THE EVOLUTION OF TOTAL SOLUBLE PROTEINS CONTENT DURING THE GERMINATION OF *GLYCINE MAX* L. L. BEANS UNDER THE INFLUENCE OF SOME RHIZOBACTERIAL STRAINS

M. MIHĂŞAN[1], **M. ŞTEFAN**[1]

[1]Universitatea Alexandru Ioan Cuza, Iaşi
e-mail: mariusmihasan@gmail.com

Utilization of rhizobacteria in order to increase the productivity may be a viable alternative to organic fertilizers. The main goal is to reduce the pollution and to preserve the environment in the spirit of an ecological agriculture. Because soybean represent a crop of major economic importance, a study was performed to analyze the impact of some rhizobacteria isolated from soybean roots on the on the germination process of Glycine max L. beans, taking into account the total soluble protein content and germination rate. The results showed that in the early stages, rhizobacteria has an inhibitory effect on the germination process.

***Key words**: plant growth promoting rhizobacteria, soybean, germination, proteins*

The positive effect of rhizobacteria for the plant growth and development is a well documented phenomenon. Some strains of plant growth-promoting rhizobacteria (PGPR) can effectively colonize plant roots and stimulate the growth of plants by direct or indirect effects [11]. The direct effect of PGPR includes providing the host plants with bacteria synthesized phytohormones [3], fixed nitrogen [7] phosphorus and iron solubilized from the soil [5]. The indirect effect on plant growth occurs when PGPR reduces or prevents the harmful effects of one or more phytopathogenic organisms [13] by producing a great variety of substances like antibiotics, siderophores and many types of enzymes (chitinase, protease, lipase, β l,3 glucanase etc. [6].

This positive effect of rhizobacteria has a direct application into the agriculture: utilization of isolated bacteria from crop plant's rhizosphere for productivity increase. In this way, PGPR are turned into an viable alternative to organic fertilizers, eliminating the need for artificial enrichment of soils with chemical compunds and reducing pollution. Considering the benefits of intensive agriculture practice in our time and the negative impact of chemical fertilizers and pesticides against the environment, usage of PGPR as biofertilizers is one of the most promising biotechnologies for growing the primary production with less quantity of chemical fertilizers (Lemanceau, Alabouvette, 1993, quoted by Orazova, 1999 [9]).

Although this biotechnology has so much to offer, the mechanism of interaction between the plant and the microorganism has yet to be cleared out. It is yet unknown when, during the vegetative cycle of the plant, the interactions with the microorganisms are more effective, when these interactions are desirable and when are not.

In this context, the main goal of this study is to establish if the interaction between different rhizobacteria strains with *Glycine max* L. seeds have a positive effect on the in-vitro germination process.

MATERIAL AND METHODS

Bacterial strains and growth conditions. Several bacterial strains were isolated from the roots of *Glycine max* L. on Bunt Rovira nutrient medium as described by Ştefan et all. (2006) [12]. For the seed treatment, a preculture was obtained by growing the selected strains in a mixed culture on liquid LB-medium [1] for 24 h on a orbital shaker at 280 C. 10 ml of this preculture was used to inoculate 1L of LB medium, and the culture was further incubated for 48 h in the same conditions.

Seed treatment and germination conditions. The *Glycine max* L. seeds (control and probe) were surface sterilized by washing with ethanol for 3 min, then twice with sterilized distillated water for 3 min. The probe seeds were inoculated with a mixture of rhizobacterial strains. For germination, the seeds were placed in Petri dishes (approx. 20 seeds/dish) and kept at room temperature. Samples were taken at different time points and kept at -200C till processing.

Soluble protein extraction. Approx. 1 gr. of material was homogenized in an mortar using broken glass, and resuspended in a total of 10 ml TRIS-HCl 0,1 M, pH 7,5. The insoluble debris was removed by centrifugation at 13000 rpm/30 min in a refrigerated Heraeus table centrifuge. Total protein contend was assayed by the dye-binding method of Bradford [2] using the Roti-Quant reagent from Roth (Karlsruhe, Germany).

SDS-PAGE. The protein content analysis was done using SDS-PAGE [8] on 5-20 % gradient gels. The gels were casted according to [1] using a Sigma gradient maker and an TV400YK (Scie-Plas, UK) electrophoresis module (20 cm in height, 20,5 cm in length and 1 mm thick). Approximately 125 µg proteins were mixed with SDS loading buffer (50 mM DTT, 2% SDS, 0,1% bromphenol blue, 10 % glycerol), boiled for 5 min at 950 C and then loaded on the gel. The gel was run at 150 V/gel for 30 min and then at 300 V/gel for approx. 3 hours. Protein staining was achieved using the standard Coomasie Brillant Blue R 250 method [10].

Gel densitometry and molecular weight determination. The stained gels were photographed and quantified using ImageQuant TL from GE Healthcare. The Sigma Wide Range protein marker was run in parallel with the samples and used to construct a curve from which the molecular weight of the target protein was determined.

Statistical analyses. Protein concentration was determined in triplicates. For each sample the mean, standard deviation and standard error was calculated. The statistical significance of the differences between samples were tested using the T-test (Fowler J., Cohen L. , Jarvis P., 2000).

RESULTS AND DISCUTIONS

The seeds of *Glycine max* L. are very rich in proteins, these bio-molecules being the main resource which must be mobilized during the germination process. So besides the usual counting of germinated and not-germinated seeds we also considered the soluble protein content as an indicator of the germination process. As it can be noticed in *Fig. 1*, during the germination process, the protein content in the untreated seeds is slowly decreasing. This, we consider as being normal, as the seeds have to produce from proteins different non-proteic compounds (sugars for cell walls, lipids for membranes, etc) before the photosynthetic processes begins. In the rhizobacteria-treated seeds, the soluble-protein content is maintained at the same level during the first 168 h of germination.

The differences of total soluble protein concentrations between treated and untreated seeds are statistically insignificant in the early stages of germination (72 and 120 hours). At 172 hours soluble protein content in untreated seeds is lower than in the treated samples (120,24 ± 14,02 mg/gr compared to 161,75 ± 25,66 mg/gr), the recorded differences being statistically significant. This would indicate a slight delay in the recruiting speed of deposited proteins in the treated seeds, probably due to a rhizobacteria effect.

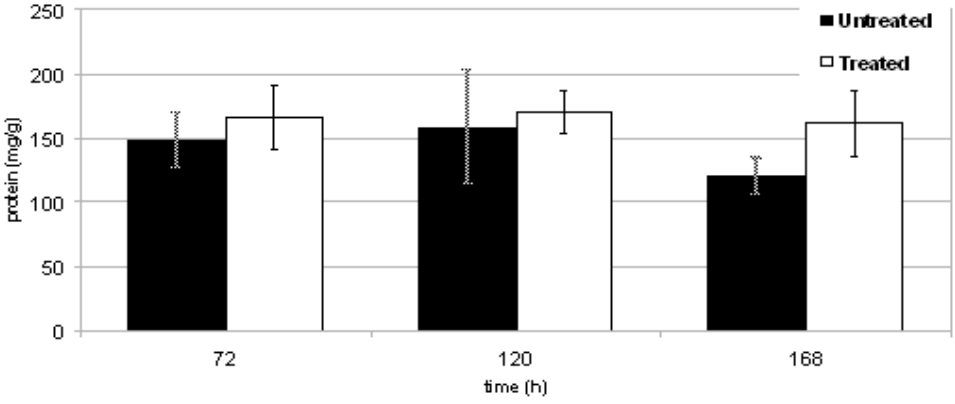

Figure 1 **Soluble protein content evolution during the germination process of *Glycine max* L. seeds. (black – untreated seeds, white - rhizobacteria treated seeds)**

The qualitative analysis of proteins in the treated and untreated seeds during the germination process shows high abundance of proteins fractions in both samples (*fig. 2*). The germination process is a highly complex one, requiring the recruiting of some deposited proteins and de-novo synthesis of others. Still, three main differences can be observed.

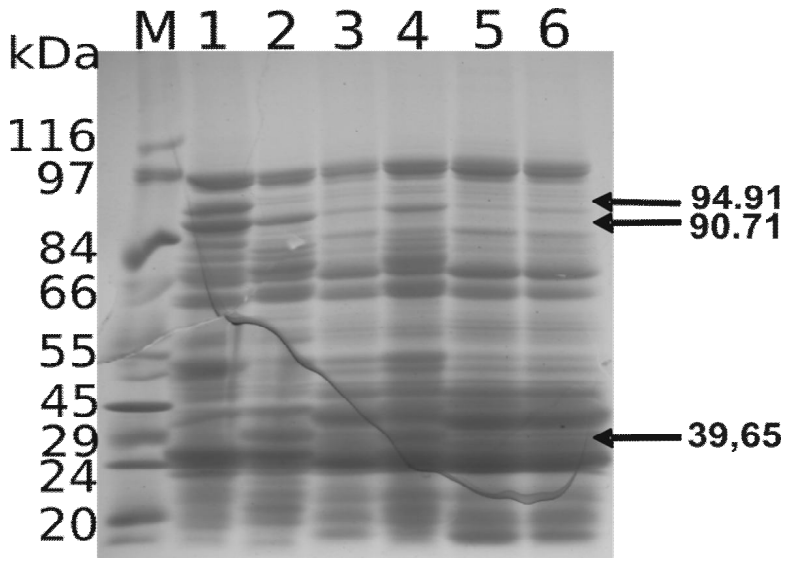

Figure 2 **SDS-PAGE of total soluble proteins. On the right the molecular weight in kDa of the main three differences that could be observed. On each lane 125 micrograms proteins were loaded as follows:** M. Sigma Wide Range protein marker; 1. Untreated seeds, 72 hours of incubation; 2. Rhizobacteria treated seeds, 72 hours of incubation; 3. Untreated seeds, 120 hours of incubation; 4. Rhizobacteria treated seeds, 120 hours of incubation; 5. Untreated seeds, 168 hours of incubation; 6. Rhizobacteria treated seeds, 168 hours of incubation

One difference, easily noticed, is the fraction corresponding to a molecular weight of 94.91 kDa. A thick band is present in the untreated seeds harvested after 72 hours of germination. In the rest of the samples, this band is replaced by a double band with much lower intensity (*fig. 3*).

The second noticeable difference is in the range of 90 kDa. The fraction corresponding to a molecular weight of 90.71 kDa it is highly abundant in the untreated seeds after 72 hours of incubation. During the germination process, the intensity of this band is decreasing, faster in the un-treated seeds and slower in the rhizobacteria seeds (*fig. 4*). This behaviour is somehow similar to the 94.91 kDa fraction and indicates a delay in the germination process. Probably these two fractions are deposited proteins which are recruited and transformed in other compounds, but the recruiting takes place with different speeds: faster in the untreated seeds and slower in the rhizobacteria-treated seeds.

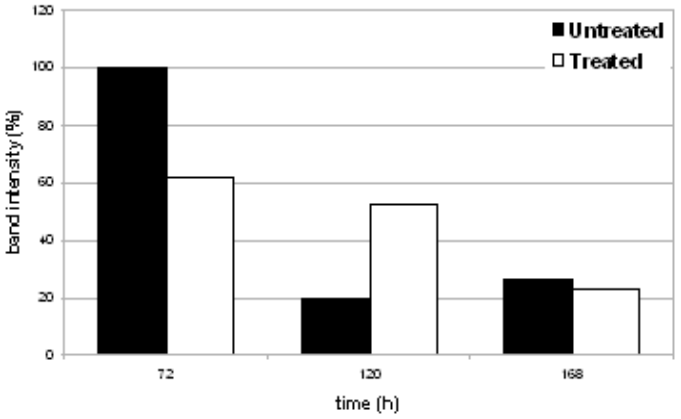

Figure 3 **The intensity of fraction 94.91 kDa. during the germination process**
(black – untreated seeds, white – rhizobacteria-treated seeds)

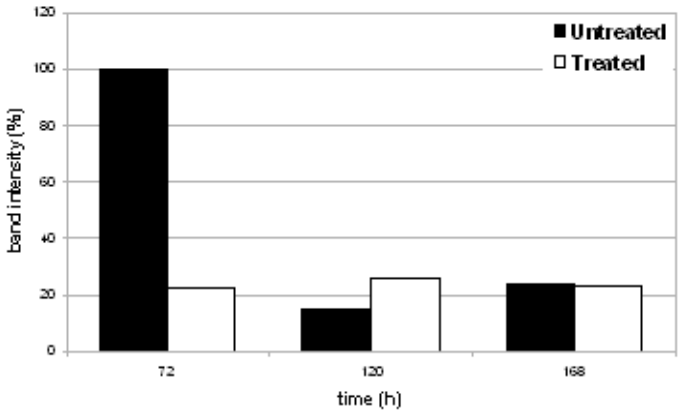

Figure 4 **The intensity of fraction 90.71 kDa. during the germination process**
(black – untreated seeds, white – rhizobacteria-treated seeds)

The last noticeable difference concern the 40 kDa range, where the fraction corresponding to a molecular weight of 39,65 behaves apparently different then the 94.91 kDa and 90.71 kDa fractions. The 39,65 kDa fraction is more abundant in the rhizobacteria-treated samples after 72 hours and decreases during the germination process. In the untreated samples, the intensity of this band is low from the start and it is maintained low during the whole process (*fig. 5*). One explanation could be that the untreated seeds are in more later stages of the germination process, stages in which the 39,65 kDa fraction is depleted, while the rhizobacteria-treated seeds are in a earlier stages when the mobilization of the 39,65 kDa is taking place.

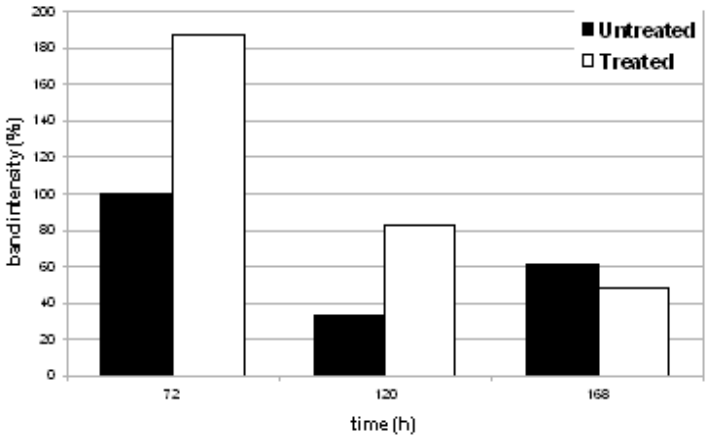

Figure 5 **The intensity of fraction 39.65 kDa. during the germination process**
(black – untreated seeds, white – rhizobacteria-treated seeds)

The data presented so far would indicate that the rhizobacteria treatment of the seeds before the germination would induce a delay in the protein metabolism. The germination yield, presented in *table 1* indicates the same thing, a delay of the whole germination process.

Tabelul 1

The germination yield

Time (hours)	Sample	Germination yield
72	Untreated seeds	73.4
	Treated seeds	54.32
96	Untreated seeds	86.64
	Treated seeds	75.81
120	Untreated seeds	88.99
	Treated seeds	71.74
144	Untreated seeds	96.92
	Treated seeds	81.08

Such a delay is, in way, expected, as in the in-vitro conditions used, the rhizobacteria are depleted of nutrients and grow only on compounds provided by the seeds. In this way, the seed itself is depleted of different nutrients, growth factors, vitamins and the germination process is inhibited.

CONCLUSIONS

The overall conclusion of this work is that in the in-vitro conditions the rhizobacteria inhibit the germination of *Glycine max* L. seeds. This would suggest that maybe it is more advisable to inoculate the seeds with rhizobacteria in a different moment, probably after the germination process is completed and the small plant is fully functional. A question is still open regarding this inhibition: does it also takes place also in the normal, field conditions, where the bacteria may have use some other nutrients sources?

BIBLIOGRAPHY

1. Ausubel, M. F., Brent R., Kingston, E. R. , Moore, D. D., Seidman, J. G., Smith, A. J., Struhl, K., 2002 - *Short Protocols in Molecular Biology*, John Wiley & Sons, vol. I, 1-3;10-25 – 10-27.
2. Bradford, M. 1976. - *A Rapid and Sensitive Method for the Quantization of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding.* Anal. Biochem, 72, 248-254.
3. Chabot, R., Antoun, H., M.P., 1996 – *Growth promotion of maize and lettuce by phosphate solubilizing Rhizobium leguminosarum biovar phaseoli*, Plant Soil, 184:311-321.
4. Fouler, J., Cohen L. Janis P., 1998 - *Practical statistics for field biology*, 2nd edition, New-York, Wiley
5. Glick, B.R., Pattern, C.L., Holguin, G., Penrose, D.M., 1999.- Biochemical *and Genetic Mechanisms used by Plant Growth Promoting Bacteria,* Imperial College Press, London.
6. Jacobson. C. B., J. J. Pasternak and B. R. Glick. 1994 - *Partial purification and characterization of 1-aminocyclopropane-1-carbooxylate deaminase from the plant growth promoting rhizobacterium Pseudomonas putida GR 12-2*. Can. J. Microbiol. 40:1019-1025.
7. Kennedy, I.R., Pereg-Gerk, L.L., Wood, C., Deaker, R., Gilchrist, K., & Katupitiya, S. 1997 - *Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between Azospirillum and wheat*. – Plant Soil, 194: 65-79.
8. Laemmli, U.K. 1970 - *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature, 227, 680-685.
9. Orazova, M.K., Polyanskaya, L.M., Zviagintsev, D.G., 1999 – *The structure of the microbial community in the barley root zone*, Microbiology, vol. 68, No. 1, 109-115.
10. Sambrook J, Fritsch E.F., Maniatis T. (1989). *Molecular Cloning - A Laboratory Manual*. Cold Spring Harbour Laboratory Press, vol. I, 18-55 – 18-57.
11. Sylvia, D. M., Fuhrmann, J.J., Hartel, P.G., & Zuberer, D.A. 1999 - *Principles and applications of soil microbiology*. Prentice Hall Inc., Upper Saddle River, New Jersey.
12. Ştefan Marius, Zenovia Olteanu, Oprică Lăcrămioara, Dunca Simona, Maniu Călin, Zamfirache Maria Magdalena, 2006 - Effects *of PGPR on the Growth of soybean (Glycine max (L.) MERR.),* IV Balkan Botanical Congress, Sofia, 20 - 26 June 2006, Book of abstracts, 144.
13. Zahir, Z. A., Muhammad Arshad, Frankenberger, W. T., Jr., 2003 - *Plant growth promoting rhizobacteria: applications and perspectives in agriculture*. Advances in Agronomy, 2003 (Vol. 81).