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Integrated use of Consortia-based Microbial Inoculants and Nutrient Complex Stimulates the Rhizosphere Microbiome and Soybean Productivity

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ABSTRACT

Background: The effectiveness of soybean inoculants in field conditions may be improved through the joint application of rhizobia and plant growth-promoting bacteria (PGPB). Plant nutrients may also contribute to the higher efficacy of microbial inoculants as well as the growth and development of plants.

Methods: A field experiment was performed to evaluate the responses of the rhizomicrobiome and soybean to seed treatments with multiple bacterial strains (*Bradyrhizobium japonicum*, *Bacillus subtilis*, *Bacillus megaterium* and *Azotobacter chroococcum*), applied individually or in consortia, with and without nutrient complex (S, Mg, Mn, Fe, Zn, Cu, B and Mo).

Result: Seed treatments with microbial consortia of *Br. japonicum*, *B. subtilis*/*B. megaterium*, *A. chroococcum* strains and nutrients had the highest effect on the abundance of total bacteria, nitrogen-fixing bacteria, actinomycetes and activity of dehidrogenase in the soybean rhizosphere. The highest effect on plant height, plant weight, pod number, pod weight, seed number and seed weight was obtained from treatment with *Br. japonicum* strains with nutrients, followed by co-inoculation with *B. megaterium* and *A. chroococcum*. In comparison with the control and *Bradyrhizobium* single inoculation, a statistically significant increase in the seed yield was recorded in treatment with *Br. japonicum*, *B. megaterium*, *A. chroococcum* and nutrients, reflecting the highest increase in protein and oil yield. The interactive effects of microbial consortia and nutrients could be used as promising seed technology for sustainable soybean cropping.

Key words: *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Glycine max*, Micronutrients, Rhizomicrobiome, Seed yield.

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is among the most important cultivated crops worldwide due to its high agro-economic value and diverse utility in the feed and food, pharmaceutical and other industries. Global consumption of chemical fertilizers and pesticides for agricultural productivity to meet the global demand of the increasing world population continues to rise, despite their harmful impacts on the environment and human health (Devi *et al.*, 2022). In order to reduce the use of agrochemicals, increase organic farming and achieve sustainable soybean production under environmental, climatic and economic challenges, researchers have investigated different alternatives, including the use of microbial inoculants (O'Callaghan *et al.*, 2022).

Seed inoculation with nitrogen-fixing *Bradyrhizobium* strains improves nodulation, plant properties, seed yield and yield quality while satisfying soybean nitrogen demands (Nakei *et al.*, 2022). Besides rhizobia, *Bacillus* and *Azotobacter* strains are the most commercially utilized plant growth-promoting bacteria (PGPB) in the production of field and vegetable crops (Gómez-Godínez *et al.*, 2023). Their use as biofertilizers is based on nitrogen fixation, solubilization of nutrients, production of siderophores and synthesis of plant hormones, which have significant effects on soil and crop productivity (Aloo *et al.*, 2022; Virk *et al.*, 2022). Additionally, *Bacillus* strains can be used as biopesticides and improve the health of plant-soil systems *via* the production of various antimicrobial compounds involved in the biocontrol of plant pathogens (Miljaković *et al.*, 2020; Kannan *et al.*, 2021).

The effectiveness of microbial inoculants depends upon their ability to survive and multiply in soils, colonize plant roots and perform their beneficial actions in the complex plant-soil system (Liu *et al.*, 2022). However, various abiotic and biotic factors, as well as improper management practices, can obstruct the inoculation outcome in the field

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(Kong *et al.*, 2018). Therefore, it is necessary to develop microbial formulations that will improve the actions of each component and comprehend the issues affecting their application (Herrmann and Lesueur, 2013). In this regard, one of the most promising approaches is the use of consortia-based microbial inoculants (Khan, 2022). Furthermore, the application of nutrient complexes through soil, foliar and seed treatments provides the essential elements for growth, development and metabolism of both microorganisms and plants, ensuring their maximum uptake and a minimum loss to the environment (Farooq *et al.*, 2012). A deficiency of macro- and micronutrients in the soil combined with the inappropriate application of fertilizers may result in micronutrient malnutrition (Arabhanvi *et al.*, 2015). Nevertheless, knowledge on the integrated use of microbial inoculants and nutrients for sustainable agricultural activities and the effects of such formulations is still poorly documented. In particular, the responses of the rhizosphere microbiome and soybean plant to consortia of rhizobia and other PGPB combined with nutrients have not yet been well explored. Understanding such interactions should contribute to improving the effectiveness of inoculants in field conditions through the joint application of microbial consortia and plant nutrients. Therefore, we examined the effect of a microbial consortia consisting of nitrogen-fixing *Bradyrhizobium japonicum* strains in different combinations with plant growth-promoting strains of *Azotobacter chroococcum*, *Bacillus subtilis* and *Bacillus megaterium*) and a nutrient complex (S, Mg, Mn, Fe, Zn, Cu, B and Mo) on the abundance and activity of microorganisms in the rhizosphere, soybean growth and development, as well as the quantity and quality of seed yield under field conditions.

MATERIALS AND METHODS

The experiment was conducted at the Rimski šanèevi experimental field (45°19′N, 19°50′E), Institute of Field and Vegetable Crops (IFVC, Novi Sad, Serbia), during the 2021 growing season. The soil was classified as Haplic Chernozem (FAO, 2015). It belonged to a group of humus soils with a slightly alkaline pH reaction, a low content of available phosphorus and a high content of available potassium (Table 1). The mean temperature and precipitation sum at the experimental field were 19.2°C and 319.1 mm, respectively.

Bacterial strains from the culture collection of the Section for Microbiological Preparations (IFVC) were used as inoculants. They were cultured in their respective liquid media: yeast extract manitol medium for *Bradyrhizobium* strains, nutrient medium for *Bacillus* strains and Burk's nitrogen-free medium for *Azotobacter* strains (Hi Media Laboratories Pvt. Limited, Mumbai, India). The chemical composition of the nutrient complex was as follows (% m/m): S- 5.2; Mg- 3; Mn- 1.5; Fe- 1; Zn- 1; Cu- 0.5; B- 0.3; Mo- 0.01.

Seeds of the soybean cultivar Apolo (maturity group I) were obtained from the Legume Department (IFVC). The following seed treatments were tested: 1. control (without bacterial mixture and nutrient compex); 2. *Bradyrhizobium japonicum* (BJ1, BJ2, BJ4, BJ6, BJ7, BJ8); 3. *Bacillus subtilis* (B5, B7, B13, B32); 4. *Bacillus megaterium* (B8, B12, B15, B17); 5. *Br. japonicum* + *B. subtilis*; 6. *Br. japonicum* + *B. megaterium*; 7. *B. subtilis* + *Azotobacter chroococcum*; 8. *B. megaterium* + *A. chroococcum*; 9. *Br. japonicum* + *B. subtilis* + *A. chroococcum*; 10. *Br. japonicum* + *B. megaterium* + *A. chroococcum*; 11. *Br. japonicum* + nutrient complex; 12. *Br. japonicum* + *B. subtilis* + nutrient complex; 13. *Br. japonicum* + *B. megaterium* + nutrient complex; 14. *Br. japonicum* + *B. subtilis* + *A. chroococcum* + nutrient complex; 15. *Br. japonicum* + *B. megaterium* + *A. chroococcum* + nutrient complex. Seeds were treated just before sowing by applying a liquid bacterial inoculum (10^9 CFU/ml) and nutrient complex to the seeds using sterilized peat as a carrier. A total of 40 ml of mixture (bacterial inoculum with or without nutrient complex) on peat was applied per treatment, while the volume of individual components in each mixture was equal. Agrochemicals were not applied. The experiment was carried out in three replications in a split-plot design and the plot size was 5 × 3 m.

The effect of seed treatments on microbial abundance and dehydrogenase activity in soybean rhizosphere was determined at full flowering (R2) and full maturity (R8), using the indirect dilution plate method (Trolldenier, 1996) on appropriate nutrient media (Hi Media Laboratories Pvt. Limited, Mumbai, India). Microbial abundance included: total bacteria (10^{-7}; soil agar), free-living N_2-fixers (10^{-6}; nitrogen-free medium), actinomycetes (10^{-4}; synthetic agar) and fungi (10^{-4}; Czapek-Dox agar). Dehydrogenase activity (DHA) was determined spectrophotometrically according to the method with triphenyltetrazolium chloride (TTC) (EN ISO 23753-1:2019). All microbial analyses were performed in three replicates and the values from two samplings were averaged.

The effect of seed treatments on plant height, plant weight, pod number, pod weight, seed number, seed weight and seed yield was determined at full maturity (R8) of soybean. Ten plants were randomly collected from each plot for biometric measurements and yield structure components and the average value for all samplings per plant was calculated. The seed yield from the plots per 1 ha was calculated (based on the 14% moisture content). The content of protein and oil in seeds was determined with the near-infrared spectroscopy (NIRS) method using an Antaris II FT NIR device (Thermo Fisher Scientific, Waltham, MA, USA),

Table 1: Soil properties (0-30 cm).

Soil pH (1 mol/L KCl)	Humus (%)	Total N (%)	AL-P_2O_5 (mg/100 g)	Al-K_2O (mg/100 g)
7.36	3.23	0.221	6.8	25.5

while protein and oil yields were calculated from seed yield and the percentage of a given component in seeds.

The data were statistically processed (StatSoft Inc., Tulsa, USA) using the analysis of variance (ANOVA) statistical method, followed by mean separation according to the Fisher's LSD test (P<0.05).

RESULTS AND DISCUSSION

Soil microbial communities have a crucial role in nutrient cycling, organic matter decomposition and many other processes that are necessary for the growth and development of plants and ecosystem functioning (Bender *et al.*, 2016). The results of this study indicate a significant influence of seed treatments on the total bacteria, free nitrogen-fixing bacteria, actinomycetes and dehydrogenase activity (Table 2). Applied treatments mostly had a positive effect on the microbial parameters and the increase compared to control ranged as follows: total bacteria (6-111%), N_2-fixers (15-181%), fungi (7-117%), actinomycetes (15-186%) and dehydrogenase (2-110%). The greatest proliferation of total bacteria, N_2-fixers, actinomycetes and dehydrogenase was obtained by applying consortia-based inoculants with nutrient complex, while the highest abundance of fungi was recorded after *Br. japonicum* + nutrients treatment. Numerous reports suggest that microbial inoculants can influence indigenous microbial communities, thus promoting free-living nitrogen-fixing bacteria as well as other beneficial bacteria or fungi (Trabelsi and Mhamdi, 2013). Furthermore, Bana *et al.* (2022) obtained significant improvements in microbial enzyme activity with micronutrient-supplemented fertilizer (N, P, K, Fe, Zn and B). Similarly, Egamberdieva *et al.* (2018) indicated the positive effect of nutrients (N, P and Mg) on rhizosphere colonization, bacterial proliferation and symbiotic performance of rhizobia inoculated soybean.

The use of microbial inoculants, especially with nutrients, increases microbial abundance and activity, suggesting their potential to enhance soil fertility. Microbial inoculants have the ability to improve nutrient availability and nutrient uptake, promote the health of soil and crops and contribute to high yields in sustainable ways. Beneficial microorganisms improve the biological, chemical and physical properties of soil and, subsequently, the productivity of agricultural crops. In this study, soybean parameters, namely plant height and weight, pod number and weight, seed number and seed weight, as well as protein yield, were significantly influenced by seed treatments (Table 3 and 4).

A higher effectiveness versus control was observed for most applied treatments, which resulted in the following improvements: plant height (1-23%), plant weight (1-51%), pod number (1-96%), pod weight (2-100%), seed number (5-79%), seed weight (7-102%), seed yield (6-25%), protein content (1-4%), protein yield (0.3-29%), oil content (0.4-3%) and oil yield (2-26%). The highest improvements in plant weight, pod number and weight, seed number and weight in relation to the control were observed with *Br. japonicum* + nutrients and *B. megaterium* + *A. chroococcum*. Seed treatments with inoculants and nutrient complex had a primacy over single and combined inoculants in the case of soybean seed yield, as well as protein and oil yield, while *Br. japonicum* + *B. megaterium* + *A. chroococcum* + nutrients had the best effect. The individual application of *Br. japonicum* strains, as well as their combination with *B. subtilis*, *A. chroococcum* and nutrients, had the equally best

Table 2: Effect of seed treatments on rhizosphere microbiome.

Seed treatment	Total bacteria ($\times 10^7$ CFU g^{-1})	Free N_2 fixers ($\times 10^6$ CFU g^{-1})	Fungi ($\times 10^4$ CFU g^{-1})	Actinomycetes ($\times 10^4$ CFU g^{-1})	Dehydrogenase (mU g^{-1})
Control	220.15^{f}	111.37^{g}	20.07^{b}	15.02^{cd}	5.67^{cde}
BJ	383.47^{abc}	176.82^{d-g}	22.25^{b}	23.00^{bcd}	6.31^{b-e}
BS	374.17^{abc}	223.67^{b-e}	18.42^{b}	19.43^{bcd}	8.99^{abc}
BM	356.32^{abc}	247.23^{a-d}	19.73^{b}	17.22^{cd}	8.31^{a-d}
BJ + BS	367.78^{abc}	185.57^{d-g}	18.95^{b}	14.23^{d}	7.18^{b-e}
BJ + BM	355.07^{abc}	186.18^{d-g}	13.35^{b}	20.85^{bcd}	4.91^{de}
BS + AC	286.10^{c-f}	247.42^{a-d}	23.92^{ab}	31.10^{abc}	7.87^{bcd}
BM + AC	242.20^{def}	225.17^{b-e}	32.38^{ab}	29.02^{a-d}	7.12^{b-e}
BJ + BS + AC	233.43^{ef}	128.60^{fg}	21.57^{b}	17.68^{bcd}	4.21^{e}
BJ + BM + AC	354.77^{a-d}	158.78^{efg}	27.77^{ab}	12.65^{d}	5.76^{cde}
BJ + Ns	334.00^{b-e}	196.70^{c-f}	43.48^{a}	25.07^{bcd}	9.88^{ab}
BJ + BS + Ns	402.82^{ab}	217.07^{b-e}	22.50^{b}	28.50^{a-d}	8.84^{abc}
BJ + BM + Ns	356.65^{abc}	273.92^{abc}	26.75^{ab}	34.22^{ab}	7.29^{b-e}
BJ + BS + AC + Ns	407.10^{ab}	313.45^{a}	30.12^{ab}	43.02^{a}	9.51^{ab}
BJ + BM + AC + Ns	464.77^{a}	290.50^{ab}	24.15^{ab}	29.13^{a-d}	11.88^{a}
P-value	0.0042	0.0006	0.4611	0.0408	0.0113

Means with different lowercase letters in the same column are significantly different (P<0.05, Fisher's LSD test); CFU- Colony forming unit; BJ- *Bradyrhizobium japonicum*; BS- *Bacillus subtilis*; BM- *Bacillus megaterium*; AC- *Azotobacter chroococcum*; Ns- Nutrients.

effect on the protein content. The highest and significant increase, compared to the control, in the oil content was obtained from inoculation with *B. megaterium*. Similarly, Moretti *et al.* (2020) described that the inoculation with bacterial consortia (*Br. japonicum*, *Br. diazoefficiens*, *B. subtilis* and *Azospirillum brasilense*) increased grain yield and quality of soybean under field conditions when compared to the single inoculation with *Bradyrhizobium*. In a meta-analysis of studies from 1987 to 2018, Zeffa *et al.* (2020) reported that co-inoculation of soybean with *Bradyrhizobium* and PGPB resulted in a significant increase in nodule number and biomass, root and shoot biomass, whereas no significant increase was observed in shoot nitrogen content and grain yield. The significant effects of nutrients on soybean yield and its components were also proven (Kobraee and Shamsi, 2013). Among their broad structural and functional roles, nutrients may affect nitrogen fixation in both legumes and non-legumes at various stages,

Table 3: Effect of seed treatments on soybean productivity.

Seed treatment	Plant height (cm plant^{-1})	Plant weight (g plant^{-1})	Pod number (no plant^{-1})	Pod weight (g plant^{-1})	Seed number (no plant^{-1})	Seed weight (g plant^{-1})	Seed yield (t ha^{-1})
Control	65.00^{d}	20.23^{de}	26.67^{def}	10.57^{e}	54.67^{ef}	7.03^{f}	3.75^{cd}
BJ	71.67^{a-d}	21.53^{b-e}	31.33^{cde}	13.63^{cde}	65.33^{cde}	9.30^{c-f}	3.99^{bcd}
BS	78.67^{a}	24.77^{bcd}	36.67^{bc}	15.67^{cd}	70.67^{cde}	10.10^{cde}	4.24^{a-d}
BM	66.33^{cd}	17.93^{e}	22.33^{f}	10.80^{e}	45.33^{f}	7.50^{ef}	4.09^{bcd}
BJ + BS	68.00^{bcd}	20.83^{cde}	25.67^{def}	12.87^{de}	57.67^{def}	8.83^{def}	4.19^{a-d}
BJ + BM	72.33^{a-d}	26.10^{abc}	36.00^{bc}	17.07^{bc}	78.33^{bc}	12.03^{abc}	4.08^{bcd}
BS + AC	77.67^{ab}	26.87^{ab}	31.67^{cde}	16.40^{cd}	69.33^{cde}	11.23^{bcd}	4.07^{bcd}
BM + AC	72.00^{a-d}	30.40^{a}	40.67^{b}	20.37^{ab}	95.33^{ab}	14.23^{a}	4.29^{abc}
BJ + BS + AC	72.00^{a-d}	23.87^{bcd}	32.67^{b-e}	14.23^{cde}	65.00^{cde}	9.40^{c-f}	3.67^{d}
BJ + BM + AC	65.67^{cd}	22.53^{b-e}	34.33^{bcd}	13.80^{cde}	75.33^{cd}	9.63^{c-f}	3.73^{cd}
BJ + Ns	75.33^{abc}	30.63^{a}	52.33^{a}	21.10^{a}	97.67^{a}	13.73^{ab}	4.34^{ab}
BJ + BS + Ns	67.33^{cd}	20.43^{de}	25.33^{ef}	13.47^{cde}	53.00^{ef}	9.17^{c-f}	4.43^{ab}
BJ + BM + Ns	66.67^{cd}	23.97^{bcd}	31.33^{cde}	16.07^{cd}	70.33^{cde}	11.61^{a-d}	4.36^{ab}
BJ + BS + AC + Ns	79.67^{a}	24.10^{bcd}	36.67^{bc}	15.53^{cd}	76.67^{c}	10.53^{cd}	4.13^{a-d}
BJ + BM + AC + Ns	73.33^{a-d}	22.13^{b-e}	27.00^{def}	14.83^{cd}	63.00^{c-f}	10.83^{bcd}	4.70^{a}
P-value	0.0444	0.0014	0.0000	0.0002	0.0000	0.0008	0.066

Means with different lowercase letters in the same column are significantly different ($P<0.05$, Fisher's LSD test);
BJ- *Bradyrhizobium japonicum*; BS- *Bacillus subtilis*; BM- *Bacillus megaterium*; AC- *Azotobacter chroococcum*; Ns- Nutrients.

Table 4: Effect of seed treatments on chemical seed composition and protein and oil yield.

Seed treatment	Protein content (% DM)	Protein yield (kg ha^{-1})	Oil content (% DM)	Oil yield (kg ha^{-1})
Control	41.65^{c}	1564^{d}	20.36^{ab}	766^{b}
BJ	43.41^{a}	1733^{bcd}	20.85^{ab}	833^{ab}
BS	42.63^{abc}	1805^{ab}	20.68^{ab}	877^{ab}
BM	42.34^{abc}	1732^{bcd}	21.04^{a}	862^{ab}
BJ + BS	42.25^{abc}	1771^{bcd}	20.84^{ab}	873^{ab}
BJ + BM	42.09^{bc}	1714^{bcd}	20.83^{ab}	850^{ab}
BS + AC	42.61^{abc}	1728^{bcd}	20.84^{ab}	851^{ab}
BM + AC	42.44^{abc}	1818^{ab}	20.66^{ab}	885^{ab}
BJ + BS + AC	42.22^{abc}	1551^{d}	20.95^{ab}	769^{b}
BJ + BM + AC	42.07^{bc}	1569^{cd}	20.95^{ab}	781^{b}
BJ + Ns	42.50^{abc}	1841^{ab}	20.46^{ab}	892^{ab}
BJ + BS + Ns	43.34^{a}	1921^{ab}	20.24^{b}	898^{ab}
BJ + BM + Ns	42.90^{ab}	1869^{ab}	20.51^{ab}	893^{ab}
BJ + BS + AC + Ns	43.33^{a}	1790^{abc}	20.35^{ab}	840^{ab}
BJ + BM + AC + Ns	42.82^{abc}	2014^{a}	20.45^{ab}	962^{a}
P-value	0.1550	0.0085	0.5506	0.3574

Means with different lowercase letters in the same column are significantly different ($P<0.05$, Fisher's LSD test); DM- Dry matter;
BJ- *Bradyrhizobium japonicum*; BS- *Bacillus subtilis*; BM- *Bacillus megaterium*; AC- *Azotobacter chroococcum*; Ns- Nutrients.

including infection, nodule formation and function and plant growth (Weisany *et al.*, 2013). Co-inoculation of soybean with *Bradyrhizobium japonicum* and *Azospirillum brasilense* in combination with foliar application of Co and Mo increased pod number, grain number per pod, weight of 100 grains and grain yield (Barbosa *et al.*, 2023). Moreover, Jarecki *et al.* (2016) found that combined application of *Bradyrhizobium* and a complex of nutrients (Mg, S, B, Cu, Mn, Mo and Zn) increased plant height, pod number and thousand seed weight, while soybean seed yield was significantly higher both in individual and combined treatments as compared to the control. The current study clearly demonstrated that nutrients and their interactions have an important effect on the performance of the inoculation strains on soybean plants. It has been reported that the uptake of main nutrients by plants depends on their available forms and their interactions with other nutrients (Leidi and Rodríguez-Navarro, 2000). Similar findings were reported by Chen *et al.* (2017), who observed that a higher supply of micronutrients significantly increased biomass and uptake of macronutrients in soybean plants through the improved interactions of rhizobia and arbuscular mycorrhizal fungi (AMF).

CONCLUSION

The use of consortia-based microbial strains in combination with a nutrient complex could help avoid rhizosphere competence, improve the survival and performance of microbial inoculants, enhance nutrient uptake, increase plant growth and yield and mitigate plant responses to abiotic and biotic stresses. The combination of these two approaches could be integrated to achieve maximum effectiveness and significantly improve crop yields, especially in organic production. Further studies are needed to confirm the efficacy of such formulations in different environmental conditions and for different cultivars.

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