THE EFFECT OF COMBINED APPLICATION OF *B. JAPONICUM* AND *P. PUTIDA* WITH MINERAL AND ORGANIC FERTILIZATION ON PROLINE CONTENTS IN SOYBEAN UNDER PHOSPHORUS INSUFFICIENCY AND MODERATE DROUGHT

INFLUENȚA APLICĂRII COMBINATE A RIZOBACTERIILOR *B. JAPONICUM* ŞI *P. PUTIDA* PE FONDAL DE FERTILIZARE MINERALĂ ŞI ORGANICĂ ASUPRA CONȚINUTULUI DE PROLINĂ LA SOIA ÎN CONDIȚII INSUFICIENTE DE FOSFOR ŞI SECETĂ MODERATĂ

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Abstract. *The use of plant growth promoting rhizobacteria (PGPR) is considered a strategy to improve plant tolerance in hostile environments. However, its underlying mechanisms are not completly understood under phosphorus (P) insufficiency and water deficit conditions. In the present study, a vegetation vessels experiment was conducted to assess the combined effects of Bradyrhizobium japonicum inoculation with Pseudomonas putida in conjunction with P and organic fertilization on proline contents of soybean (Glycine max L.) plants grown in P-deficient soil and subjected to moderate drought. The experimental results demonstrated that the application of both strains significantly changed proline contents in leaves and roots, especially under moderate drought and P insufficiency compared to the inoculation with the symbiotic B. japonicum strain alone. The rhizobacteria strains application without fertilization or with cattle manure increased proline accumulation in leaves under drought but did not affect significantly this parameter in well-watered plants. By contrast, integrated use of isolates decreased proline concentration in roots of soybean with mineral P and organic fertilization regardless of soil moisture level. In conclusion, the combined use of B. japonicum and P. putida is efficient approach to improve soybean growth and drought tolerance through altering proline contents, especially in soil with P insufficiency as well as under application of organic fertilizer.*

Key words: drought, manure, phosphorus, proline, rhizobacteria, soybean.

Rezumat. *Aplicarea bacteriilor promotoare a creşterii este considerată strategie de ameliorare a toleranței plantelor la factorii nefavorabili de mediu, însă mecanismele ce determină rezistența cauzată de bacteriile benefice sunt insuficient elucidate în condiții deficitului de P şi umiditate. În prezentul studiu, s-a organizat o experiență în vase de vegetație care a avut ca scop evaluarea efectului combinat al bacteriilor Bradyrhizobium japonicum şi Pseudomonas putida, aplicate pe fondalul fertilizării cu P sau îngrăşăminte organice asupra conținutului de prolină la plantele de soia (Glycine max L.) supuse secetei moderate. Rezultatele experimentale au arătat că aplicarea împreună a*

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ambelor tulpini bacteriene au modificat semnificativ conținutul de prolină în frunze şi rădăcini, în mod special la plantele supuse concomitent secetei moderate şi insuficienței de P în raport cu plantele inoculate numai cu tulpina B. japonicum. Influența tulpinilor rizobacteriene asupra plantelor fără fertilizare sau fertilizate cu bălegar de vite cornute a majorat acumularea prolinei în frunze în condiții de secetă, dar n-a influentat semnificativ acest indice la plantele bine irigate. Utilizarea combinată a acestor tulpini dimpotrivă a diminuat semnificativ concentrația prolinei în rădăcinile soii fertilizate cu P mineral sau fertilizate organic indiferent de nivelul de umiditate a solului. În concluzie, aplicarea combinată a bacteriilor B. japonicum şi P. putida este opțiune eficientă de îmbunătățire a creşterii soii şi de majorare a toleranței la secetă determinată, în particular la plantele cultivate pe solul cu insuficiență de fosfați mobili cât şi la plantele fertilizate cu îngrăşăminte organice.

Cuvinte cheie: fosfor, prolină, rizobacterii, secet*ă*, soia.

INTRODUCTION

Abiotic stresses such as drought and phosphorus deficiency are main constraints of agricultural production and therefore remain a big problem of food insecurity and sustainability of agriculture (Dimkpa *et al*., 2009; Egamberdiyeva *et al*., 2016). Legumes are more vulnerable to unfavorable conditions than cereal crops. Soybean (*Glycine max* L.) is an agronomically and nutritionally important legume crop because it is a major source of protein and vegetable oils in many countries, however, its production is restricted by drought (Devi and Sinclair, 2013) and P deficiency (Vance *et al*., 2003). Maintaining high plant productivity under environmental stresses is important challenge facing sustainable agriculture (Gill and Tuteja, 2010). Therefore, it is necessary to find out approaches to improve the productivity and tolerance of crops to abiotic stress factors. The use of plant growth-promoting bacteria (PGPR) and symbiotic microorganisms may prove useful in developing strategies to promote plant growth and enhance tolerance of crops in hostile environments (Khan *et al*., 2006). One of the efficient mechanisms of crop tolerance is osmotic adjustment through the accumulation of active/compatible solutes in plant tissues that enable plants to improve water status (Ashraf and Foolad, 2007; Ashraf, 2010). Amongst osmoprotectants proline has essential functions in plant metabolism including acquiring plant resistance to drought conditions. Glick *et al*. (1998) showed that under different stresses using PGPR such as *Pseudomonas fluorescence* can alleviate the adverse effects of stress on plant growth. Likewise, Egamberdieva *et al*. (2016) established improved drought tolerance of lupine due to application of rhizobium inoculants. It is proposed the use of more than single PGPR in biofertilizer preparation could be better option over a single bacterium to bring synergistic effect of nutrient mobilization, enhanced efficacy, stability and increase crops productivity (Abdah_ALah *et al*., 2017). It is necessary to be mentioned, the use of rhizobacteria along with chemical and organic fertilizers may serve as an effective option for enhancing crop tolerance and productivity, as well as nutrients efficiency.

Although there are reports regarding beneficial effects of species *Pseudomonas* sp. and *B. japonicum* sp. on plant growth under unfavorable environmental conditions, it is still not completely clear to what extent does these rhizobacteria species impact accumulations of key osmoprotectants, in particular proline, in soybean plants cultivated under P-deficit soils and drought conditions.

The aim of this study was to assess the effect of combined application of *Bradyrhizobium japonicum* and *Pseudomonas putida* in conjunction with mineral or organic fertilization on proline contents and growth of soybean under P insufficiency and moderate drought conditions.

MATERIAL AND METHOD

In a controlled pot culture experiment, soybean (*Glycine max* L. cv. Horboveanca) plants were inoculated with *B. japonicum* (denoted as Rh*)* alone as reference treatment and other set of plants was inoculated with *B. japonicum* in combination with soil applied *P. putida* (denoted as PP). The soil used in this study was a carbonated chernoziom mixed with sand in order to create P insufficiency condition. These rhizobacteria treatments were tested in conjunction with plant fertilization: without P fertilization (P0), fertilized with 100 mg P/kg soil (P100) and application of cattle manure (M) at rate 20 g/kg of soil. Soybean seeds before inoculation were surface-sterilized in 70% ethanol and then rinsed five times with sterile distilled water. Four replicate pots were used per treatment (n=4). Two levels of soil moisture were installed as normal 70% WHC (water holding capacity) and drought (35% WHC) imposed at flowering stage for 12 days. Water-stressed plants and their corresponding non-stressed controls were harvested on 12^{th} day of exposure to drought. Relative water content (RWC) was measured at the end of drought. After harvest, roots, nodules and leaves were weighed separately to determine fresh weight, and then placed in an oven to dry at 60°C until a constant dry weight was obtained. Also, the numbers of nodules were recorded. The proline content in plant tissues was estimated by spectrophotometrically analysis at 520 nm of the ninhydrin reaction, according to Bates *et al*. (1973). Data were subjected to varying means of analysis and categorized using the “least significant difference” test in the Statistic program 7 at 0.05 probability level. Statistics values were presented as means ± SE of three replicates.

RESULTS AND DISCUSSIONS

A compatible osmolyte such as proline, glycine or betaine plays an important role in plant tolerance to stress factors through osmotic adjustment (Ashraf, 2010). In this study, we examined the effect of combined application of two rhizobacteria species *Bradyrhizobium japonicum* and *Pseudomonas putida* on proline contents in leaves and roots of soybean in relation to types of fertilization and soil moisture conditions.

Experimental data of the effect of rhizobacteria on proline concentration in leaves under P insufficiency and moderate drought are presented in figure 1 (A,B). Proline accumulation was higher in leaves of plants subjected to water deficit as compared with well-irrigated plants. However, combined effects of *B.*

japonicum and *P. putida* on proline accumulation in leaves were significant only under low soil water regimes (fig. 1A). Similarly, Lobato *et al*. (2008) revealed an accumulation of proline in leaves of *Glycine max* grown under water deficit conditions. In the present investigation, there was no significant difference between rhizobacteria treatments in well-watered plants. Hence, in conditions of P insufficiency and moderate drought, the combined application of both rhizobacteria increased the content of proline in leaves approximately by 2 fold compared to single application of *B. japonicum* (fig. 1A). Our results of enhanced proline accumulation in drought stressed plants corroborate with results of Egamberdieva *et al*. (2017a) for lupine. Data regarding the osmolyte accumulation in roots of plants grown under normal irrigation and drought are shown in fig. 1B.

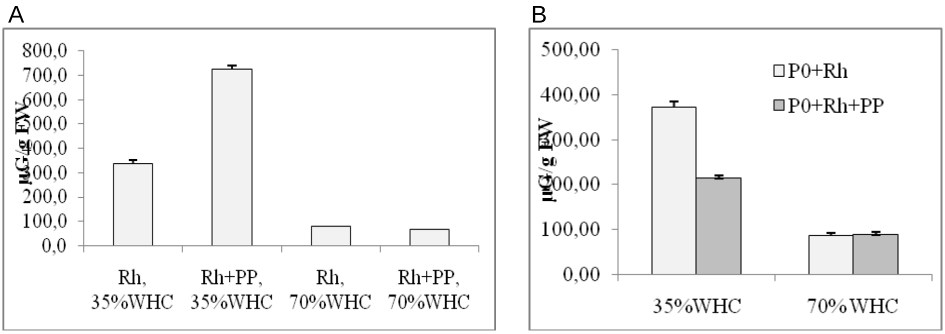

Fig. 1 The effect of *Bradyrhizobium japonicum* (Rh) applied alone or in combination with *Pseudomonas putida* (PP) on proline content in leaves (A) and roots (B) under no fertilization (P0) and moderate drought. WHC – water holding capacity. Columns are means ± SE.

In the treatment with both strains application the decrease in proline concentration in roots was observed (by 73.5%) due to combined use of strains compared with treatment of *B. japonicum* alone at the low moisture level (35% WHC), but there was no difference between bacteria treatments in well-watered plants (fig. 1B). Therefore, the highest concentration was registered in leaves of plants grown under P deficiency and subjected to moderate drought. A high level of proline enables the plants to maintain an osmotic balance when growing under low water potentials (Ashraf, 2010). In this regard we agree with literature findings that the combined use of tested rhizobacteria could compensate the drought effects and improve plant development through enhanced production of proline, amino acids, and soluble sugars and provided for better absorption of water and nutrients from soil (Vardharajula *et al*., 2011). Hence, the accumulation of proline in leaves under combined application of *B. japonicum* and *P. putida* plays positive influence in osmotic adjustments of plant tissues under unfavorable moisture of soil and low P supply.

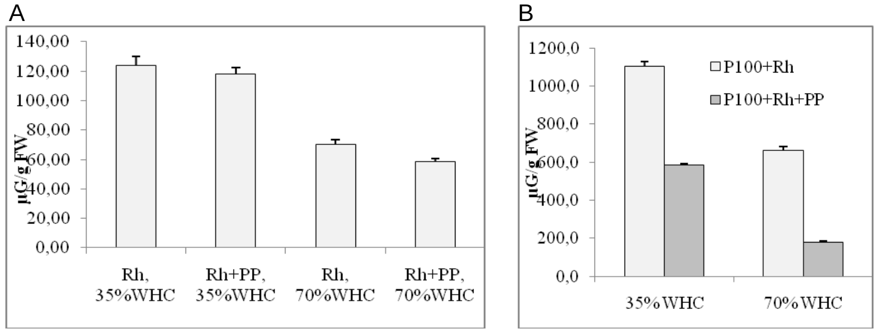

Fig. 2 The effect of *Bradyrhizobium japonicum* (Rh) applied alone or in combination with *Pseudomonas putida* (PP) on proline content in leaves (A) and roots (B) under phosphorus fertilization (P100) and moderate drought. WHC – water holding capacity. Columns are means ± SE.

According to literature data the effect of PGPR has been examined, as a rule, under low soil fertility. Unfortunately, there is scarce information regarding integrated use of these bacteria strains with chemical P fertilizer application, especially under water deficit conditions. In the present study it was tested a treatment of rhizobacteria application with P fertilization. The current experimental results revealed that adequate phosphorus nutrition (100 mg P/kg soil) decreased concentration of proline in leaves (fig. 2A) compared to plants cultivated under P deficiency (fig. 1A) regardless of rhizobacteria application. However, the combined use of *B. japonicum* and *P. putida* had less impact on proline content in leaves of soybean fertilized with mineral phosphorus. Thus, under adequate P nutrition and normal moisture (fig. 2A) dual inoculations decreased proline level in leaves by 20.2% as compared to single inoculated plants with *B. japonicum*. This could indicate that moderate drought affected to a lesser extent the accumulation pattern of this osmolyte when P nutrition is sufficient. The most significant effect of inoculates applied on tissues parameter was found in root tissue in both soil moisture regimes (fig. 2B). Combined application of tested strains decreased this parameter in roots in both water soil regimes. Experimental results revealed a remarkably decrease (3,6 fold) of proline in normal moisture conditions and by 1,9 fold in roots of plants grown under water deficit (fig. 2B). Also, Liu *et al*. (2015) suggested that P fertilization significantly decreased proline concentration in water-stressed *Fargesia rufa*. Again, plants subjected to insufficiency of water registered higher proline concentrations in leaves and roots than in well irrigated plants (fig. 2A,B).

There are investigations reporting a considerable impact of integrated application of PGPR and organic fertilization on growth and productivity of crops cultivated under no stress conditions as was demonstrated by Krey and coworkers (2011). In our trail, it was included treatment of integrated use of rhizobacteria and manure fertilization in order to finding out their interaction effect on soybean under moderate drought. The patterns of proline accumulation in leaves and roots affected by rhizobacteria and soil moisture conditions under organic fertilization are shown in figure 3A and B. Under moderate drought, plants with both bacteria strains showed a slight increase of proline accumulation in leaves as compared to reference plants (*B. japonicum* alone). It was revealed that proline accumulation in leaves increased by 35.7% in plants treated with *B. japonicum* and *P. putida* under temporary drought. Likewise, Agami *et al.* (2016) demonstrated a significant improvement in proline accumulation in *Ocimum basilicum* L. after administration of PGPR (*Azotobacter chroococcum* A101), resulting in enhanced water uptake, water use efficiency and photosynthetic efficiency. However, in treatments with organic fertilization, there was no difference between strains regarding concentration of this osmolyte in leaves of plants grown under normal irrigation regime (fig. 3A).

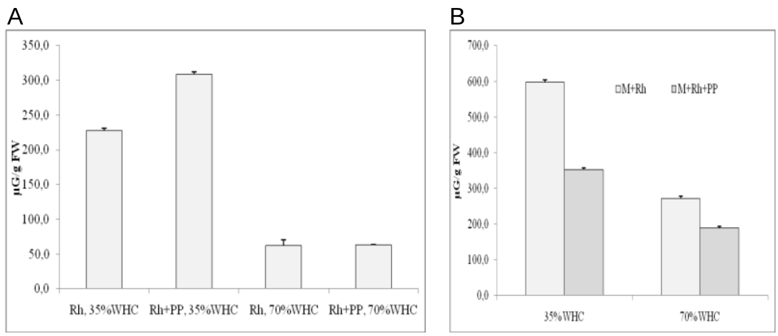

Fig. 3 The proline content in leaves (A) and roots (B) of soybean plants inoculated with *Bradyrhizobium japonicum* alone (Rh) and together with *Pseudomonas putida* (PP) in conjunction with manure fertilization under drought. WHC – water holding capacity. Columns are means ± SE.

Comparing with leaves the changes of this metabolite were more evidently in roots. Particularly, the proline accumulation decreased in roots after rhizobacteria application (*B. japonicum* combined with *P. putida*) of plants fertilized with manure and subjected to temporary drought (fig. 3B). Accordingly, these rhizobacteria diminished the concentration of proline in roots by 74.8%. The reason for this result is that combined application of two rhizobacteria increased significantly root growth, nodulation rate as well as leaf vigour that provided more favourable physiological conditions for a higher utilization of proline in different physiological processes. Similar influence was registered in well-irrigated plants. Therefore, we suggested that physiological state of plants was less affected by abiotic factors. Likewise, some researches obtain similar tolerance enhancement under stress environments with other bacterial species (Estévez *et al.*, 2010; Egamberdieva *et al.*, 2004; 2017a; 2017b). Ashraf (2010) concluded that drought stress is an essential limiting abiotic factor for plant growth and crop productivity. The application of PGPR has been found an effective biotechnology option to increase productivity of crops under drought conditions (Vardharajula *et al*., 2011).

The summary parameter to estimate the influence of rhizobacteria, fertilization and abiotic factors is considered plant growth. Plant growth was estimated using dry matter accumulation. The experimental results regarding the effect of studied biotic and abiotic factors on soybean growth are presented in table 1. The dry matter weights of plants were significantly inhibited by moderate drought. Under combined abiotic factors P insufficiency and water deficit soybean registered the lowest value (4.87 g/plant) of primary productivity. However, the combined application of tested rhizobacteria under such unfavorable conditions increased plant growth by 12.7% compared to inoculation with *B. japonicum* alone. Similar impact was observed under well-watered condition. Egamberdieva *et al*. (2017a) also revealed an enhancement in the dry weight of lupine (*Lupinus angustifolius* L.) plants treated with the bacterium *Bradyrhizobium* under drought stress. In our study, the moderate increase of plant productivity due to rhizobacteria inoculation was because plants were subjected to short drought. However, the better impact of bacteria application occurred when crops were grown in stressful conditions for prolonged periods (Egamberdiyeva and Hoflich, 2004).

In current investigation, the combined use of these strains in conjunction with mineral P fertilization inhibited beneficial impact of bacteria (Table 1). It was found out that growth response to rhizobacteria application was more pronounced under organic fertilization than under mineral P fertilization. Hence, the highest increase of soybean production was recorded in response to inoculation with both strains under manure fertilization in well irrigated plants (70% WHC). Also, the combined use of rhizobacteria promoted better plant growth under low soil moisture level (35% WHC) where that parameter increased by 14.3% in comparison to single *B. japonicum* inoculation.

Table 1

Effect of rhizobacteria (*B. japonicum* and *P. putida*) in conjunction with fertilization on dry matter weight of soybean in relation to soil moisture level

Treatments	Soil moisture (%)	P insufficiency		P fertilization		Manure fertilization	
		g/plant	SE	g/plant	SE	g/plant	SE
B. japonicum	70% WHC	7.71	0.24	14.89	0.16	10.39	0.27
B. japonicum+ P. putida		8.66	0.19	15.63	0.16	11.22	0.16
B. japonicum	35% WHC	4.87	0.09	8.66	0.35	7.05	0.14
B. japonicum+ P. putida		5.49	0.12	9.23	0.35	8.06	0.13

WHC - water holding capacity, SE - standard error. Data presented are the means ± SE (n = 4).

Probably, organic matter of cattle manure facilitated proliferation of microorganisms in soil and promotes higher activities of tested bacteria which in turn enhanced plant vigour and performance especially under limited water conditions. It is necessary to note that under manure fertilization leaves of inoculated plants with both bacteria strains grown in water limited conditions had higher RWC compared to those of inoculated plants only with *B. japonicum*. Likewise, plants inoculated with both rhizobacteria strains had more nodules and vigorous roots development than treatment with *B. japonicum* alone (data not shown). In general, plants with combined use of *B. japonicum* and *P. putida* had higher biomass production than plants treated with single inoculation with *B. japonicum.* We supposed that that effect was probably indirect due to rhizobacteria enhancement of nutrients uptake and assimilation in particular of P because plants also had higher P concentrations in leaves and roots than single inoculated plants (Rotaru, 2018). In addition, beneficial effects of rhizobacteria could be due to the presence of other activities. For instance, PGPR facilitate synthesis of some plant-growth promoting elicitors such as IAA, cytokines, axuins under normal as well as under drought conditions, improve synthesis of photosynthetic pigments, some antioxidant enzymes and antioxidants under stress conditions which contributed to stimulation of plant growth induced by rhizobacteria (Egamberdieva *et al.*, 2016). Therefore, this study clearly demonstrated the beneficial impact of combined use of these bacterial strains in soybean productivity under well-watered as well as under moderate drought condition. Thus, the combined application of *Bradyrhizobium japonicum* and *Pseudomonas putida* strains is more efficient for improving the growth of soybean cultivated on P-deficient soil or with organic fertilization under normal moisture as well as under moderate drought when compared with inoculation of *B. japonicum* alone. Experimental results find out that the combined use of *B. japonicum* with *P. putida* evidently had impact on proline contents in leaves and roots of soybean. Regarding leaves, bacterium biofertilizers significantly affected the proline contents under drought and there were not remarkable changes in well-watered plants. However, when the plants were inoculated with *B. japonicum* along with *P. putida* application the extent of growth suppression was decreased and plants had greater dry weights than treated plants with *B. japonicum* alone.

CONCLUSIONS

1. The combined use of bacterial strains (*B. japonicum* and *P. putida*) affected proline contents in soybean irrespective of type of fertilization and soil moisture conditions. The positive effects of the microbial use on tolerance of drought-stressed soybean, cultivated under P insufficiency and water deficit conditions could be through increasing contents of osmotic compounds.

2. Combined application of both bacteria strains showed synergic effects on soybean growth under P insufficiency and moderate drought conditions as well as under manure fertilization. There is a need to perform similar experiments under

field conditions with different types of inoculants in order to improve crop productivity under hostile environments.

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