INTERACTIVE EFFECTS OF RHIZOBACTERIA INOCULATION AND PHOSPHORUS ON PROLINE AND NITROGEN CONTENTS IN ROOTS AND NODULES OF SOYBEAN GROWN UNDER CONSTANT P INSUFFICIENCY AND WATER DEFICIT

EFECTELE INTERACTIVE ALE INOCULĂRII RIZOBACTERIEI ŞI FOSFORULUI ASUPRA CONȚINUTULUI DE PROLINĂ ŞI AZOT ÎN RĂDĂCINILE ŞI NODULII DE SOIA CRESCUȚI ÎN CONDIȚII DE INSUFICIENȚĂ CONSTANTĂ DE P ŞI DEFICIT DE APĂ

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Abstract. *Plant osmolytes play an important role in plant tolerance to abiotic stress factors. A greenhouse experiment with soybean was carried out with N-fixer rhizobacteria Bradyrhizobium japonicum applied singly or in combination with P (20 and 100 mg kg^{-1} dry soil) and two soil moisture regimes: 70% of whole holding capacity (WHC) as control and water stress 35% WHC. All plants were grown under same moisture conditions until the flowering stage. Water deficit conditions were imposed at flowering stage of plants for 12 days. Plants cultivated under the combined effects of P deficiency and drought exhibited the highest proline accumulation. Soybean inoculated with B. japonicum showed increased root proline concentrations compared to uninoculated plants subjected to temporary water deficit. Integrated use of inoculation and a moderate dose of P had a synergic effect on free proline accumulation in roots. The results revealed that combined influence of drought and P insufficiency significantly increased proline concentrations in nodules rather than in roots. Integrated use of rhizobacteria strain and a moderate dose of P decreased free amino acid accumulation in nodules under drought. There was a synergic interaction between the rhizobacteria strain and P in terms of leaves and stems nitrogen contents of soybean. In conclusion, soybean inoculation with B. japonicum in conjunction with P fertilization partially attenuates the adverse effects of constant low P availability and temporary drought on plants.*

Keywords: *Bradyrhizobium japonicum*, soybean, phosphorus, proline, nitrogen, soil moisture

Rezumat. *Compuşii din plante cu acțiune osmotică au rol important în formarea tolerantei plantelor la factorii de stres abiotic. A fost organizat un experiment cu plantele de soia cu aplicarea rizobacteriilor Bradyrhizobium japonicum atât separată cât şi in combinare cu fosforul (20 si 100 mg P/kg sol) şi cultivate la două regimuri de umiditate a solului - 70% din capacitatea totală*

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de apă (CTA) a solului şi 35% CTA. Toate plantele au fost crescute la umiditatea optimă până la faza înfloritului. Deficitul de apă din sol a fost declanşat în faza înfloritului deplin pe o perioadă de 12 zile. Cea mai mare acumulare de prolină s-a înregistrat la plantele cultivate la insuficiența de fosfor şi supuse deficitului de umiditate. Plantele de soia inoculate cu tulpina de B. japonicum au arătat un conținut sporit de prolină în rădăcini comparativ cu plantele din varianta control. Aplicarea integrată a rizobacteriilor şi P a manifestat acțiune sinergică la nivel de conținut de prolina în rădăcini. Rezultatele experimentale au demonstrat că seceta şi insuficiența de fosfor au majorat mai pronunțat concentrația de prolină în nodozități decât în rădăcini. Utilizarea combinata a rizobacteriilor şi P în doză moderată a micşorat acest indice în nodozități la plantele supuse secetei temporare. S-a stabilit un efect sinergic la administrarea combinată a rizobacteriilor B. japonicum şi P asupra conținutului de azot în frunze şi tulpini. In concluzie, inocularea semințelor cu tulpina B. japonicum in combinare cu fertilizarea cu P contribuie la atenuarea parțială a efectelor secetei şi deficitului de P asupra plantelor de soia.

Cuvinte cheie: Insuficiență de fosfor, prolină, *Bradyrhizobium japonicum*, soia, umiditate

INTRODCUTION

Among the environmental stresses, drought stress (Khan *et al.*, 2018; Reddy *et al.,* 2004) along with phosphorus deficiency (Lynch, 2007) are the most adverse factors for legumes growth and productivity. It is estimated that drought stress may cause a 50% loss in crop plants (Kasim *et al.,* 2013). Investigations demonstrated water deficiency affects plants at physiological, biochemical and metabolic levels such as osmoprotectants (Glick, 2014). Likewise, the P insuffiency induces damage in crops physiology diminishing their tolerance to unfavorable environmental conditions (Jin *et al.,* 2006). Among legumes, soybean (*Glycine max* L.) is a crop that is one of the major plant protein and vegetable oil resources and plays an important role in agricultural sustainability. This species is very sensitive to water deficit, especially during flowering and onset of pods (R3) (Liu *et al.*, 2017). In addition, Sa-T and Israel (1995) reported that P deficiency diminished some physiological parameters: especially nitrogen assimilation in soybean plants under normal irrigation regime.

Therefore, these two adverse environmental conditions could seriously affect soybean production in many countries. There are studies, which demonstrated that the P supplementation leads to improvement of nitrogen assimilation and drought resistance of soybean (Jin *et al.,* 2006). Plant adaptation to environmental stresses is associated with metabolic adjustments that lead to the accumulation of several compatible organic solutes like sugars, polyamines, betaine, quaternary ammonium compounds, carbohydrates, proline and other amino acids (Hashem *et al.,* 2015). It was well documented that proline is accumulated in many plant species under environmental stress conditions as an osmoregulator (Alexieva *et al.*, 2001; Ashraf, 2010). The accumulation of this metabolite in plant tissues contributes to water homeostasis and improvement of plant growth under unfavorable conditions.

Nowadays large varieties of microorganisms' strains are used worldwide with the aim to enhance crop productivity and their resistance to abiotic stresses (Bhattacharya *et al.,* 2012; Khan *et al.,* 2018). It is documented that under normal ecological conditions soybean obtains nitrogen through biological nitrogen fixation by establishing association with rhizobia (Bulgarelli *et al.,* 2017). However, several studies demonstrated that N-fixer bacteria increased plant growth and N uptake under deficit irrigation (Liu *et al*., 2017). Rhizobacteria symbiosis could improve the legume plant nitrogen status by increasing the nutrient uptake and N-fixation to satisfy plants and nodules development (Bulgarelli *et al*., 2017). Low P availability persists in many soils, which together with inadequate compatible rhizobacteria strain to a particular legume result into low tolerance to abiotic factors and poor productivity of plants. To our knowledge, however, proline accumulation and nitrogen contents in soybean plants as influenced by rhizobacteria alone or in combination with P fertilization under water stress have not yet been well documented. Therefore, this study was to investigate the interactive effects of rhizobacteria *Bradyrhizobium japonicum* and P on proline contents in roots and nodules as well as nitrogen concentrations of soybean cultivated on soil of low P availability and temporary drought.

MATERIAL AND METHODS

Soybean (*Glycine max* L. Merrill, cv Horboveanca) plants inoculated with *Bradyrhizobium japonicum* 646 (Rh denoted in the text) alone or in conjunction with P application were grown at two soil moisture levels: normal as 70% WHC (water holding capacity) and temporary drought - 35% WHC. The soil used for the experiment was chernoziom carbonated with low P availability (18 mg P/kg soil) and mixed with sand in order to create P insufficiency conditions. Uninoculated plants served as control treatment. Before planting, the P was applied in soil at rate 0; 20 mg P/kg soil and 100 mg P/kg soil. Before sowing, the soybean seeds were thoroughly mixed with *B. japonicum* inoculants (10^8 cfu/mL). All plants from each treatment were grown under normal soil moisture until the beginning of flowering (R1) stage. After that ~~a~~ half of plants were subjected to temporary water deficit for 12 days. Moisture content of each pot was weighed every 3 days during the whole processing procedure, and was maintained at 70% of the maximum water-holding capacity. The experiment was in a completely randomized design and four replications were performed for flowering- stage and four for maturity grain stage, for each of the treatments. Free proline in roots and nodules was quantified spectrophotometrically according to Bates *et al* (1973). The Kjeldahl method was used to measure nitrogen content of plant samples. The obtained data were analyzed by using Statistic 7 program. Values were presented as means with standard errors (SE) from three independent treatments. The differences in the means were determined by the least significant difference (LSD) (P=0.05) test.

RESULTS AND DISCUSSIONS

In this study, an effort was made to assess the influence of inoculation of soybean with *B. japonicum* alone and in combination with phosphorus on proline accumulation in roots and nodules and nitrogen content in soybean plants grown under constant low P availability and temporary drought.

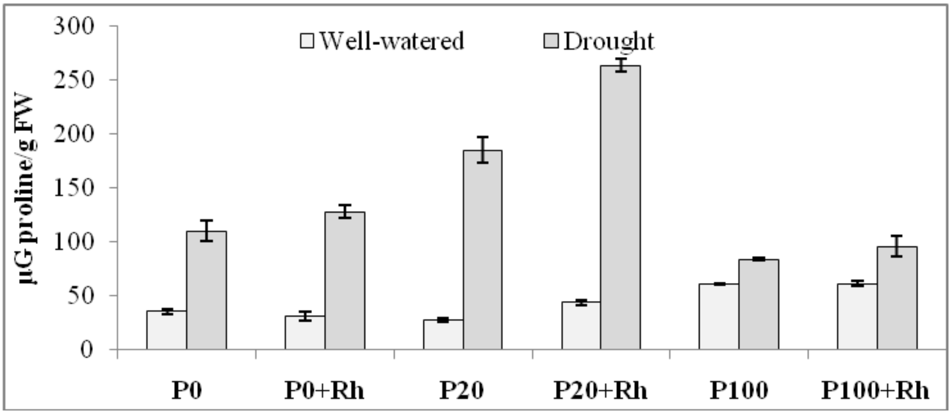

Fig. 1 The effect of *Bradyrhizobium japonicum* (Rh) applied alone or in combination with phosphorus (P) on proline content in roots under moderate drought. Columns are means ± SE.

Experimental results revealed that rhizobacteria and soil humidity had an impact on the pattern of proline concentrations in soybean roots. The contents of proline in soybean roots in relation to treatments and soil moisture level are shown in figure 1. It was noticed that imposition of drought stress caused an accumulation of proline in roots. Plants exposure to drought resulted in a multifold increase in proline concentration in roots tissues of uninoculated (3,1 fold) and inoculated (up to 6 fold) treatments indicating that proline was produced by the plants as response to the stress and inoculation with rhizobacteria further improved stress response of the plants in terms of proline content in roots. The soybean inoculation with *B. japonicum* alone under low P availability but well watered conditions did not change significantly this parameter in roots compared to uninoculated treatment. Similarly, the inoculation with *B. japonicum* in conjunction with P supplementation at a higher rate (100 mg/kg soil) did not influence the proline accumulation in roots for well-watered soybean plants. However, the seed inoculation with *B. japonicum* increased the proline concentration by 16,4% in comparison to uninoculated plants under drought conditions. Under water deficit conditions it was registered a significant increase of proline concentration in roots in treatment with a moderate dose of P in conjunction with seeds bacteria inoculation where this parameter increased by 42.2% compared to reference treatment (P20). Plants accumulating higher contents of free proline show increased stress tolerance (Ashraf, 2010). The increase of proline in drought stressed soybean plants corroborate with the findings of other studies for wheat (Jatav *et al.,* 2012) and for lupine (Egamberdieva *et al.*, 2017). Shukla *et al* (2015) have reported that priming *Triticum aestivum* L. with *T. harzianum* improved drought stress tolerance by mediating enhanced synthesis and accumulation of proline, thereby conferring tolerance to drought stress. Our study's results reflect increased accumulation of proline in inoculated soybean plants ~~as~~ compared to uninoculated plants. A similar observation was reported by Vardharajula *et al* (2011) for maize, where *Bacillus* spp. improved plant growth and tolerance to drought stress via enhanced production of proline, amino acids and soluble sugars. It seems that bacteria strain application could attenuate the drought effects and improve water status in plants. We assume that integrated application of P and rhizobacteria provided better conditions for proline synthesis which in turn induced plant tolerance to drought. Plants subjected to stressed conditions show higher activity of proline synthesizing enzymes while ~~as~~ the activity of catabolizing enzymes is down regulated (Iqbal *et al*., 2015; Khan *et al.,* 2015). The increase in proline accumulation due to rhizobacteria inoculation may occur because of its direct impact on the metabolizing machinery. In addition, the obtained results could be explained due to the fact that nitrogen-fixer bacteria improve nitrogen (N) nutrition in crops which was associated with more synthesis of amino acids, particularly proline which is an amino acid rich in nitrogen.

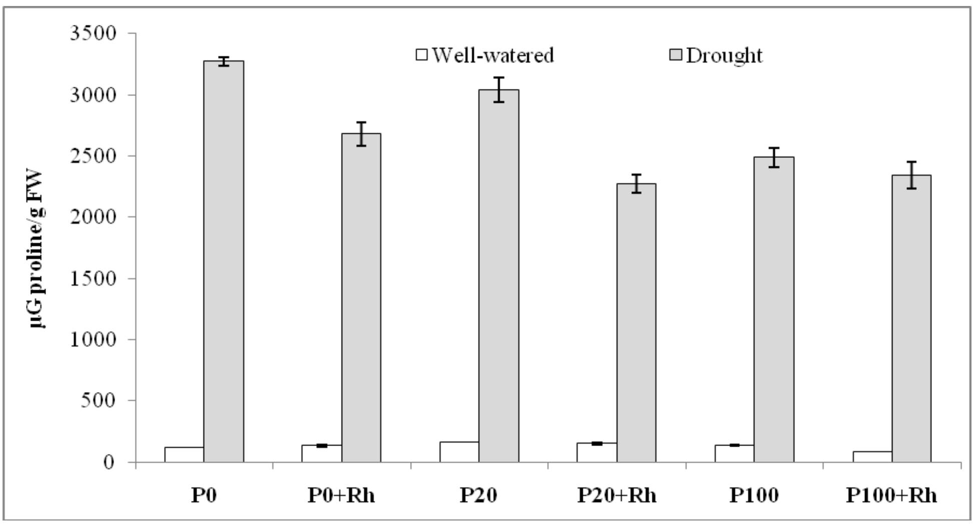

Fig. 2 The effect of *Bradyrhizobium japonicum* (Rh) applied alone or in combination with phosphorus (P) on proline content in nodules under moderate drought. Columns are means ± SE.

In our previous study (Rotaru, 2018) we did not evaluate the changes of proline in nodules of soybean in relation to rhizobacteria inoculation. It is necessary to emphasize that nodules are more sensitive to water deficit and P deficiency than other soybean organs (Getachew, 2014). The contents of proline in nodules in relation to treatments and soil moisture levels are shown in Figure 2. Experimental results of this research revealed that proline accumulation in nodules was higher than in roots. A significant increase was observed in the proline contents of nodules of soybean plants growing under water stress in contrast to nodules of plants grown under normal soil moisture conditions. We suggest that a higher increase of proline in nodules rather than ~~in~~ roots demonstrated that nodules are more susceptible to water deficit condition. Generally, the interactive effect of P insufficiency and water deficit leads to the highest level of proline concentration in nodules. The application of *B. japonicum* in conjunction with P decreased the proline concentrations by 33.8% and 6.1% in treatments with moderate and high dose of P, respectively (fig. 2). The highest accumulation of this metabolite in nodules under P insufficiency might be due to the fact that nodules are the primary sinks of P and the nodules tissues are abundant in nitrogen source which maintained the proline synthesis at a higher level. Moreover, the increased accumulation of proline showed the efficacy of inoculation treatments in maintaining osmotic potential, which may contribute to cellular adaptation to abiotic stress. In this respect, our findings are corroborated ~~to~~ with those related in studies performed by Sharifi *et al* (2011). Similar results were reported by Egamberdieva *et al* (2017), who observed an improving lupine drought tolerance following treatment with *Bradyrhizobium* species. In this research, it was observed that application of rhizobacteria isolate decreased the proline content in nodules of plants subjected to temporary water deficit and low P supply. However, plants fertilization with a moderate rate of P (20 mg/kg soil) increased proline accumulation in nodules by 34.9% ~~of~~ for well-watered plants compared to unfertilized ones. The application of *B. japonicum* together with a moderate dose of P fertilizer did not significantly change this parameter. The fertilizer application alone at high dose (100 mg P/kg soil) less increased this parameter to a smaller extent in nodules, by only 13.4%. In contrast, the integrated use of rhizobacteria and higher dose of P diminished the concentration of proline in nodules by 56.2%. Perhaps, plants in this treatment were not in stressed state and thereby the proline accumulation decreased. In addition, this result could be explained partially by the biological dilution factor because nodules were better developed compared to those of plants grown under suboptimal P nutrition conditions.

Normal plant growth and the potential to attenuate the adverse effects of unfavorable environments correlated with nitrogen status of crops. It is well known that nitrogen is a major component of the proline molecules and plays an important role in photosynthesis and tolerance of crops. Additionally, certain investigations showed that proline plays an important role of improving energy generation and storage by influencing the nitrogen metabolism (Hashem *et al*., 2015).

Table 1

Interactive effect of *B. japonicum* (Rh*) and* P supplementation on nitrogen contents in soybean plant parts in relation to soil moisture. Data presented are the means ± SE (n = 3)

Treatments	Leaves	Stems	Roots	Nodules
	Normal irrigation, 70% WHC			
P0	4.64 ± 0.26	1.82 ± 0.17	2.74 ± 0.10	4.67 ± 0.09
P0+Rh	4.88 ± 0.17	1.93 ± 0.06	2.72 ± 0.07	5.30 ± 0.13
P20	4.07 ± 0.18	1.45 ± 0.09	2.36 ± 0.11	5.12 ± 0.11
P20+Rh	4.41 ± 0.25	1.54 ± 0.05	2.24 ± 0.11	4.78 ± 0.07
P100	3.37 ± 0.14	1.10 ± 0.05	2.11 ± 0.04	4.65 ± 0.02
P100+Rh	3.96 ± 0.28	1.38 ± 0.06	2.16 ±0.10	4.98 ± 0.05
Water deficit, 35% WHC				
P0	4.45 ± 0.22	1.97 ± 0.07	2.52 ± 0.01	*
P0+Rh	4.23 ± 0.10	2.0 ± 0.09	2.59 ± 0.02	*
P20	4.12 ± 0.17	2.01 ± 0.16	2.38 ± 0.010	5.36 ± 0.03
P20+Rh	3.99 ± 0.10	2.0 ± 0.10	2.31 ± 0.03	5.39 ± 0.14
P100	3.28 ± 0.12	1.24 ± 0.13	2.09 ± 0.01	5.69 ± 0.12
P100+Rh	3.63 ± 0.13	1.39 ± 0.10	2.12 ± 0.04	5.91 ± 0.09

**Insufficiency of nodules mass*

It was well known that seed inoculation with *Bradyrhizobium japonicum* species contributes to improving nitrogen nutrition of soybean plants. However, its magnitude effect depends on environmental conditions, in particular by the P availability for plants and soil moisture regime. The experimental results regarding the effect of this bacterium applied alone or in conjunction with P on nitrogen contents in different parts of plants under normal soil moisture (70% WHC) and temporary water deficit (35% WHC) are presented in table 1. The inoculation increased this parameter by 5.2% in leaves of plants cultivated under P insufficiency and well-watered conditions compared to uninoculated ones. However, the application of *B. japonicum* in conjunction with P at rate 100 mg/kg soil considerably increased the nitrogen content in leaves by 17.5% compared to reference plants. Likewise, the highest increase of this physiological trait by 25.4% was observed in stems. Evidently, these results demonstrate that soybean displays a positive response to P fertilization. Hence, study's data are in concordance with results of other research (Getachew, 2014). There were not significant changes in nitrogen contents in roots due to rhizobacteria strain application irrespective of P treatments under normal soil moisture level. Regarding nodules, under normal irrigation regime the application of *B. japonicum* increased N content in nodules of plants cultivated in P-deficit soil. The same trend was observed in experiments with soybean by Sa-T and Israel (1995). The application of P at rate 20 mg/kg soil increased nutrient concentration in the symbiotic apparatus by 9.6% compared to unfertilized plants.

Basically, the rhizobacteria inoculation of plants subjected to temporary drought did not have a remarkable impact on nitrogen status of soybean plants. Although, it is necessary to note that there was a slight improvement of nitrogen status in leaves and stems by 10.7% and 12.1% under low soil moisture due to combined use of rhizobacteria and P supplementation (tab. 1). Concerning the nitrogen contents in roots and nodules, there were not registered changes in any studied treatments under water limited conditions. Probably, the water deficit in soil restricted the positive influence of rhizobacteria and the symbiotic system activity was reduced along with decreasing the mineral nitrogen uptake by roots.

CONCLUSIONS

On basis of experimental data we suggested that inoculation with *B. japonicum* could partially compensate the drought effects and improve soybean plant development through enhanced production of proline, probably, other osmolytes too and result in better absorption of water and nutrients from soil. Improved osmolyte proline concentration in roots by integrated use of rhizobacteria *B. japonicum* and P fertilization could have a beneficial impact on root physiological activity by enhancing root system access to more nutrients and water from soil.

Thus, experimental data of this study revealed that rhizobacteria application in conjunction with adequate phosphorus nutrition improved nitrogen content in soybean under not water stress plants, but *B. japonicum* strain did not significantly change nitrogen status in roots and nodules under drought conditions.

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