PLANT GROWTH PROMOTING RHIZOBACTERIA CAN INHIBIT THE *IN VITRO* GERMINATION OF *GLYCINE MAX* L. SEEDS

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Abstract. Utilization of plant growth promoting rhizobacteria (PGPR) 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. seeds, taking into account the aspartate- and alanin-aminotransferase activities. The results showed that in the early stages, rhizobacteria has an inhibitory effect on the germination process.

INTRODUCTION

Our understanding of the biology, biochemistry, and genetic development of roots has considerably improved during the last decade (Benfey and Scheres, 2000). In contrast, the processes mediated by roots in the rhizosphere (the field of action around, on and within a root - Lynch 1990, Pinton et al. 2001) are not yet well understood (Hawes et al., 2000).

Plant growth promoting rhizobacteria (PGPR) are root colonizing microorganisms which are known to be in constant communication with plants roots. Also, some strains of plant growth-promoting rhizobacteria can effectively stimulate the growth of plants by direct or indirect effects (Sylvia et al. 1999).

The mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include: 1. the ability to produce or change the concentration of the plant hormones indoleacetic acid (IAA; Mordukhova et al., 1991), gibberellic acid (Mahmoud et al., 1984), cytokinins (Tien et al., 1979) and ethylene (Arshad and Frankenberger, 1991; Glick et al., 1999); 2. asymbiotic N_2 fixation (Kennedy et al., 1997); 3. antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982), β-1,3-glucanase, chitinases (Renwick et al., 1991), antibiotics and cyanide; 4. solubilization of mineral phosphates and other nutrients (De Freitas et al., 1997). Many of the studies with PGPR show plant growth promotion, but only under gnotobiotic conditions (Glick et al., 1999) or in potting media (Fuhrmann and Wollum, 1989) where these bacteria do not compete with the normal array of soil microorganisms.

The use of PGPR as biofertilizers is one of the most promising biotechnologies to improve primary production with low inputs in fertilizers, through any of the many mechanisms possible: biocontrol, nutrient mobilization, phytohormone production and nitrogen fixation. (Lemanceau and Alabouvette, 1993, Glick, 1995; Gutiérrez et al., 1996).

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. It was studied two enzymes involved in protein metabolism (aspartate- and alanin-aminotransferase) and one biologic stress marker (superoxide-dismutase).

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 al. (2006). For the seed treatment, a preculture was obtained by growing the selected strains in a mixed culture on liquid LB-medium (Ausubel et al., 2002) for 24 h on a orbital shaker at 28^0 C. 10 ml of this preculture was used to inoculate 1L of LB medium, the culture being 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 beans/dish) using sterile filter paper and kept at room temperature (Photo 1). Samples were taken at different time points and kept at -20^0C till processing.

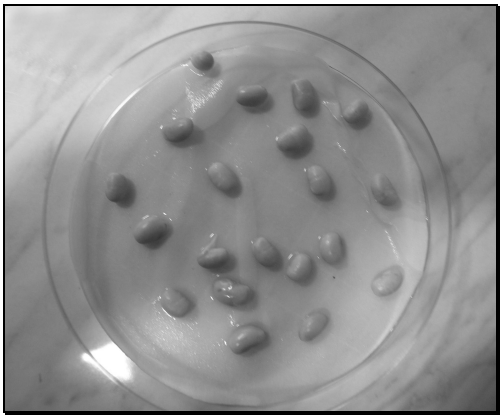

Photo 1 - *Glycine max* L. beans

Enzyme extract preparation. 1 gram of germinated beans was homogenized using a mortar and pestle for 3 min and resuspended in 10 ml of chilled TrisHCl 0,1 M ph 7.5. After 10 min of extraction at room temperature, the homogenized was separated of insoluble cellular debris by centrifugation for 15 min at 4000 rpm using a Hettich Universal 320 centrifuge. The clear supernatant was used as enzyme source.

Soluble protein content was assayed by the dye-binding Bradford method using the Roti-Quant reagent from Roth (Karlsruhe, Germany).

SOD assay was done photometrically using the method described by Winterbourn et al. (1975). Enzyme activity was expressed as USOD/min/mg protein, following the indications of Artenie et al. (2008).

Aspartate- and Alanin-aminotransferase assay were conducted using the method described by Artenie et al. (1981) which employ 2,4-d*initrophenylhydrazine* to quantify the reaction products. Enzyme activity was expressed as microM piruvic acid/min/mg protein. All optical densities were determined using a Beckman-Coulter DU-730 Life Sciences spectrophotometer.

Statistical analyses. Enzyme assays were determined in triplicates. For each sample the mean, standard deviation and standard error was calculated. The statistical significance of the differences between samples was tested using the T-test (Fowler et al., 2000).

RESULTS AND DISCUTIONS

The seeds of *Glycine max* L. are very rich in proteins, these bio-molecules being one of the main resources which must be mobilized during the germination process. Utilization of protein as C and N sources requires the presence of proteolytic enzymes and aminoacid transferases such as aspartate- and alanin-aminotransferase. The latter ones are necessary in order to link the protein metabolism to all the other metabolic pathways in the cell (Mathews et al., 2000). This is why we considered the activity of these enzymes as a key indicator for the protein metabolism in the germination process under the influence of PGPR.

Our previous observations showed that the germination process is slowed down by the presence of PGPR (Mihăşan and Ştefan, 2008) in the *in vitro* conditions (Photo 2). This could be due to a stressing effect induced by PGPR or due to a competition for nutrient resources between plant and inoculated rhizobacteria. In order to answer this question the activity of aspartate-aminotrasferase, alanin-aminotransferase and SOD were measured in several points during the *in vitro* germination.

The evolution of **aspartate-aminotrasferase activity** during the *in vitro* germination can be observed in Fig. 1.

In the first stages of germination (72 hours), both the untreated and PGPR treated seeds express the same level of activity for this enzyme. At 120 hours from inoculation, the level of

aspartate-aminotransferase activity is higher in the un-inoculated seeds (30,38 ± 0,672 microM/min/mg protein) compared to PGPR treated ones (25,46 ± 4,12 microM/min/mg protein). This difference is statistically significant (p=0.03) and shows that the proteins are faster metabolized in the case of un-treated seeds. During the germination, the seeds have to produce from proteins different non-proteic compounds (sugars for cell walls, lipids for membranes, etc) before the photosynthetic processes begins (Mathews et al., 2000), so we presume that the recorded delay in protein metabolism of PGPR treated seeds would lead to a decrease of the germination processes.

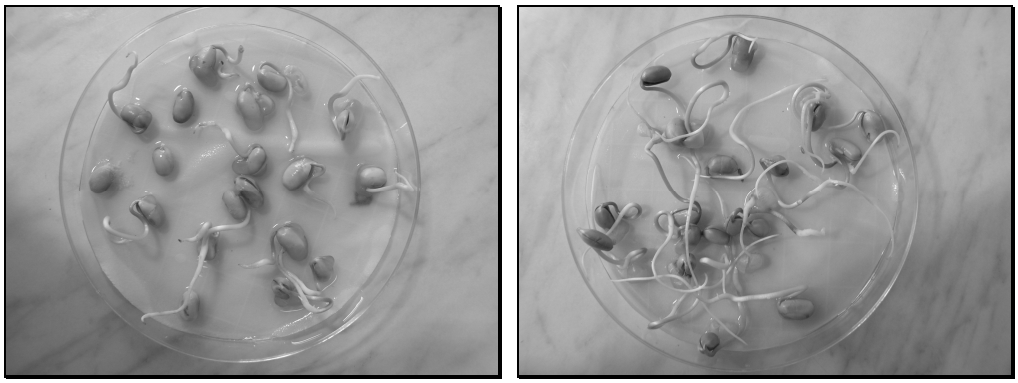

Photo 2 – Soybean germination: left-untreated seeds, right-PGPR treated seeds (at 120 hours since inoculation)

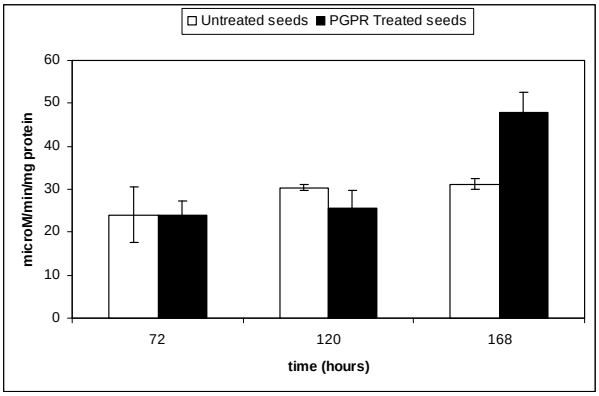

Fig. 1 - Aspartat aminotransferase activity during the germination process of *Glycine max* L. seeds (white – untreated seeds, black - PGPR treated seeds)

This hypothesis is also sustained by the level of aspartate aminotransferase activity recorded at 168 hours. At this point, the PGPR treated seeds shows a higher activity of this enzyme (47,92 ± 4,58 microM/min/mg protein) compared with untreated seeds (31,17 ± 1,282 microM/min/mg protein). The recorded differences are statistically significant (p=0.0005) and

can prove that untreated seeds are approaching the end of the germination, fact proved also by the development of the first green leaves. The PGPR treated seeds show a more intense protein metabolism, as they are in the earlier stages of germination (at 168 hours the green leaves are not fully developed and thereby photosynthesis cannot take place).

The evolution of **alanin aminotransferase activity** during the germination of soybean seeds (Fig. 2) demonstrates the same general idea of a PGPR inhibitory effect in *in vitro* conditions.

So, if at 72 hours recorded differences between the enzymatic activities were not statistically significant, at 120 hours the untreated seeds show an more active protein metabolism (471,94 ± 67,32 microM/min/mg protein) compared with the PGPR treated ones (288,27 ± 83,98 microM/min/mg protein). The recorded differences are statistically significant (p=0.001).

As in the case of previously discussed enzyme, at 168 hours the alanin aminotransferase activity records an increase for PGPR treated seeds (507,80 ± 26,02 microM/min/mg protein) and a decrease for the untreated ones (307,93 ± 27,66 microM/min/mg protein). The differences are again statistically significant (p=0.03), sustaining the hypothesis of an inhibitory effect induced by the presence and activity of PGPR used for seed inoculation.

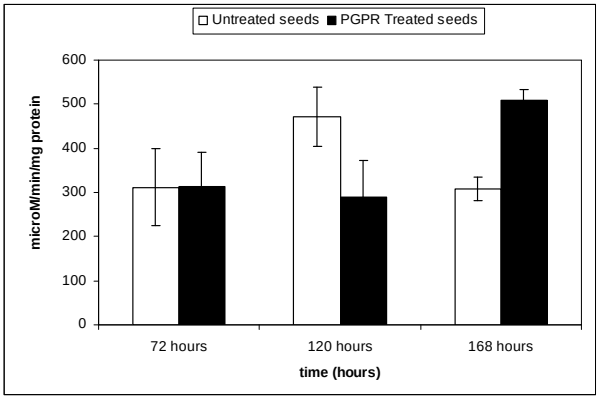

Fig. 2 - Alanin aminotransferase activity during the germination process of *Glycine max* L. seeds (white – untreated seeds, black - PGPR treated seeds)

This results presented so far are in good concordance with our previously studies regarding the total soluble protein content evolution during the *in vitro* germination of soybean seeds (Mihăşan and Ştefan, 2008).

The reported delay in the germination of PGPR treated seeds can be explained taking into account two possible aspects. First of all the inhibitory effect could be the result of an oxidative stress induced by the presence and activity of PGPR used for seed inoculation. The second possible explanation is a competition for nutrient resources between soybean seeds and rhizobacteria. In order to establish the cause of the germination delay we also studied an oxidative stress marker enzyme: superoxide-dismutase (SOD), the result being presented in Fig. 3.

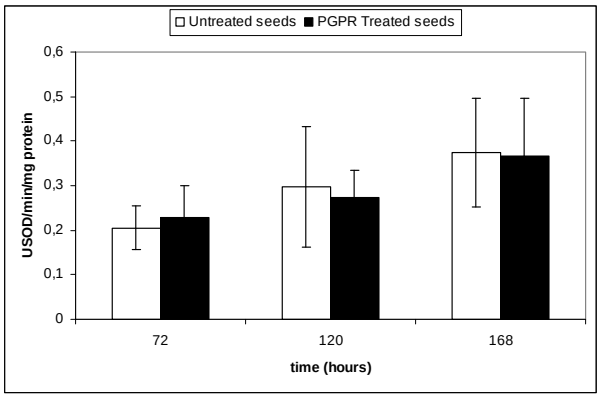

Fig. 3 - SOD activity during the germination process of *Glycine max* L. seeds (white – untreated seeds, black - PGPR treated seeds)

Although apparently an increase in SOD activity can be observed during the germination processes for both untreated and PGPR treated seeds, no statistically significant differences were recorded. This would indicate that the oxidative stress is at the same level for test and control disregarding the presence of PGPR. So we can presume that the germination delay recorded in the case of PGPR treated seeds are due to a competition for nutrient competition between inoculated rhizobacteria and soybean seeds. In our *in vitro* condition the seeds were hydrated using distillated sterile water and the support was plain sterile filter paper. This means that the nutrients are very scarce and thereby the only nutrient source for rhizobacterial development remains the soybean seeds.

CONCLUSIONS

The overall conclusion of this work is that in the *in vitro* conditions the rhizobacteria inhibit the germination of *Glycine max* L. seeds probably due to a nutrient competition. 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?

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