RHIZOSPHERE BACTERIA HELP PROTEIN ACCUMULATION IN SOYBEAN SEEDS

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Keywords: rhizosphere bacteria, soybean, protein, lipids, soluble carbohydrates
Abstract: The use of rhizobacteria as biofertilizers is one of the most promising biotechnologies to improve primary production with low inputs in fertilizers. In this context, the main goal of this study was to establish if the interaction between different rhizobacteria strains with *Glycine max* L. plants have a positive effect on the total soluble protein, carbohydrates and lipids content of soybeans. Our results revealed that there are no significant differences between the seeds produced by inoculated plants and those produced by the non-inoculated plants regarding soluble reducing carbohydrate content, lipid content and relative humidity. In the case of soluble protein content, rhizobacteria inoculated plants produce beans that contain a greater amount of soluble protein per gram. No qualitative differences could be shown, making our tested rhizobacteria strains an appealing strategy for improving soybean protein production.

INTRODUCTION

Soybean (*Gylcine max* (L.) Merr.) is a subtropical legume; temperatures of 25 to 30^0 C are optimal for its growth, nodulation and N2 fixation.

Soybean is one of the most important sources of edible oil and protein in the world (Li et al, 2004). Soy products are the main ingredients in many meat and dairy substitutes. They are also used to make soy sauce, and the oil is used in many industrial applications.

Rhizobacteria are root colonizing microorganisms which are known to be in constant communication with plants roots. Previous studies have shown that some roots-associated bacteria called *plant growth-promoting rhizobacteria* (PGPR) can stimulate growth and development of soybean plants (Glick et al., 1999). The beneficial effects of the PGPR are direct plant growth promotion, mobilization of insoluble nutrients (e.g. phosphate) resulting enhancement of uptake by the plant (Lifshitz et al., 1987), and production of antibiotics toxic to soil-borne pathogens (De-Ming and Alexander, 1988). Co-inoculation studies with PGPR and *B. japonicum* have also demonstrated that increased soybean plant root and shoot weight, grain yield, plant vigour, nodulation and nitrogen fixation can result from the presence of the PGPR (Lie and Alexander, 1988; Verma et al., 1986; Yahalom et al., 1987).

Plant-microorganism interactions are very complex and often have important consequences for the plants. For example, host-pathogen interactions are detrimental to one of the two organisms involved. In compatible interactions, plant disease develops. In incompatible interactions, a resistant host plant establishes a set of defense mechanisms against the pathogen, collectively referred to as Systemic Acquired Resistance (SAR) - Mithöfer, 2002. Some bacteria that live in the rhizosphere are able to modify nodule formation and biological nitrogen fixation (BNF) when they are co-inoculated with rhizobia (De Freitas et al., 1997). However, the mechanisms used by these bacteria to produce the effects mentioned, are not well-understood. The phytohormones are implicated in nodule formation in one way or another (Hirsch, et al., 1997). Common mechanisms used by rhizobacteria to alter nodule formation or biological nitrogen fixation include the release of phytohormones such as auxins, gibberellins, cytokinins and ethylene, or the alteration of endogen levels in the plant (Hirsch, et al., 1997). The effects of some phytohormones are indirect, as they stimulate root growth, providing further sites for infection and nodulation. Systemic induction of secondary metabolites such as flavonoids are implicated in these bacterial effects (Andrade, et al., 1998). A further well-studied mechanism is the ability of some PGPRs to reduce disease caused by foliar pathogens, by triggering a plant-mediated resistance mechanism: Induced Systemic Resistance (ISR) (Van Loon, et al., 1998).

Many studies involving PGPR showed plant growth promotion, but only under gnotobiotic conditions (Glick, et al., 1995) or in potting media (Fuhrmann and Wollum, 1989), where these bacteria do not compete with the normal array of soil microorganisms. PGPR must be rhizosphere competent and able to survive in soil. Traits associated with rhizosphere competence and survival in soil include an ability to tolerate a reasonable range of abiotic factors including temperature, pH and moisture (Sylvia et al., 1998).

The use of PGPR as biofertilizers (preparations of microorganism(s) that may be a partial or complete substitute for chemical fertilization) is one of the most promising biotechnologies to improve primary production with low inputs in fertilizers (Bashan 1998), through any of the many mechanisms possible: biocontrol, nutrient mobilization, phytohormone production and nitrogen fixation (Glick, et al., 1995). Usage of isolated bacteria from crop plant’s rhizosphere for productivity increase may be an alternative to organic fertilizers (Compant et al. 2005). The main goal is to reduce the pollution and to preserve the environment in the spirit of an ecological agriculture.

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. plants have a positive effect on the total soluble protein, carbohydrates and lipids content of soybeans.

MATERIALS 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 (Stefan et al., 2006). Numerous recent studies show a promising trend in the field of inoculation technology. Mixed inoculants (combinations of microorganisms) that interact synergistically are currently being devised (Bashan 1998). Microbial studies performed without plants indicate that some mixtures allow the bacteria to interact wit h each other synergistically, providing nutrients, removing inhibitory products, and stimulating each other through physical or biochemical activities that may enhance some beneficial aspects of their physiology, like nitrogen fixation. It still has to be demonstrated that these bacterial synergistic effects also benefit plant growth.

For soybean 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.

Plant cultivation and inoculation in field conditions

The inoculation of soybean seeds was carried out with a mixture of rhizobacteria strains obtained as described above (final culture density - 64 x 106 CFU/ml). The soybeans seeds (Pioneer PR91M10/91M10) inoculated or non-inoculated with rhizobacteria were planted using a small experimental seeding machine.

The experiment was conducted during 2008 within Ezareni Didactic Farm of Iasi, in a cambic cernoziom soil with adobe clay texture and good fertility, moderate humus and highly nitrogen content, moderate mobile phosphor supply, highly potassium content and a very low acid reaction, almost neutral.

The plants were growth in ecological conditions, without using organic fertilizers and pesticides. After harvesting, the beans were collected and further analysed.

Soluble proteins 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 for quantification of protein content and for SDS-PAGE.

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

SDS-PAGE. The protein content analysis was done using SDS-PAGE on 5-20 % gradient gels. The gels were casted according to (Ausubel et al., 2002) 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 95^0 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 (Sambrook et al., 1989).

Soluble carbohydrate content – was assayed using 3,5-dinitrosalicilic acid method as described by (Artenie et al., 2008) and an UV-VIS spectrophotometer (Beckman Coulter DU 720 Life Sciences). The results were expressed in g glucose/100 g analyzed material.

Lipid content – the dried seeds were extracted with a Soxhlet apparatus followed by a gravimetric measurement (Artenie et al., 1981).

Statistical analyses. All assays were done 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 (Fouler et al., 1998).

RESULTS AND DISCUTIONS

The economic value of *Glycine max* L. seeds reside in their high protein and lipid content. Together, lipids and protein content account for about 60 % of dry soybeans by weight; protein at 40 % and oil at 20 %. The remainder consists of 35 % carbohydrate.

Soybeans contain significant amounts of all the essential amino acids for humans making it a primary ingredient in many processed foods, including dairy product substitutes. According to the Food and Drug Administration USA, "soy protein products can be good substitutes for animal products because, unlike some other beans, soy offers a "complete" protein profile (Henkel et al., 2000).

In order to improve the production of soybean proteins several strategies were developed: usage of chemical or organic fertilizers, pesticides, developing of transgenic soybean plants (Hinchee et al., 1988). Most of them have a negative impact on the environment. That’s why the present study is focused on the development of environmental friendly methods for improving the soybean protein production using rhizobacteria as biofertilizers (Bashan, 1998).

Our previous observations showed that some rhizobacterial strains isolated from soybean roots have a positive effect on plant growth and development processes in field conditions (Stefan et al., 2008). In the present study we aimed to investigate the rhizobacteria effects on the quality of the harvested beans. Several biochemical indices for the nutritional value of beans were taken into account including total soluble protein content, soluble carbohydrates and lipids content. The results of our tests are presented in Table 1.

Table 1 –Biochemical parameters of the experimental variants used in this study

Biochemical parameters	Non-inoculated control (mean ± standard error)	Inoculated sample (mean ± standard error)	P(T<=t) two-tail
Soluble protein content (mg/g)	425,52 ± 6,22	438,13 ± 3,87	0,002
Soluble reducing carbohydrate (mg glucose/g)	2,44 ± 0,087	2,53 ± 0,08	0,47
Lipid content (%)	22,08 ± 0,39	21,76 ± 0,28	0,52
Relative humidity (%)	6,02 ± 0,06	6,13 ± 0,077	0,31

As it can be seen from the data presented, there are no significant differences between the seeds produced by inoculated plants and those produced by the non-inoculated plants regarding soluble reducing carbohydrate content, lipid content and relative humidity.

In the case of **soluble protein content**, rhizobacteria inoculated plants produce beans that contain a greater amount of soluble protein per gram. Although small, the recorded difference between sample and control is statistically significant as proved by the results of the “t” test ($p<0.002$) – Fig. 1. Considering an average soybean production of 1.5 T/ha, the above mentioned difference can be translated into an increase of protein production with aprox. 19 kg of soybean protein/ha.

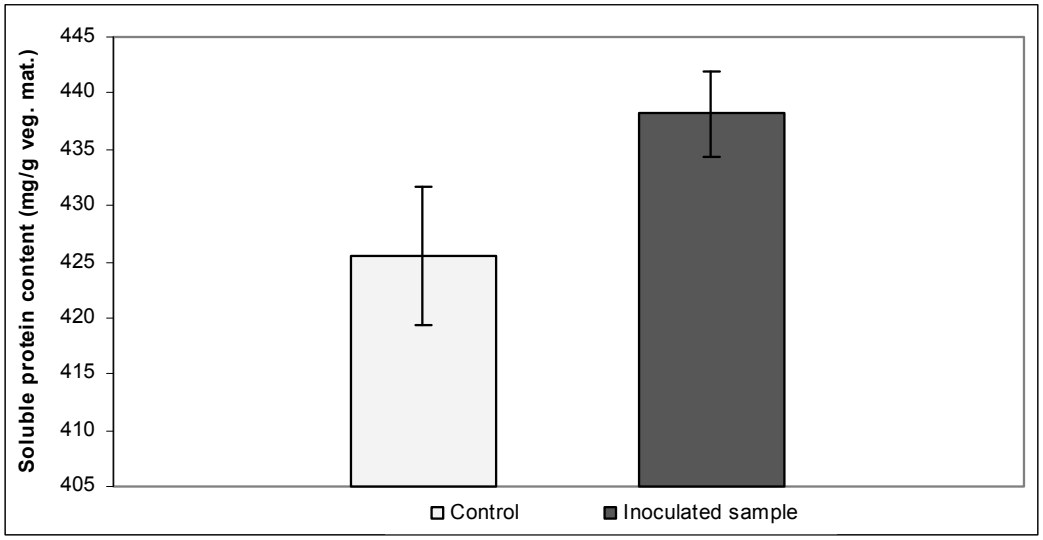

Fig. 1 – Total soluble protein content of beans produce by inoculated and non-inoculated soybean plants

Such improvement in total protein production can be achieved also by amplification of various genes in the genome of transgenic soybean plants (Li et al., 2004) or by usage of important amounts of chemical or organic fertilizers. Both alternatives have their own disadvantages: usage of GMO (genetically modified organisms) is an idea strongly rejected by the public opinion; chemical fertilizers have a well establish negative impact on the environment. A better alternative would be than to use rhizobacteria as biofertilizers. A question arises: does this bacteria influence only the plant growth by mobilizing nutrients from the soil (Sylvia et al., 1999) or they induce some qualitative modification in the protein pattern? In order to asset this problem an SDS-PAGE electrophoresis was performed with the protein extracts (Fig. 2).

As it can be seen from Fig. 2, there are no significant qualitative differences between the protein electrophoresis pattern of seeds produced by non-inoculated and rhizobacteria inoculated soybean plants. In this case the tested rhizobacterial strains help protein accumulation only by a mechanism which involves probably only an increased availability and uptake of nutrients from soil.

CONCLUSIONS

The overall conclusion is that in field conditions rhizosphere bacteria isolated from soybean plants roots can stimulate protein accumulation in soybean seeds. Our test recorded only quantitative differences regarding total soluble protein content between seeds obtain from inoculated and non-inoculated soybean plants. No qualitative differences could be shown, making our tested rhizobacteria strains an appealing strategy for improving soybean protein production.

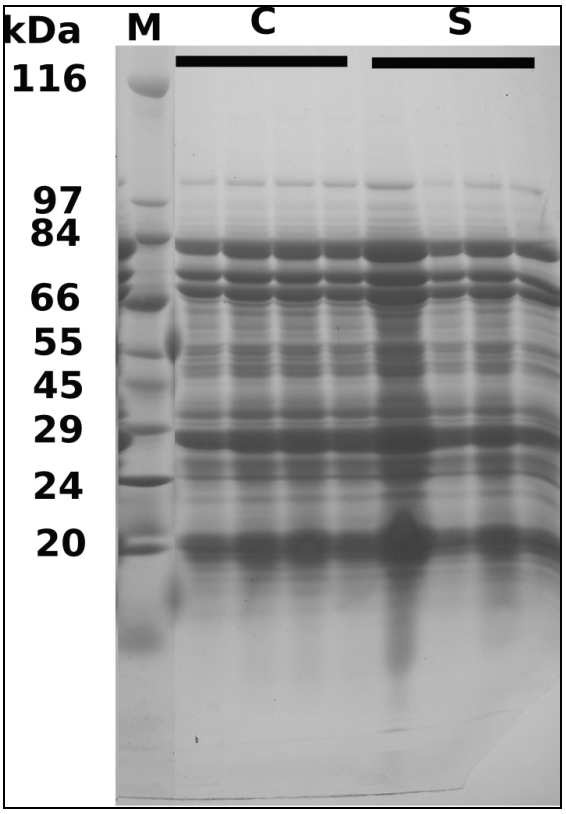

Fig. 2 - SDS-PAGE of total soluble proteins: M-Sigma Wide Range protein marker; C-non-inoculated control; S-rhizobacteria inoculated sample (125 micrograms proteins were loaded on each lane**)**

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ACKNOWLEDGMENTS
This work was supported by the CNCSIS Grant, IDEI 440, Nr. 249/01.10.2007.