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Original scientific paper

Isoflavone content and antioxidant activity of soybean inoculated with plant-growth promoting rhizobacteria

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Abstract: Plant-growth promoting rhizobacteria (PGPR) elicit activation of the phenylpropanoid pathway in plants, which leads to phenolics production and enhanced antioxidant capacity. The purpose of this work was to assess the antioxidant activity of soybean plants, *Glycine max* L., inoculated with PGPR (isolates of *Azotobacter chroococcum, Streptomyces* sp. and mixture of these) during plant development, as well as the yield of inoculated soybean plants. PGPR applied in the experiment stimulated flavonoids and isoflavone synthesis, which enhanced the non-enzymatic antioxidant ability of the soybean plants. Moreover, PGPRs stimulated the accumulation of daidzein and genistin in soybean seedlings (5-fold and 2-fold compared to the control values, respectively). The mixture of PGPRs had a positive impact on the antioxidant activity (10–20 % higher activity) and yield components of soybean, which proposed this inoculum as possibly a potent bio-fertilizer in soybean production.

Keywords: glycine max; phenylalanine ammonia-lyase (PAL); plant-growth promoting rhizobacteria (PGPR); phenolics.

INTRODUCTION

Plant growth-promoting bacteria (PGPB) stimulate plant growth, increase yield, reduce pathogen infection, and reduce the impact of abiotic or biotic plant stress.[1] In many cases, plant–microbe associations enhance the defense capacity of the plant and effectively ward off a broad spectrum of pathogens.[2]

Plant growth-promoting rhizobacteria are capable of reducing disease incidence in the aboveground plant parts through a plant-mediated defense mechanism, known as induced systemic resistance (ISR). Rhizobacteria-mediated ISR

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is effective against a broad spectrum of plant pathogens; including oomycetes, fungi, bacteria, viruses, and even insect herbivores.[3] This suggests that by inducing ISR, the beneficial microorganisms confer broad-spectrum resistance in plants.[4]

One of the first reactions of plants to various changes in the environment is the production of reactive oxygen species (ROS), primarily superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), in the tissue that is exposed to the stimuli.[5] The two processes production and neutralization of ROS always occur in cells in a simultaneous manner. Rapid alterations in the ROS levels or change in the balance between production and scavenging rates would result in the generation of a signal and response of the plant to stimuli.[6] Plant–microbe interactions induce a biphasic ROS production in plants, consisting of a low amplitude first phase, followed by a much higher and sustained accumulation during the second phase.[7] In the case of symbiotic interactions, ROS have also been observed, but a suppression of the second wave of ROS seems to occur.[8] The second response or lack of response is thought to play an important signaling role in the activation of plant defense. In order to avoid ROS accumulation leading to cell death, organisms have evolved enzymatic and non-enzymatic antioxidant mechanisms constantly generating and abolishing ROS.[6]

Phenylalanine ammonia-lyase (PAL) is the entry-point enzyme into the phenylpropanoid pathway responsible for the synthesis of plant phenylpropanoids or phenolics, many of which play important roles in plant defense and present the most important non-enzymatic antioxidants.[9] Soybean represents an important source of isoflavones, the antioxidant activity of which is related to the number of hydroxyl groups present in their chemical structure. The antioxidant capacity of isoflavones decreases with glycosylation or replacement of the hydroxyl group by methoxy group. *In vitro*, isoflavones can prevent lipid peroxidation due to their metal chelating and radical scavenging capacity.[10] They are synthesized by isoflavone synthase *via* the phenylpropanoid pathway, but the genetic regulation of isoflavone biosynthesis in plants is not well understood.

Although simultaneous induction of growth and accumulation of secondary metabolites are rare in nature, the use of PGPR to increase the levels of some secondary metabolites becomes very popular in organic production. For this reason, there is great interest in finding effective biotechnological methods to obtain consistent and reproducible induction of these secondary metabolites in soybean plants.[11]

The purpose of this study was to investigate the effect of PGPR, isolates of *Azotobacter chroococcum* and *Streptomyces* sp., as well as mixture of these bacteria, on the non-enzymatic antioxidant system of soybean plants when applied in field conditions, in order to test the potential of these PGPR to induce accumulation of phenolic compounds in soybean plants. Secondary, the aim was to assess possible effect of these PGPR on soybean yield components.

EXPERIMENTAL

Details related to material and experimental design are given in Supplementary material to this paper.

Inoculum characteristics and preparation

The bacterial strains in this experiment were isolates from natural populations from different soil types from northern Serbia: humoglay (pH 7.2), pseudoglay (pH 5), chernozem (pH 7) and wheat straw compost (pH 8.9). Due to different characteristics of these soils, the applied bacteria have some metabolic capabilities that suggest their ability to survive in different environmental conditions. Characteristics of each isolate are presented in Table I.

TABLE I. Characteristics of the PGPR isolates applied in the experiment: morphology, Gram strain, spore formation, origin and most significant alignment

Isolate	Morphology	Gram	Spores	Source – soil	Alignment
RC	Cocci–rods	+	–	Humoglay	*A. chroococcum*
Č	Cocci–rods	+	–	Chernozem	*A. chroococcum*
PS	Cocci–rods	+	–	Pseudoglay	*A. chroococcum*
No. 5	Filamentous	+	–	Chernozem	*Streptomyces* sp.
No. 7	Filamentous	+	–	Humoglay	*Streptomyces* sp.
9K	Filamentous	+	–	Wheat straw compost	*Streptomyces* sp.

Isolates of *A. chroococcum* were isolated by employing serial dilution plate technique using Fjodor nitrogen-free medium (incubation for 48 h at 28 °C). The plates were checked for *A. chroococcum* growth and pigmentation. The isolated colonies of *A. chroococcum* were re-streaked for purification and the pure isolates were maintained on the same medium. Subsequently, the isolated colonies were inoculated in a 250 mL conical flask containing 100 mL Fjodor broth and incubated at 28 °C under shaking at 150 rpm (environmental shaker, Incubator ES 20/60, BioSan) for two days. After the incubation period, the inoculum was adjusted at 10^6 cell mL^{-1} (optical density (*OD*) at 600nm, $A_{0.625} = 10^8$ cell) and was ready for application.

Streptomyces sp. strains were isolated using synthetic agar, by employing the serial dilution plate technique. The media were solidified with 1.5 % agar and pH 7 was adjusted with 1 M NaOH or HCl before autoclaving at 121 °C for 15 min. The plates were incubated for 7–14 days at 28 °C and then checked for sporulation (optical microscope, Olympus, KHC, Japan). Selected *Streptomyces* sp. strains were grown on the same medium. The plates were incubated at 20±2 °C, and sporulation results were recorded after 14 days by visual assessment. The plates were flooded with sterile, distilled water and the resulting spore suspension was harvested. The spore suspension was adjusted to $>1\times10^7$ spores ml^{-1}, determined by spore counting in Neubauer chamber. Inoculum of *A. chroococcum* (AB) consisted of isolates RC, Č and PS (1:1 volume ratio), while inoculum of *Streptomyces* sp. (S) consisted of isolates No. 5, 7 and 9K. Inoculum MIX was prepared from AB and S inoculums (1:1 volume ratio).

Biochemical analyses

The phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity was determined according to Gerasimova *et al.*[9] and expressed in U g^{-1} fresh weight.

Total content of phenolics of an acetone extract of dry leaves and roots was determined by the Folin–Ciocalteu procedure[12] and are expressed as mg catechin g^{-1} dry weight. The amount of flavonoids in a methanolic extract of dry leaves and roots was performed by the

method described by Markham[13] and are expressed as mg rutine g^{-1} dry weight. The contents of proanthocyanidins were determined by the method of Hagerman *et al.*[12] and are expressed as mg leukoanthocyanidins g^{-1} dry weight. The contents of phenolic compounds are given in mg g^{-1}.

Total potential antioxidant activity of the investigated dry material extracts was assessed based on their scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. The change in the optical density of the DPPH radicals was monitored according to Washida *et al.*[14] and given as percent of neutralized radicals.

Analyses of the PAL activity, total phenolics, flavonoids, proanthocyanidins, and DPPH radical scavenging activity were spectrophotometrically performed using a UV/Vis spectrophotometer model 6105, Jenway, Dunmon, UK.

HPLC analysis of soybean leaves (I, II, and III sampling period) and mature seeds (IV sampling period) were performed following the protocol described in Yuan *et al.*[15] An Agilent model 1100 HPLC equipped with binar pump, degaser, autosampler and diode array detector (DAD) was used to separate, identify and quantify isoflavones. Separation of these compounds was achieved using a 5 μm Zorbax SB C18 reversed phase HPLC column (150 mm×4.6 mm) with a Zorbax SB C18 guard column. Mobile phase gradients were formed between two degassed solvents. Solvent A was 1 vol. % acetic acid in water and solvent B 100 % acetonitrile. The gradient conditions were: 0–5 min 15 % B; 5–44 min from 15 to 35 % B; 44–45 min from 35 to 15 % B, 45–50 min 15 % B. A post separation period of 20 min was applied. The column temperature was 25 °C, the solvent flow rate was 0.6 mL min^{-1} and the injection volume was 10 μL. The spectra were collected between 240 and 400 nm by DAD and components in the eluate were detected at 260 nm. Isoflavones were identified by retention times, by comparison of UV spectra with those of standard compounds and from literature data.

Aglycones were quantified from three five-point regression curves ($R \geq 0.9998$) obtained using the corresponding standards (daidzein, glycitein, genistein). The standards of isoflavones, including daidzin and genistin, were purchased from ChromaDex (Irvine, CA, USA), daidzein from Fluka (Buchs, Switzerland), genistein from Serva (Heidelberg, Germany) and glycitein from Aldrich (Steinheim, Germany). Actual concentrations of isoflavones in glycoside forms were calculated from the regression curve of the corresponding aglycones, after applying corrections for differences in molecular weight between aglycones and glycosides and are expressed as mg g^{-1} dry weight.

Yield components

Yield components were recorded in various stages of development: number of nodules per root (II period of sampling), number of pods per plant (III period of sampling), mass of 1000 seeds and seed yield per ha (the end of experiment).

Statistical analyses

All results represent a mean of three experiments performed in three years. Assessed values of the biochemical (performed in 6 replicas) and morphological analyses were expressed as means ± standard error of determinations made in triplicates and tested by ANOVA followed by comparison of the means by the Duncan multiple range test ($P < 0.05$). Relationships between some biochemical parameters were assessed by correlation analyses and are expressed by the Pearson coefficient of correlation (r) and coefficient of determination (r^2). Data were analyzed using Statistica for Windows, version 12.6.

RESULTS AND DISCUSSION

This work was performed to reveal how isolates of *A. chroococcum* and *Streptomyces* sp. affect the antioxidant properties of soybean as a hallmark of plant response to various environmental stimuli. In order to achieve better viability of inoculums under field conditions, three isolates of *A. chroococcum* and *Streptomyces* sp. that are adapted to ecologically different habitats (Table I) were used for the preparation of the inoculums.

In response to abiotic and biotic stresses, such as pathogen attacks, UV irradiation, mechanical wounding, and light, PAL induces phenylpropanoid biosynthesis.[16] PAL has been extensively studied in plants because of its decisive function in the biosynthesis of many secondary metabolites[17] and it is thought to be responsible for many essential functions, including establishing mechanical support, production of pigments, such as anthocyanins, and signaling with flavonoid nodulation factors.[18]

The analyzed soybean plants had similar PAL activity in the leaves throughout the vegetation. Only AB and S inoculums stimulated PAL activity in soybean roots in III period of sampling (Fig. 1).

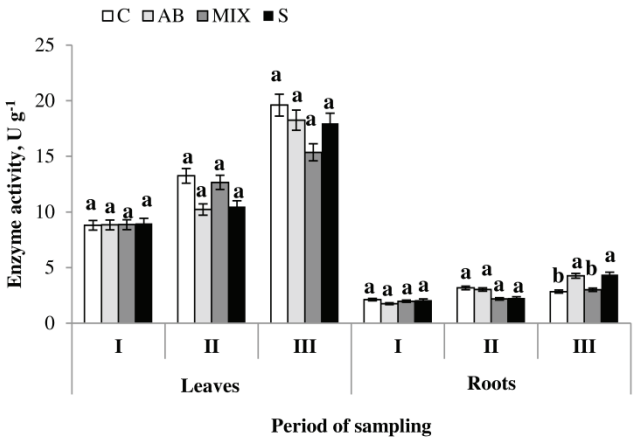

Fig. 1. Phenylalanine ammonia-lyaseactivity in soybean leaves and roots inoculated with PGPR and grown under field conditions. C – control, AB – *A. chroococcum* inoculum, S – *Streptomyces* sp. inoculum, MIX – mixture of AB and S. The results represent mean of 3 years of research, the bars represent standard error. The results marked with different letters differ significantly at $P < 0.05$ (Duncan's test).

Leaves of these plants had significantly higher amounts of total flavonoids in period II (Fig. 2B) and proanthocyanidin contents in period III of the sampling (Fig. 2C). Furthermore, a significant increase in the total phenolic content was recorded in III of the sampling in leaves of soybean plants inoculated with S inoculum period (Fig. 2A) and it was in positive correlation with the PAL activity

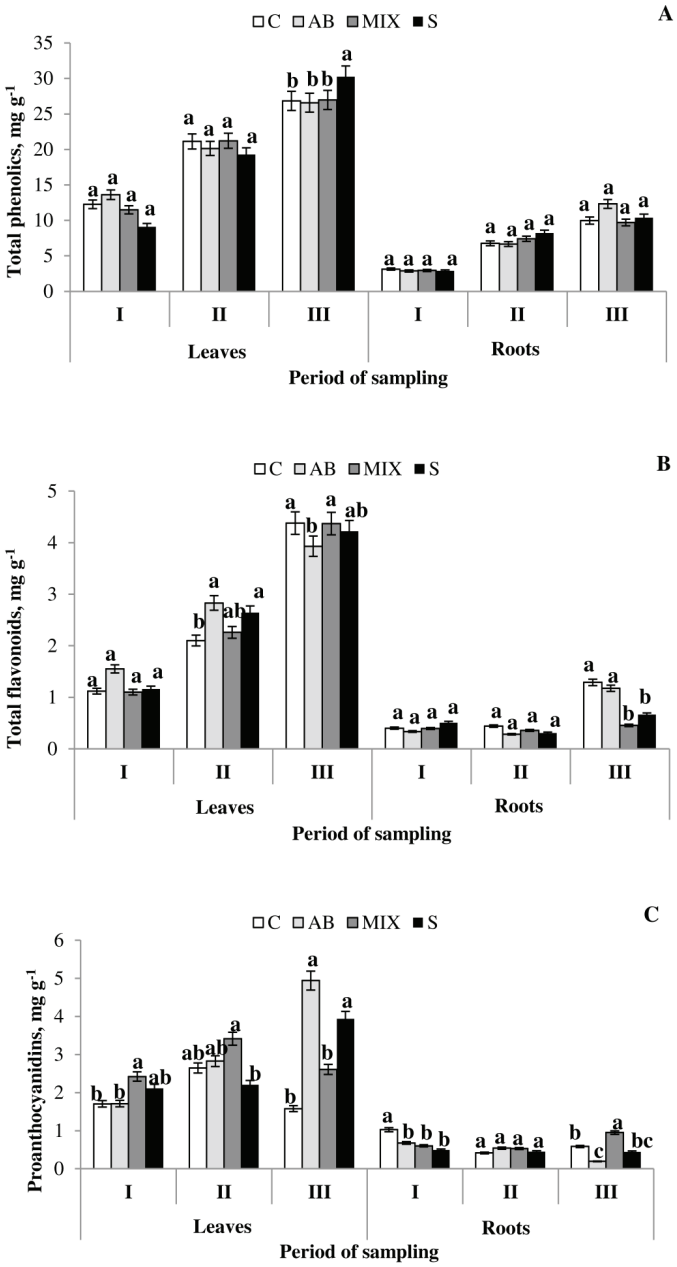

Fig. 2. The contents of total phenolics (A), flavonoids (B) and proanthocyanidins (C) in soybean leaves inoculated with PGPR and grown under field conditions. C – control, AB – *A. chroococcum* inoculum, S – *Streptomyces* sp. inoculum, MIX – mixture of AB and S. The results represent the mean of 3 years of research, bars represent the standard error. Results marked with different letters differ significantly at $P<0.05$ (Duncan's test).

(r = 0.99, r^2 = 0.98). As is shown in Fig. 2, plant from the MIX treatment had similar total flavonoid content as those from the S treatment. At the first two sampling periods, soybean plants inoculated with MIX had significantly higher content of proanthocyanidins in the leaves and the same was assessed in period III in the roots of these plants (Fig. 2C), compared to other treatments and the control. The obtained results showed that the proanthocyanidins content in leaves were mostly stimulated by coupled inoculum in most of the vegetation stages, after which in the seed beginning stage, their content were invariable (Fig. 2C).

Proanthocyanidins are oligomeric and polymeric end products of the flavonoid biosynthetic pathway, their major role in plants is to provide protection against microbial pathogens, insect pests and larger herbivores.[19] However, their content in roots of inoculated plants was not stimulated by applied growth-promoting rhizobacteria (Fig. 2C).

The DPPH-scavenging test refers to the non-enzymatic antioxidant ability of plant extracts. The DPPH-test revealed that leaf extracts of plants inoculated with S inoculum had a greater ability to scavenge the DPPH-radical in sampling periods I and III, as did the MIX inoculum (Table II). The results of the DPPH-test were in positive correlation with the total phenolics content in soybean leaves treated with PGPR (r = 0.91–0.99, r^2 = 0.93–0.97).

TABLE II. DPPH-radical scavenging activity of soybean leaves and roots inoculated with PGPR and grown under field conditions. C – control, AB – *A. chroococcum* inoculum, S – *Streptomyces* sp. inoculum, MIX – mixture of AB and S. The results marked with different letters differ significantly at $P < 0.05$ (Duncan's test)

Sample	Sampling period	Scavenging activity, %			
		C	AB	MIX	S
Leaves	I	51.3 ± 0.9^{b}	54.5 ± 0.5^{ab}	58.3 ± 0.5^{a}	45.8 ± 0.1^{c}
	II	57.4 ± 0.8^{d}	65.4 ± 0.6^{b}	60.4 ± 0.7^{c}	71.5 ± 0.4^{a}
	III	52.9 ± 0.8^{ab}	55.1 ± 0.3^{a}	40.8 ± 0.1^{d}	49.0 ± 0.3^{c}
Roots	I	63.0 ± 1.1^{c}	67.1 ± 1.1^{b}	72.1 ± 0.6^{a}	75.8 ± 0.7^{a}
	II	61.4 ± 1.0^{b}	34.6 ± 0.1^{c}	61.1 ± 0.9^{b}	63.7 ± 0.4^{a}
	III	64.4 ± 1.2^{b}	68.2 ± 0.7^{a}	57.6 ± 0.4^{c}	64.3 ± 0.4^{b}

The DPPH-scavenging activity of soybean roots inoculated with the S and MIX inoculums significantly depended on the contents of total flavonoids (r = 0.98–0.99, r^2 = 0.96–0.99) and proanthocyanidin (r = 0.89–0.98, r^2 = 0.70––0.98). Roots of soybean plants inoculated with AB had significantly lower DPPH-scavenging activity (sampling period II) in comparison to the control and other inoculums (Table II).

Isoflavones seem to have a variety of roles, such as precursors to defense compounds (phytoalexins), which inhibit the growth of various microbes, and as endogenous regulators of auxin transport in roots. Synthesis of isoflavones could be induced by wounding[20] and by *Bradyrhizobium japonicum*.[21] Soy and soy

products may contain three types of isoflavones in four chemical forms: 1) the aglycones daidzein, genistein and glycitein; 2) the *β*-glycosides daidzin, genistin, and glycitin; 3) the acetyl-*β*-glycosides 6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin and 6″-*O*-acetylglycitin; and 4) the malonylglycosides 6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin and 6″-*O*-malonylglycitin.[20] According to Algar *et al.*,[22] accumulation of malonyldaidzin represents the most effective way to store defense compounds for immediate use upon stress challenge, which could point to possible stress-mitigating effect of PGPR during the first two phases of development of the investigated soybean plants, as well as in the seed. According to the obtained results, the control plants had higher amount of isoflavone malonyl-*β*-glycosides in the second trifoliate stage of development than the inoculated plants.

The levels of the soy isoflavones are affected by genetic and environmental factors and by the mode of cultivation.[11] There are only few other authors[11,22–24] that performed similar experiments and they also assessed the difference in isoflavone accumulation and composition after PGPR inoculation in soybean cell cultures, seedlings and seeds, however, there was no data about the isoflavone content and antioxidant properties of PGPR-inoculated soybean throughout the ontogenesis of soybean plants. According to different authors, application of PGPR stimulates the innate antioxidant capacity of plants exposed to stress conditions (drought and salinity).[25,26]

Plants from the AB treatment had high total isoflavone contents in the leaves through all three sampling periods (Fig. 3D). At the full bloom period, the control plants had the highest and at the seed beginning stage, the lowest total isoflavone content in comparison to the treatments.

In the early PGPR–plant interaction (the second trifoliate), the treated plants had higher daidzein contents in comparison to that in the control plants (Fig. 3A). Plants inoculated with S inoculums had the highest daidzein contents in the second trifoliate stage and in seeds (Fig. 3A). Glycitin and malonylglycitein contents accumulated throughout vegetation yet, their content in seeds did not differ among the treatments (Fig. 3B). As for the genistein group of isoflavones, plants treated with S and MIX inoculums had higher genistin contents in leaves compared to AB and control in the I and III sampling periods (Fig. 3C).The mass of 1000 seeds were the highest in S treatment (150.5 g) and the highest amount of seeds per ha was in the MIX treatment 5.0 t ha^{-1}, while others had significantly lower seed yield (4.0–4.1 t ha^{-1}) (Supplementary material, Table S-I). According to Aung *et al.*[27], co-inoculation with *B. japonicum* and *Azospirillum* sp. also stimulated yield components in soybean.

Comparison of the results from isoflavone and yield analyses clearly shows that in the full bloom period plants from the S treatment had the highest number of nodules per plant, but the lowest total isoflavone content, which indicates that

the higher isoflavone content is not provoked by nodulation. The total isoflavone content was the highest in seed beginning stage due to the intensive process of seed formation. If the treatments are compared, plants from the MIX treatment had the lowest isoflavone content in seeds, but the highest in leaves in the seed formation stage. In addition, these plants had the highest number of pods per plant and yield, which possibly explains that an intensive synthesis of these phenolics was demanded by filling a larger number of pods (Table S-I of the Supplementary material).

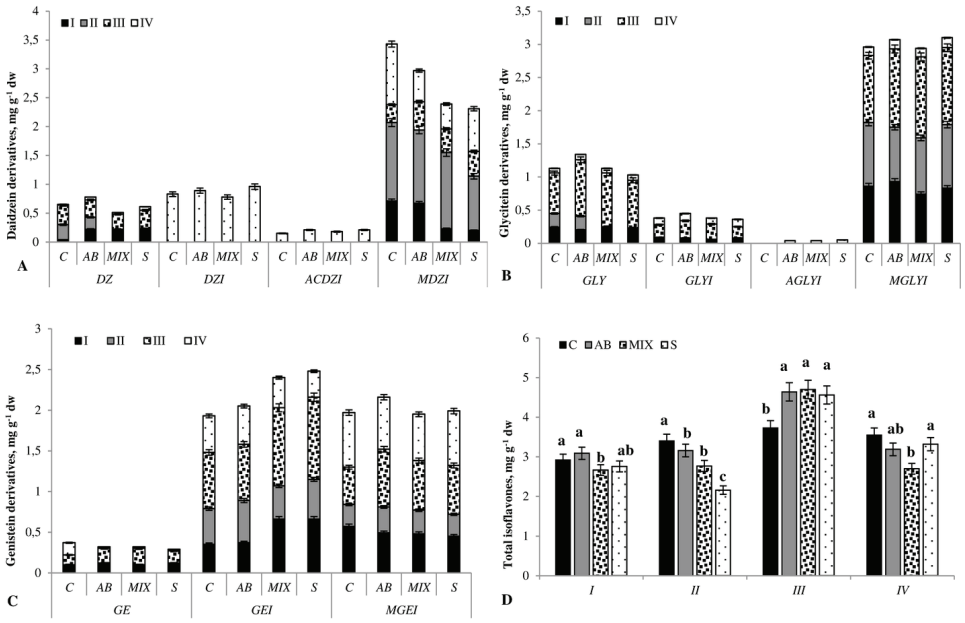

Fig. 3. A: Daidzein (DZ – daidzein, DZI – daidzin, ACDZI – acetyldaidzin, MDZI – malonyldaidzin). B: Glycitein (GLY – glycitein, GLYI – glycitin, AGLY – acetylglycitin, MGLY – malonylglycitin). C: Genistein (GE – genistein, GEI – genistin, MGEI – malonylgenistin). D: Total isoflavones (TIF) contents in soybean leaves (I–III) and seeds (IV) inoculated with PGPR and grown under field conditions. C - control, AB - *A. chroococcum* inoculum, S – *Streptomyces* sp. inoculum, MIX – mixture of AB and S. Results represent mean of 3 years of research, bars represent standard error. Results marked with different letters differ significantly at $P < 0.05$ (Duncan's test).

CONCLUSION

According to obtained results, it could be concluded that *Azotobacter chroococcum* enhanced accumulation of isoflavones, as well as total flavonoid content in leaves of soybean plants throughout all investigated stages of development. At the seed beginning stage, *Streptomyces* sp. stimulated accumulation of total phenolics, flavonoids, proanthocyanidins and isoflavones, which could be the reason

of enhanced antioxidant ability of extracts of these plants. Furthermore, the mixture of applied PGPR induced synthesis of proanthocyanidins in the leaves at the second trifoliate and full bloom stages. All PGPR stimulated isoflavone accumulation in soybean leaves at the seed beginning stage; however, only seeds from single inoculation treatment had high isoflavone contents. Besides the positive impact on accumulation of non-enzymatic antioxidants, the mixture of *A. chroococcum* and *Streptomyces* sp. had a positive impact on the yield of soybean plants, which indicates the possibility of applying a mixture of these PGPRs as a potent bio-fertilizer in soybean production.

SUPPLEMENTARY MATERIAL

Additional data and details related to sampled material and experimental design are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

САДРЖАЈ ИЗОФЛАВОНА И АНТИОКСИДАНТНА АКТИВНОСТ СОЈЕ ТРЕТИРАНЕ СА РИЗОБАКТЕРИЈАМА КОЈЕ ПОСПЕШУЈУ РАСТ

БИЉАНА КИПРОВСКИ[1] ЂОРЂЕ МАЛЕНЧИЋ[2], СИМОНИДА ЂУРИЋ[2], МИРА БУРСАЋ[3], ЈЕЛЕНА ЦВЕЈИЋ[3] и ВЛАДИМИР СИКОРА[1]

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Микроорганизми који поспешују раст биљака (PGPR) стимулишу и синтезу фенолних једињења и побољшавају антиоксидантни капацитет биљака. У овом раду испитан је утицај инокулације изолатима *Azotobacter chroococcum*, *Actinomycetes* (*Streptomyces* sp.) и смешом изолата наведених микроорганизама на антиоксидантни систем биљака соје током развоја биљака, као и на принос инокулисаних биљака. Корисни микро организми су стимулисали синтезу флавоноида и изофлавона, који су даље утицали на повећање антиоксидантног капацитета биљака соје. Садржај даиздеина и генистина у клијанцима соје инокулисане корисним микроорганизмима био је 2–5 пута већи у поређењу са контролом. Смеша изолата корисних микроорганизама имала је повољан утицај на антиоксидантну активност биљака соје (10–20 % већа активност), као и принос семена, због чега би се дати инокулум могао препоручити као потенцијални био-фертилизатор.

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