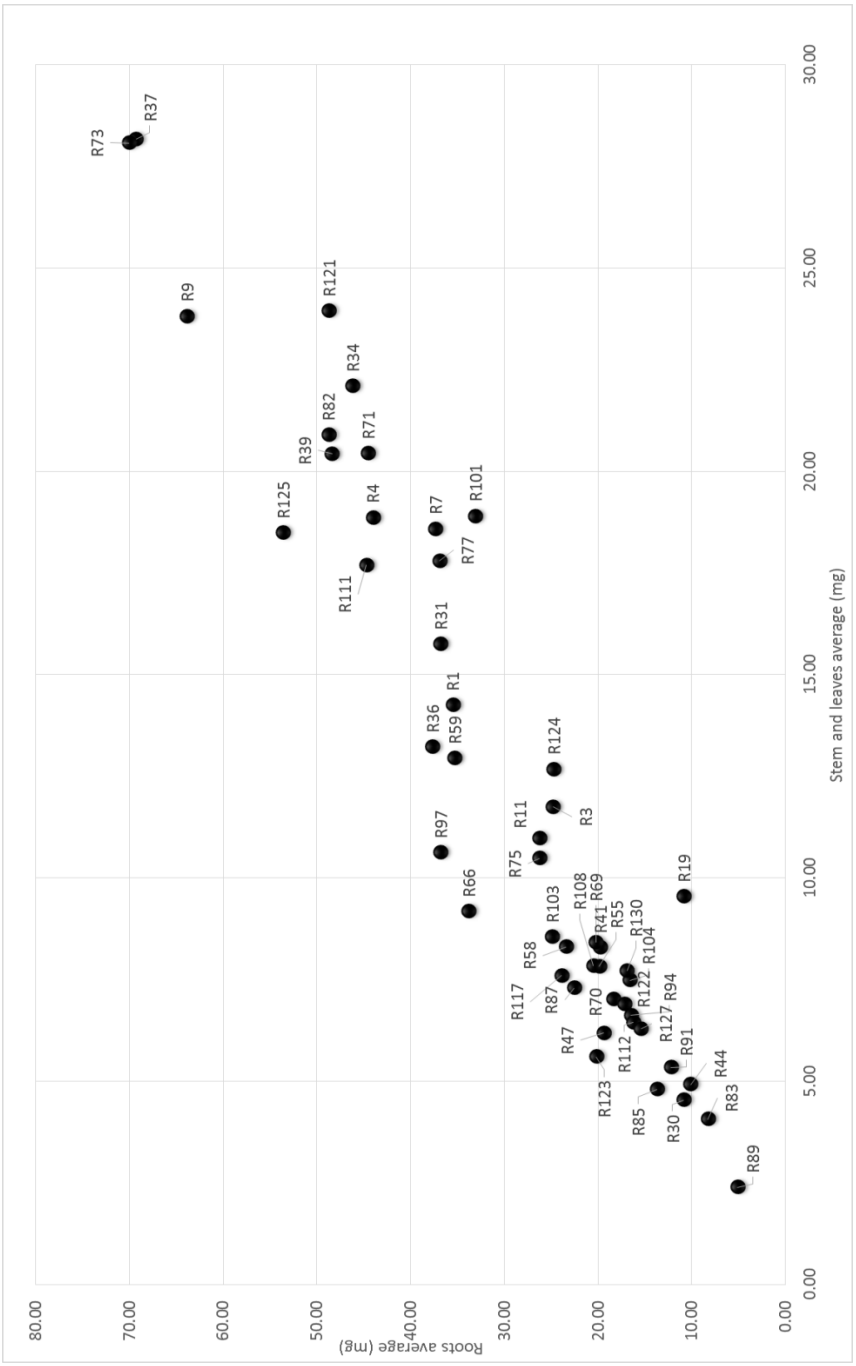

Figure 1. Scatter plot of weight distribution from the red clover plants inoculated with rhizobial isolates

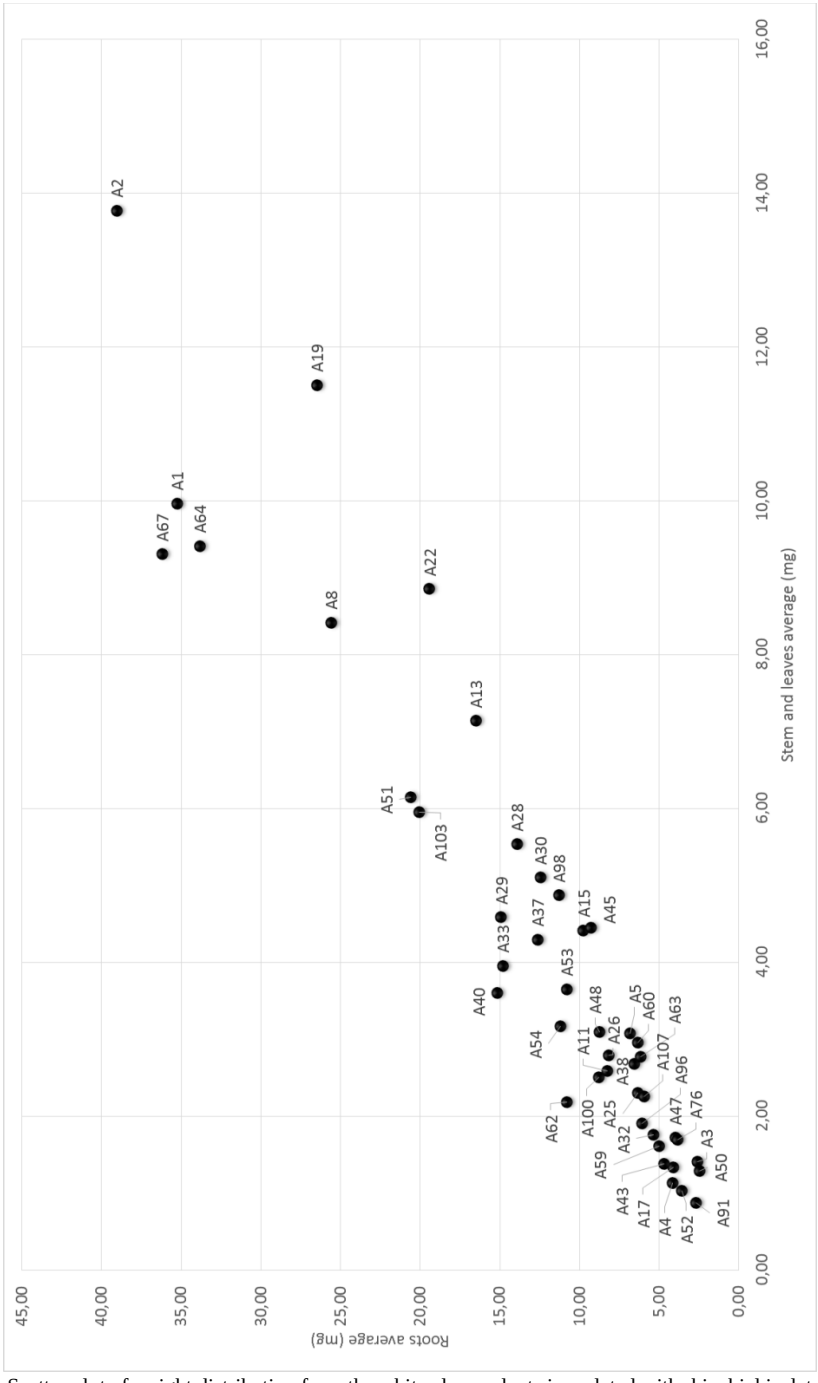

Figure 2. Scatter plot of weight distribution from the white clover plants inoculated with rhizobial isolatess

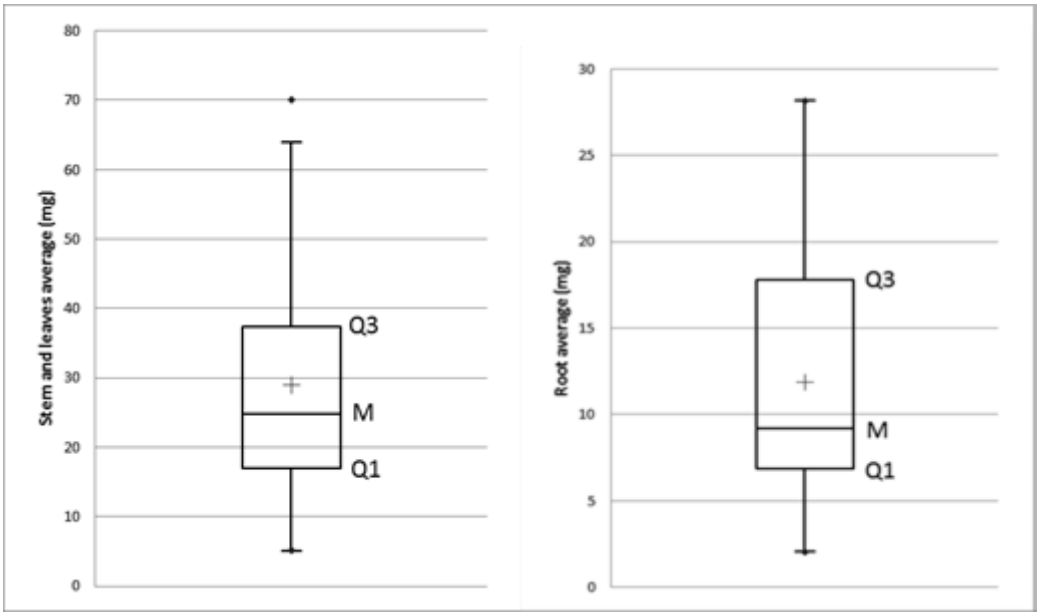

Figure 3. Boxplot charts for the overall distribution of stem and leaves (left) and roots (right) weight values from red clover plants inoculated with rhizobial isolates

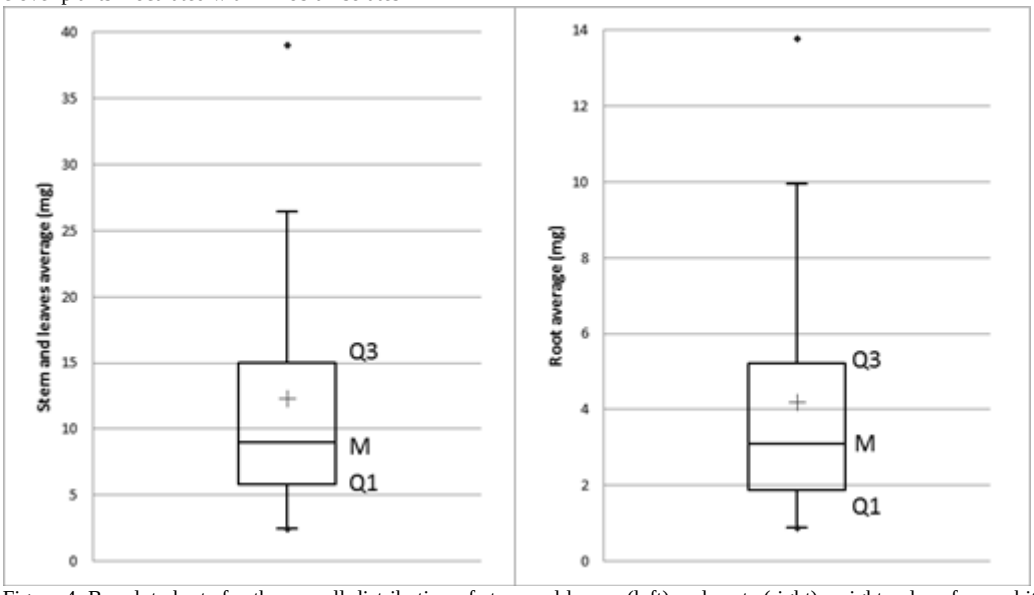

Figure 4. Boxplot charts for the overall distribution of stem and leaves (left) and roots (right) weight values from white clover plants inoculated with rhizobial isolates

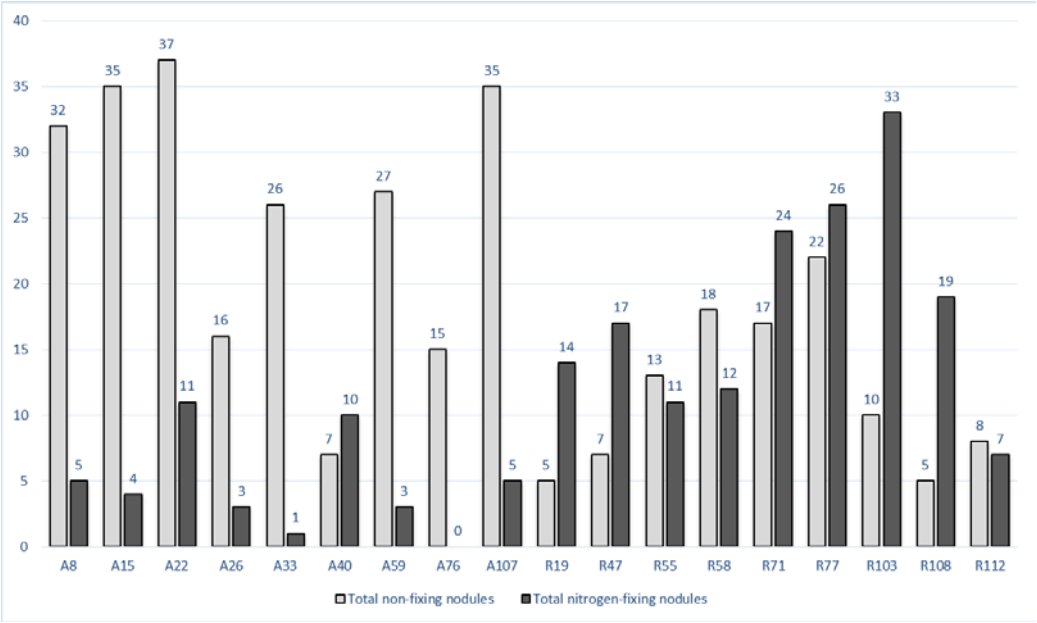

Figure 5. The total of non-fixing nodules (light color) and the total of nitrogen fixing nodules (dark color) from the roots of inoculated plants for each rhizobial isolate

The inoculated white clover plants show a high amount of non-fixing nodules, with an average of 25,55 nodules/plant and an average of nitrogen fixing nodules of 4,66 nodules/plant. The situation is reversed for the inoculated red clover plants, which show a low number of non-fixing nodules, with an average of 11,66 nodules/plant and a high amount of nitrogen-fixing nodules, with an average of 18,11 nodules/plant (Figure 5).

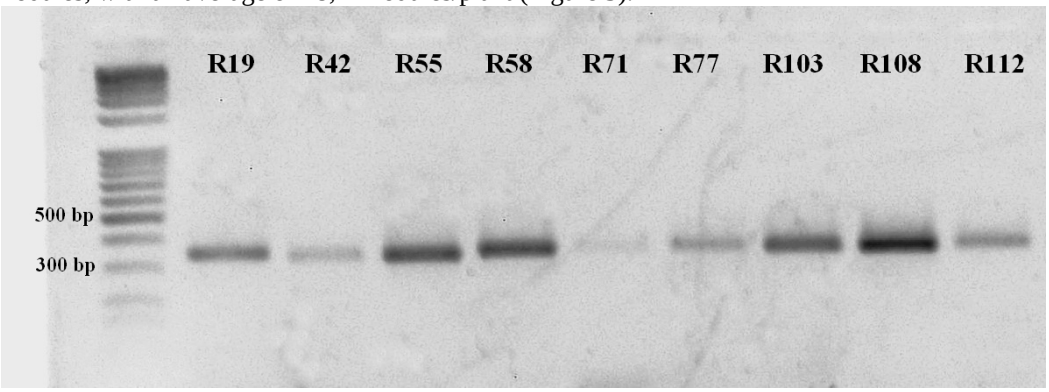

Figure 6. PCR-amplification of the *nifH* gene, a gene involved in nitrogen fixation

The 350bp-long amplified *nifH* gene confirmed the presence of bacteria in the nodules (Figure 6). This step was added as a confirmation of the presence of nitrogenase-bearing bacteria in the pink nodules and to see if the molecular biology methods can support the morphological aspects of the nodules. PCR is a qualitative method, therefore the difference in band intensity could be correlated with the initial amount of bacteria in the nodules or the initial concentration of isolated genomic DNA. A correlation between band intensity and biomass accumulation

would be unlikely. The biomass accumulation that we are interested in, would depend on the efficiency of the rhizobial isolate to fix nitrogen and not on the number of bacterial cells.

CONCLUSIONS

This study tested the nitrogen-fixing efficiency of some indigenous rhizobial isolates by determining the biomass accumulation of inoculated plants. Forty nine rhizobial isolates from red clover nodules and forty four rhizobial isolates from white clover nodules were used in this study. All the isolates belong to *Rhizobium leguminosarum* bv. *trifolii*, a nitrogen-fixing alphaproteobacteria.

Plants inoculated with the A2, R37 and R73 rhizobial isolates showed the highest amount of biomass accumulation.

On average, inoculated white clover plants had more non-fixing nodules than nitrogen fixing nodules; inoculated red clover plants had more nitrogen-fixing nodules on their roots than non-fixing nodules.

The PCR-amplification of the *nifH* gene showed the presence of nitrogen-fixing bacteria in the nodules of inoculated plants.

REFERENCES

Broughton, W. J., Dilworth, M. J., (1971): *Control of leghaemoglobin synthesis in snake beans.* The Biochemical Journal, 125, 1075–1080.

Castro-Sowinski, S., Herschkovitz, Y., Okon, Y., Jurkevitch, E., (2007): *Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms*. FEMS microbiology letters, 276, 1–11.

Catroux, G., Hartmann, A., Revellin, C., (2001*): Trends in rhizobial inoculant production and use*. Plant and Soil, 230, 21-30.

Denarie, J., Debelle, F., Rosenberg, C., (1992): *Signaling and host range variation in nodulation*. Annual Review of Microbiology, 46, 497-531

Fabiano, E., Arias, A., (1991): *Competition between a native isolate of Rhizobium leguminosarum bv trifolii and two commercial inoculant strains for nodulation of clover*. Plant and Soil, 137, 293-296.

Franche, C., Lindström, K., Elmerich, C., (2008): *Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants*. Plant and Soil, 321, 35-59

Gorgan, D. L., (2008): *Introducere în studiul filogeniei și filogeografiei moleculare*, Bioflux, 70-71

Handberg, K., Stougaard, J., (1992): *Lotus japonicus, an autogamous, diploid legume species for classical and molecular genetics*. The Plant Journal, 2(4), 487-496

Herridge, D. F., Peoples, M. B., Boddey, R. M., (2008): *Global inputs of biological nitrogen fixation in agricultural systems*. Plant and Soil, 311, 1-18

Kim, J., Rees, D. C., (1994): *Nitrogenase and biological nitrogen fixation*. Biochemistry, 33(2), 389-397

Kneip, C., Lockhart, P., Voss, C., Maier, U. G. G., (2007): *Nitrogen fixation in eukaryotes - new models for symbiosis*. BMC Evolutionary Biology, 7:55.

Laguerre, C., Nour, S. M., Macheret, V., Sanjuan, J., Drouin, P., Amarger, N., (2001): *Classification of rhizobia based on nodC and nifH gene analysis reveals a close phylogenetic relationship among Phaseolus vulgaris symbionts*. Microbiology, 147(4), 981-993

Laguerre, G., Louvrier, P., Allard, M., Amarger, N., (2003): *Compatibility of rhizobial genotypes within natural populations of Rhizobium leguminosarum biovar viciae for nodulation of host legumes*. Applied and Environmental Microbiology, 69, 2276-2283.

Lindemann, W. C., Glover, C. R., (2003): *Nitrogen fixation by legumes*. Guide A-129, College of Agriculture and Home Economics, New Mexico State University

Lindström, K., Murwira, M., Willems, A., Altier, N., (2010): *The biodiversity of beneficial microbe-host mutualism: the case of rhizobia*. Research in Microbiology, 161, 453–463.

Meade, J., Higgins, P., O'Gara, F., (1985): *Studies on the inoculation and competitiveness of a Rhizobium leguminosarum strain in soils containing indigenous rhizobia*. Applied and Environmental Microbiology, 49(4), 899-903

Streeter, J. G., (1994): *Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation*. Canadian Journal of Microbiology, 40(7), 513-522

Vance, C. P., (2001): *Symbiotic Nitrogen Fixation and Phosphorus Acquisition. Plant Nutrition in a World of Declining Renewable Resources*. Plant Physiology 127, 390–397.

Vincent, J. M., (1970): *A manual for the practical study of root-nodule bacteria*. IBP Handbook no. 15, Blackwell Scientific Publications, Oxford, England

Acknowledgment

This work was supported by the PN-II-ID-PCE-2011-3-1011/292/05.10.2011 grant; financed by the Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI).

*** XLSTAT software: http://www.xlstat.com/en/

[1]*Alexandru Ioan Cuza University of Iași, Romania*
**email: stefan.anddrei@gmail.com*
[2]*Institute of Biological Research, Iași, Romania*
[3]*Academy of Romanian Scientists*