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SOYBEAN (*GLYCINE MAX* [L] MERR.) INOCULATION WITH *BACILLUS PUMILUS* RS3 PROMOTES PLANT GROWTH AND INCREASES SEED PROTEIN YIELD: RELEVANCE FOR ENVIRONMENTALLY-FRIENDLY AGRICULTURAL APPLICATIONS

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Abstract: Fertilization is considered as one of the main sources of environmental pollution caused by agriculture. When high fertilizer rates are applied, nutrient losses take place which pollute agricultural ecosystems. Defense strategies were developed in order to minimize the environmental burden caused by agricultural pollution. One of these strategies is based on the usage of biofertilizers – microorganisms isolated from soil that can stimulate plant growth. In this context, effects of a *Bacillus pumilus* strain isolated from soybean rhizosphere on plant growth, nodulation and seed protein yield were investigated. Two factors (A x B) experiments were conducted using a "split plots design". The soybean plants have been grown in ecological conditions, without organic fertilizers and pesticides. Our results suggest that the tested rhizobacterial strain significantly increased the plant's height ($p<0.0001$), number of leaves ($p<0.0003$), foliar area ($p<0.0002$), nodulation ($p<0.0002$) and bean protein content ($p<0.05$), compared to the non-treated control. We concluded that *Bacillus pumilus* Rs3 promoted plant growth and increased seed protein yield. Our rhizobacterial strain could be an eco-friendly alternative for reducing soil pollution caused by fertilizers usage.

Keywords: rhizobacteria, soybean growth, nodulation, protein, reducing carbohydrate, biofertilizer.

1. INTRODUCTION

Soil bacteria specifically interacts with plant roots in the rhizosphere, where bacterial number is generally higher than in the free soil (Sylvia et al., 1999). The bacteria that provides benefits for the plant either form symbiotic relationships with the plant or are free-living in the soil, but found near or even within the roots (Frommel et al., 1991). Beneficial free-living soil bacteria are usually referred to as plant-growth-promoting rhizobacteria or PGPR (Kloepper et al., 1989) - a group that includes different bacterial species: *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Herbaspirillum*, *Burkholderia* and *Bacillus* (Glick, 1995). The mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include: a) ability to produce plant hormones (indoleacetic acid, gibberellic acid, cytokinins (Glick, 1999); b) synthesis of enzymes that can modulate plant growth and development (Jacobson et al., 1994); c) nitrogen fixation (Kennedy et al., 1997); d) reducing or preventing the harmful effects of one or more phytopathogenic organisms (Zahir et al., 2003) by producing ß-1,3-glucanase (Fridlender et al., 1993), chitinases (Renwick et al., 1991), antibiotics (Shanahan et al., 1992), cyanide (Flaishman et al., 1996); e) solubilization of mineral phosphates and other nutrients (De Freitas et al., 1997). Some rhizobacteria can promote plant growth indirectly by modifying nodule formation and biological nitrogen fixation (Cattelan et al., 1998). In a competition study where soybean was co-inoculated with *Bradyrhizobium japonicum* USDA 110, *Bradyrhizobium japonicum* USDA 118 and one of 17 different isolates of rhizobacteria, 3 isolates increased the number of nodules (Polonenko et al.,

1987). However, the mechanisms used by these bacteria to produce the mentioned effects are not well-understood.

Specific bacteria introduction into rhizosphere in order to promote plant growth has been the subject of an intensive research during the last decades, and this practice has become accepted in agriculture because pollution by nutrients causes many problems on the environment (Bashan, 1998). One of the main sources of these problems are fertilizers used in agriculture. Industrial production and the usage of fertilizers have led to a sharp increase in food production that has been accompanied by the population growth in almost all countries around the world. Considering the benefits of intensive agriculture practice and the negative environmental impact of chemical fertilizers and pesticides, usage of rhizobacteria as biofertilizers is one of the most promising biotechnologies for growing the primary production with less quantity of fertilizers (Bashan, 1998). Moreover, usage of bacteria isolated from crop plant's rhizosphere for productivity increase may be an eco-friendly alternative to organic nutrients (Compant et al., 2005).

Since soybean represent a crop with major economic value, pesticides and organic fertilizers are commonly applied before sowing and during vegetation period with negative impact on environment. From this point of view, the purpose of our study was to evaluate the plant growth-promoting effect of one *Bacillus pumilus* strain for assessing its biotechnological potential as biofertilizer, in order to prevent the usage of commonly applied fertilizers and pesticides.

2. MATERIALS AND METHODS

2.1. Bacterial strain and inoculant preparation

One bacterial strain: *Bacillus pumilus* Rs3 (BP Rs3) (Stefan et al., 2002) isolated from soybean rhizosphere was used to asses the effect on soybean growth promotion. The strain was previously tested for phosphate solubilization capacity using Katznelson and Bose method (Katznelson & Bose, 1959) and for antibacterial activity using disk diffusion method (Lorian, 2005) with 4 test bacteria (*Staphylococcus aureus* ATCC - 6538p, *Bacillus cereus* ATCC – 9634, *Pseudomonas aeruginosa* Ip – 5838, *Escherichia coli* ATCC – 10536). The selected rhizobacteria solubilised phosphate and presented antimicrobial activity against all tested bacteria.

Bacillus pumilus Rs3 strain was cultivated from slant material in Bunt Rovira nutrient medium (agar-agar free) and incubated in 2000 ml flasks on an orbital shaker at 210 rpm at 27^0 C. After 5 days the strain was subcultured under the same conditions as described above; the cells were adjusted to 6,4 x 10^7 cells mL^{-1} using sterilized tap water and used to inoculate soybean seeds just prior of sowing.

2.2. Plant growth

The field experiment was carried out with BP Rs3 strain used to inoculate the seeds. Soybean seeds (Pioneer PR91M10/91M10) were surface-sterilized in sodium hypochlorite (2 % solution containing 4 ml/l Tween 20) and rinsed 5 times with distilled water (Bhuvaneswari et al., 1980). Seeds were coated with the inoculum containing 6,4 x 10^7 cells mL^{-1}. The control seeds were washed with sterilized tap water. All seeds were planted using a small experimental seeding machine.

The experiment was repeated two times between April - October 2007 and May - October 2008 and sited at the Experimental Farm of the Agricultural University of Iasi, Eastern Romania ($47^o07`$N latitude, $27^o30`$E longitude), on a chambic chernozem soil - SRTS-2003 (Florea & Munteanu, 2003), or haplic chernozems after WRB, 2006 (WRB, 2006) with a clay-loamy texture and good fertility, moderate humus (3,6-3,4 %), highly nitrogen content (0,17 g/100 g soil), moderate P_2O_5 supply (12,6 mg/100 g soil), highly K_2O content (20,2 mg/100 g soil) and a very low acid reaction, almost neutral (pH = 6,6). In 2006 an area was selected for growth experiments without any agricultural practices previously performed.

The trials were designed as two factors (A x B) in a "split plots design" with four replications, each plot covering an area of 25 m^2.

After sowing, the soybean plants were monitored every week to observe the influence of tested bacterial strain on plant height, number of intact trifoliate leaves, foliar area (top leaflet basis using digital photography) and nodulation (nitrogen-fixing nodules were counted). The plants were growth in ecological conditions; no fertilizers or pesticides were applied.

2.3. Biochemical estimations

The harvested beans were used to asses the influence of tested rhizobacteria for their nutritional value by monitoring some biochemical parameters as following:

a) Total reducing monosaccharides were measured using 3,5-dinitrosalicylic acid method (Miller, 2002) with glucose monohydrate solution (30 to 300 µg/ml) as standard; absorbance was measured at 500 nm with an Jasco V 350 UV-VIS spectrophotometer; disaccharides were assayed using the same method after a preliminary hydrolysis of the aqueous extract in HCl 20 % for 5 min in boiling water; soluble polysaccharides were assayed by hydrolysis in HCl 20 % of the aqueous extract for 3 hours in boiling water, followed by total reducing monosaccharides assay; the insoluble components obtained after aqueous extraction of soluble sugars were used for insoluble polysaccharides determination by hydrolysis in HCl 20 % for 3 hours in boiling water followed by total reducing monosaccharides assay.

b) Total soluble proteins were extracted using cold phosphate buffer saline (PBS) pH 7,4 (Sambrook & Russell, 2000) in 1 g seed/10 mL buffer ratio. Protein amount was assayed by the dye-binding micro-method of Bradford using the Roti-Quant reagent from Roth (Karlsruhe, Germany). The concentration was expressed as g bovine serum albumin (BSA) per 100 g beans;

c) Electrophoresis was performed on 10 % SDS-PAGE gels (Laemmli, 1970). The gels were casted according to (Ausubel et al., 2002) using an Apelex midi (15 x 12,5 cm) electrophoresis unit. Approximately 50 µg protein were loaded per lane and visualized using standard Coomasie Brillant Blue R 250 staining method (Sambrook et al., 1989). Gel densitometry and molecular weight determination were performed using ImageQuant TL from GE Healthcare.

d) Total lipid content – approximately 20 grams of beans were dried at 105^0 C until constant weight and grinded to powder using a Waring laboratory blender; exactly 2 grams of powder were extracted with diethyl-ether for 3 hours with a Soxhlet extractor (Chen et al., 1996), followed by a gravimetric measurement of the remaining material. The differences of the powder weight before and after extraction were considered as sample's total lipid amount and expressed as g/100 g dried bean.

2.4. Statistical analysis

The results concerning biometric parameters were statistically analyzed using two-way ANOVA (ANOVA: two factors with replication) (Sokal & Rohlf, 1995). Biochemical data were analyzed using Student's t-Test. All results are expressed as means ± S.E.M. of two consecutive years' experiments; F values for which $p<0.05$ were considered as significant.

3. RESULTS

In order to evaluate the plant growth promotion effect induced by our strain (BP Rs3), we studied some biometrical indicators for both aerial (plant height, number of leaves, foliar area) and root system (number of nitrogen fixing nodules). No organic fertilizers or pesticides were applied for field studies and growth experiments.

Three sets of measurements were made during plant growth: before flowering, during flowering and fructification. Biometrical results are shown in figures 1 to 3.

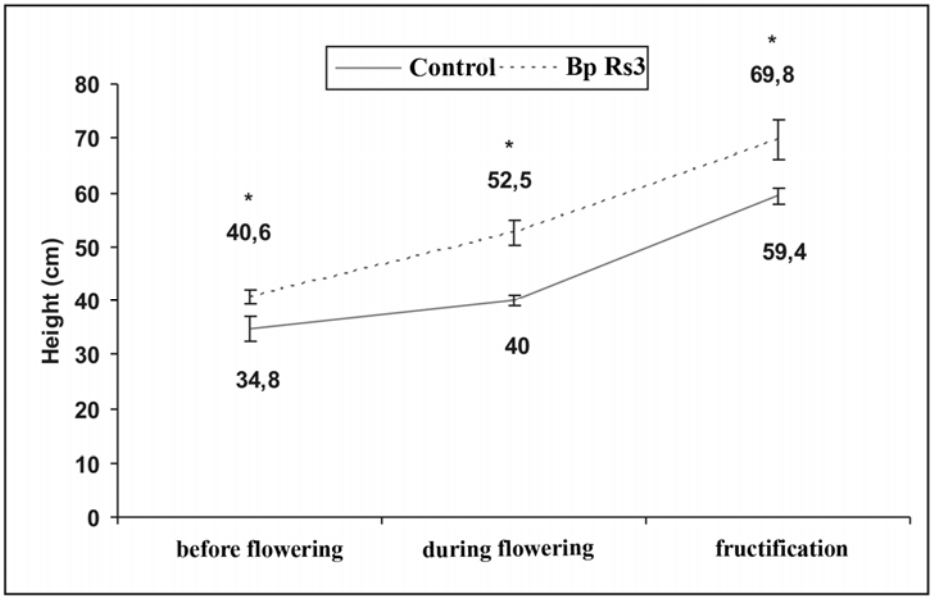

Figure 1. *Bacillus pumilus* Rs3 effect on plant height. Values are means ± S.E.M. of 2007 and 2008 field measurements; *p<0.0001 vs. non-treated control.

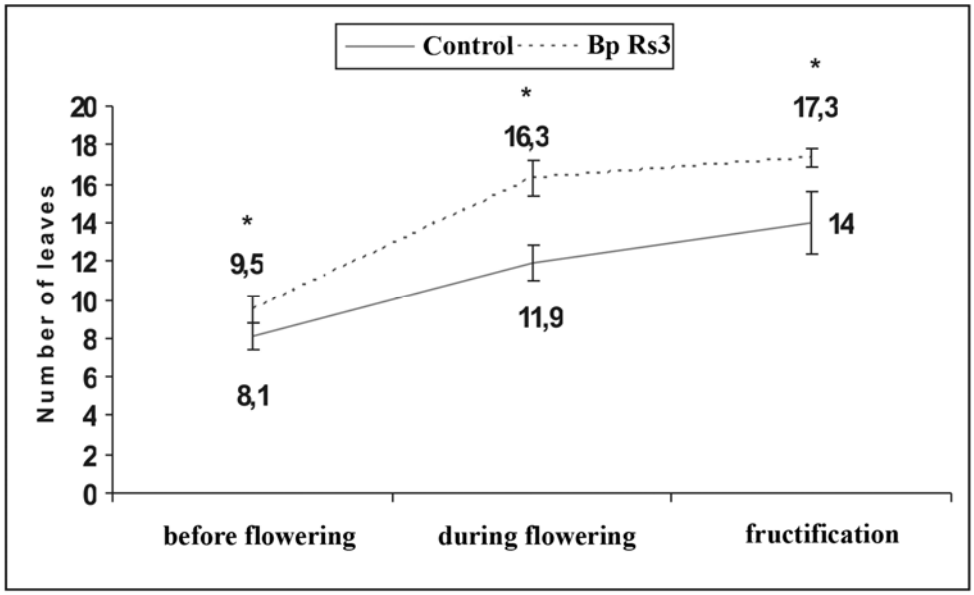

Figure 2. *Bacillus pumilus* Rs3 effect on intact trifoliate leaves number. Values are means ± S.E.M. of 2007 and 2008 field measurements; * $p<0.0003$ vs. non-treated control.

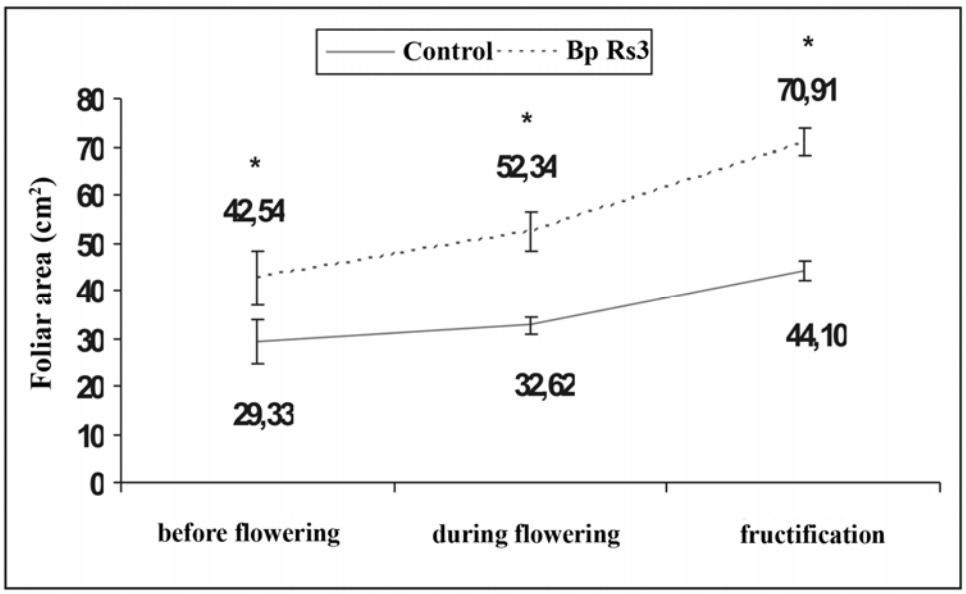

Figure 3. *Bacillus pumilus* Rs3 effect on foliar area (cm^2, top leaflet basis using digital photography). Values are means ± S.E.M. of 2007 and 2008 field measurements; * $p<0.0002$ vs. non-treated control.

Plant growth was significantly increased by inoculation with Bp Rs3. All investigated aerial system parameters were significantly increased compared with non-treated control: plant height ($F(2,1)=77.4$, $p<0.0001$, Fig. 1), number of leaves ($F(2,1)=28.64$, $p<0.0003$, Fig. 2), foliar area ($F(2,1)=16.64$, $p<0.0002$, Fig. 3). Also, BP Rs3 significantly increased the nitrogen fixing nodules number per plant ($F(2,1)=39.54$, $p<0.0002$, Fig. 4) and promoted root system growth compared with non-treated control (Photo 1).

In order to evaluate the effect of Bp Rs3 on nutritional value of harvested beans we analyzed chemical composition of soybean seeds, by assessing total soluble proteins, total lipid and carbohydrates content. Inoculation with our rhizobacterial strain induced non-significant increase of reducing carbohydrates (mono-, di-, polysaccharides) and total lipid content in harvested beans compared with non-treated control (Tab. 1).

Total amount of soluble protein was 66 % higher in beans harvested from inoculated soybean plants compared with non treated control (Tab. 1).

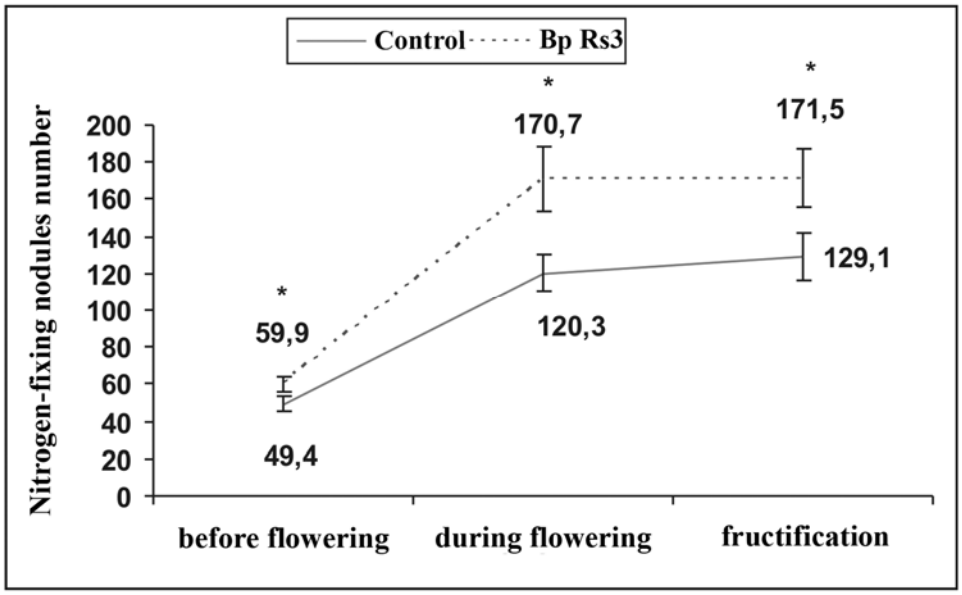

Figure 4. Effects of *Bacillus pumilus* Rs3 seed inoculation on nitrogen-fixing nodules number. Values are means ± S.E.M. of 2007 and 2008 field measurements; * p<0.0002 vs. non-treated control.

Photo 1. Soybean nitrogen-fixing root nodules: left – non-treated control; right – Bp Rs3 inoculated plant

Table 1. Effects of inoculation with Bp Rs3 on chemical composition of harvested beans

Group	**TRM**	**TRD**	**TRSP**	**TRIP**	**TL**	**TSP**
	g/100 g beans				g/100 dried beans	g BSA/100 g beans
Control	0,57 ± 0,03	8,28 ± 0,23	1,82 ± 0,24	8,14 ± 0,19	20,68 ±0,5	***3,28 ± 0,20***
Bp Rs3	0,77 ± 0,04; p = 0,11 vs. control	8,83 ± 0,09; p = 0,22 vs. control	2,064 ± 0,09; p = 0,24 vs. control	8,76 ± 0,51; p = 0,23 vs. control	21,23 ± 0,15; p = 0,38 vs. control	***4,95 ± 0,10; p = 0,006 vs. control***

TRM = Total reducing monosaccharides; TRD = Total reducing disaccharides; TRSP = Total reducing soluble polysaccharides; TRIP = Total reducing insoluble polysaccharides; TL = Total lipid; TSP = Total soluble protein

Since Bp Rs3 produced significant quantitative changes in seed protein content, a question arose regarding some possible qualitative modifications induced by inoculation with our rhizobacterial strain. As shown by the SDS-PAGE analysis (Fig. 5), inoculation of soybean plants with Bp Rs3 did not induce any detectable qualitative modifications in the seed proteins electrophoretic pattern.

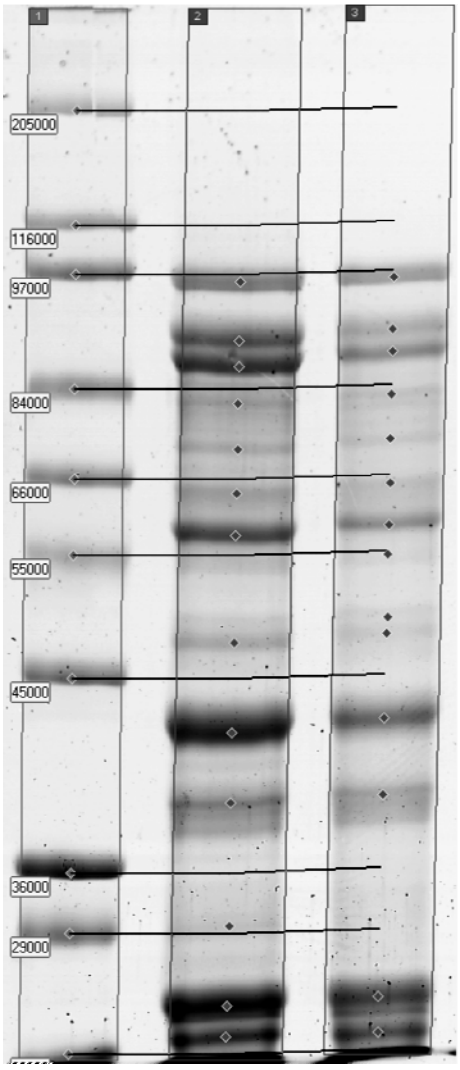

Figure 5. SDS-PAGE analysis of total soluble proteins from harvested beans. 1-Sigma wide range molecular weight marker; 2- plants inoculated with Bp Rs3; 3-non-treated control. Approx. 50 µg proteins were loaded per lane. Gel densitometry and molecular weight estimation were performed using ImageQuant TL software (GE Healthcare).

4. DISCUSSION

Persistent organic pollutants, fertilizers and pesticides in particular, represent an issue of concern in Romania both for the environment and public health protection (Ferencz & Balog, 2010). This is why ecological solutions like plant growth promoting bacteria used for increasing agricultural productivity are needed.

In the selection of plant growth promoting bacteria the final evaluations must be made under field conditions. Compared to a controlled environment as a greenhouse, the field environment is generally much more stressful and the conditions are much more complex. Thus, results obtained in greenhouse experiments do not necessarily reflect the potential for plant growth promotion in the field (Khan et al., 2009). In the present study we used one bacterial strain (*Bacillus pumilus* Rs3, isolated from soybean rhizosphere) selected for field growth experiments without applying fertilizers or pesticides.

Previous studies have reported that rhizobacteria inoculation of soybean plant produced a wide range of effects in plant development (Lee et al., 2008). Rhizobacterial strains stimulate plant growth and enhance some physiological processes (Zhang et al., 1997). In our study BP Rs3 seed inoculation significantly increased plant height, number of leaves and foliar area, inducing in this way a plant growth promoting effect, without utilization of fertilizers. It is well documented that *Bacillus* species are among the most bacteria isolated from plant tissues (Kobayashi & Palumbo, 2000). Other *Bacillus pumilus* strains (isolated from the rhizosphere of *Alnus glutinosa*) had also a strong growth-promoting activity, but in greenhouse conditions (Probanza et al., 1996), (Ramos et al., 2003). Plant-growth-promoting effects of *Bacillus pumilus* strains are induced by its capabilities to produce physiologically active gibberellins (Gutiérrez-Mañero et al., 2001). These effects are mainly derived from morphological and physiological changes of the inoculated plant roots, leading to an enhancement of water and mineral uptake.

It is important to note that in our field experiments the growth promotion provided by Bp Rs3 was apparently related to improved root development and enhanced nodulation, which resulted in better nutrient uptake capability and increased nitrogen supply. Enhanced nodulation is probably due to *Bacillus pumilus* capabilities to produce gibberellins (Gutiérrez-Mañero,Ramos-Solano & 2001). This kind of effect was previously observed and the experiments demonstrate that *Bacillus* sp. strains enhance soybean nodulation and growth under low temperature stress (Bai et al., 2003).

Although some studies involving plant growth promoting rhizobacteria inoculation revealed plants

physiological changes (stimulation of carbohydrates biosynthesis) induced by the presence of rhizobacteria (Bashan, 1998), our results showed no significant differences between Bp Rs3 inoculated seeds and non-treated control regarding reducing soluble and insoluble carbohydrates content.

Some authors reported that rhizobacteria enhanced protein concentrations in plants (Sannazzaro et al., 2006). Our data showed that Bp Rs3 inoculation increased with 66 % the total amount of seed soluble protein, probably due to stimulation of protein biosynthesis processes in soybean plants, providing in this way soybean seeds with higher nutritional value. Bp Rs3 treatment does not induce any qualitative changes of seed protein content.

For all these reasons, we concluded that our rhizobacterial strain could be an eco-friendly alternative for reducing soil pollution caused by fertilizers usage. Bp Rs3 has a viable biotechnological potential to be used as biofertilizer, in the context of sustainable development and the respect for environment (Ianos et al., 2009).

Bp Rs3 strain used in this work is a spore-forming strain isolated from soybean rhizosphere which showed growth promoting effects on soybean plants. Its spore-forming characteristic makes Bp Rs3 more adaptable to commercial formulations in future inoculant production (Liu & Sinclair, 1993). However, to determine whether this strain is suitable for development into commercial biofertilizer, further experiments are necessary, especially to verify its plant growth promoting efficacy under a wider range of field conditions and with a greater number of soybean cultivars, and to evaluate relevant safety issues. Even if Bp Rs3 was isolated from soybean rhizosphere and it seems to represent a stable component of the bacterial population in soil, further studies are needed to investigate how repeated application of this culture would affect the balance with various indigenous soil bacteria.

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