

Article

Perspectives of *Bradyrhizobium* and *Bacillus* Inoculation for Improvement of Soybean Tolerance to Water Deficit

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Abstract: The objective of this study was to analyze the response of antioxidant parameters in soybean plants inoculated with newly isolated *Bradyrhizobium japonicum* and *Bacillus subtilis* strains as single and co-inoculants under drought stress. Bacterial strains were selected according to osmotic stress tolerance (in the presence of 36% PEG 6000) in appropriate liquid media. The effect of soybean inoculation was examined in a soil pot experiment in water deficit conditions (0 and 7 days withholding water). The influence of water stress and inoculation was evaluated in soybean leaves, roots, and nodules through guaiacol peroxidase (POX), ionically cell-wall-bound peroxidase (POD) activity, and ABTS⁺ radical cation scavenging capacity, as well as parameters of N-fixation efficiency. The results showed a significant influence of inoculation on constitutive and drought-induced antioxidant and N-fixation parameters. Inoculation increased the activity of POX (up to 116, 169, and 245%), POD (up to 116, 102, and 159%), and antioxidant capacity (up to 74, 76, and 81%) in soybean leaves, roots, and nodules under water deficit, respectively. Application of bacterial strains resulted in higher shoot, root, and nodule weight and nitrogen content both in non-stressed and drought stress conditions. Overall, co-inoculation had better effects on the investigated soybean parameters compared to single inoculation. Selection and application of bacterial strains with improved tolerance to drought stress is necessary in developing inoculants that would result in enhanced crop production under unfavorable environmental conditions.

Keywords: antioxidative response; *Bacillus*; *Bradyrhizobium*; drought stress; N-fixation efficiency; soybean



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1. Introduction

The soybean (*Glycine max* (L.) Merrill) is among the most important legumes worldwide, mainly being cultivated for its high-quality protein and oil content (40% and 20%) as well as other valuable compounds such as fiber, vitamins, minerals, and antioxidants [1]. It represents nearly 60% of the world's oilseed production and contributes approximately 70% to the consumption of total protein meal [2]. Due to its versatile usage for food and feed, medical, and industrial products, soybean production is constantly increasing, and it currently occupies over 133 million hectares worldwide, with over 348 million tons produced in 2022 [3]. Furthermore, the soybean develops symbiotic relationships with its specific nitrogen-fixing rhizobia and fixes up to 300 kg N ha^{−1} annually [4]. Thus, it acquires the majority of N for its growth and yield and enriches the soil with this essential macronutrient, ensuring low requirements for mineral fertilization and benefits for the yield of successive crops in rotation [5]. The most important symbionts of the soybean are species of *Bradyrhizobium* (*Br. japonicum*, *Br. diazoefficiens*, *Br. elkanii*, and *Br. yuanmingense*), whose effective strains are commonly introduced in agricultural soils via seed inoculation [6]. However, the plant and the bacterium, as well as symbiotic efficiency, are

affected by different biotic stressors such as pathogens and pests, as well as abiotic stresses including drought, salinity, heavy metals, etc. [7].

Drought stress is the main environmental factor restricting soybean production on a global scale, reducing the yield by up to 40% annually [8]. According to Jumrani and Bhatia [9], drought during the vegetative and reproductive stages of the soybean depresses its yield by 28% and 74%, respectively. Water deficit negatively impacts soybean fitness and productivity by affecting its morphological, biochemical, and physiological traits [10,11]. Drought alters photosynthesis, transpiration, stomatal conductance, gas exchange, cell turgidity, seed germination, and reproductive development. It also impairs plant height, root diameter, fresh root biomass, root/shoot ratio, leaf area, relative water content, chlorophyll and carotenoid content, proline content, pod number, and yield [12–14]. Biological nitrogen fixation (BNF) is particularly sensitive to drought, declining even before the photosynthetic and transpiration rates [15]. Production of reactive oxygen species (ROS) is significantly increased under drought stress, causing oxidative damage to chlorophyll, DNA, carbohydrates, proteins, and lipids; increased membrane lipid peroxidation; nutrient imbalance; and even cell death [16]. The high toxicity of ROS is regulated by the antioxidant defense system, involving non-enzymatic antioxidants such as ascorbic acid, glutathione, alkaloids, carotenoids, flavonoids, tocopherols, and phenolic acids, as well as enzymatic antioxidants like superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, etc. [16,17]. Improvement of tolerance to drought stress is also connected with the synthesis and accumulation of osmotically active solutes (soluble sugars, free amino acids, and N-containing compounds). Under drought stress, these compounds promote osmotic adjustment, lowering osmotic potential and preserving plant cells and metabolic activities [18]. Drought also modifies the soil microbiome, including changes in microbial composition, assembly, and function [19,20].

To face this challenge, various conventional and modern methods have been employed, including irrigation, breeding, mulching, planting, and the application of exogenous regulators and synthetic hormones, although these techniques are time-consuming and capital-intensive [21]. Microbial inoculants, based on plant-growth-promoting rhizobacteria (PGPR), are an alternative, environment-friendly, and sustainable strategy for improving crop resilience, production, and yield quality during abiotic and biotic stresses [22]. Various PGPR, including *Bradyrhizobium* and *Bacillus* strains, produce plant hormones, exopolysaccharides, enzymes, and other secondary metabolites in response to stress, which can improve root and shoot morphology, the antioxidant defense system, and water and nutrient availability [23,24]. Inoculation outcome depends primarily on the bacterial strains and environmental conditions. The selection of novel and more drought-tolerant *Bradyrhizobium* and *Bacillus* strains through inoculation trials under water deficit is needed in order to enable early adjustments of soybean production to challenges arising from climate change. *Bradyrhizobium japonicum* strains show different tolerances to osmotic stress. More tolerant strains could have an important role in improving the antioxidant response and symbiotic effectiveness of soybeans under drought stress [25,26]. However, little is known about how the introduction of new drought-tolerant *Bacillus* strains affects symbiotic performance, plant growth, and tolerance to drought. The objective of this study was to select *Bradyrhizobium japonicum* and *Bacillus* spp. strains according to their osmotic stress tolerance and to determine their effect as single and co-inoculants on antioxidant parameters and symbiotic efficiency in soybean plants under water deficit conditions.

2. Materials and Methods

2.1. Plant Material

The experimental plant was the soybean (*Glycine max* (L.) Merrill) cultivar Galina, an early variety of the 0 maturity group. Seeds were acquired from the Legume Department, Institute of Field and Vegetable Crops, Novi Sad, Serbia (IFVCNS). The cultivar achieves a high and stable yield under different growing conditions, with a potential yield exceeding

6 tons per hectare. Galina has a medium-height shoot, overgrown with gray hairs. The grains are of moderate size with yellow seed color and yellow hilum. This variety is suitable for timely, delayed, and stubble crop planting and is recommended for human consumption.

2.2. Bacterial Strains

This study was performed with *Bradyrhizobium japonicum* and *Bacillus* spp. strains from the Collection of Laboratory for Microbiological Research (IFVCNS). *Bradyrhizobium japonicum* strains were previously isolated from soybean nodules and identified using 16S rDNA sequencing, as described by Marinković et al. [27]. Indigenous *Bacillus* strains were isolated from different agricultural soils in Serbia and characterized according to morphological, cultural, and biochemical properties [28]. Molecular identification of newly isolated *Bacillus* strains was performed by 16S rDNA sequencing as described by Miljaković et al. [29]. *Bradyrhizobium* and *Bacillus* strains were cultivated and maintained on yeast extract mannitol agar (YEMA) and nutrient agar (NA), respectively [28,30].

2.3. Osmotic Stress Tolerance

Bradyrhizobium and *Bacillus* strains were grown separately in yeast extract mannitol broth (YEMB) or nutrient broth (NB). After incubation for 72 h (*Bradyrhizobium* strains) and 48 h (*Bacillus* strains) at 28 ± 2 °C and 150 rpm (Edmund Bühler SM-30 B, Bodelshausen, Germany), 1 mL of the bacterial cultures was transferred in 50 mL of YEMB or NB with the addition of polyethylene glycol (PEG) 6000 (36% *w/v*) (Sigma Aldrich, St. Louis, MO, USA). The PEG concentration was determined on the basis of previous research [31]. Broths without PEG were used as control treatments. Strains were re-incubated for 48 h (*Bacillus* strains) and 72 h (*Bradyrhizobium* strains) and then inoculated onto NA (*Bacillus* strains) and YEMA (*Bradyrhizobium* strains) plates (dilutions 10^{-3} – 10^{-10}). The number of colony-forming units (CFUs) was counted after two (*Bacillus* strains) and five days (*Bradyrhizobium* strains) of incubation at 28 ± 2 °C and expressed as $\log_{10}$ CFU mL⁻¹. The percentage of CFUs was determined by comparing to the control, which represented 100% growth of each bacterial strain [25,31]. Two strains from each genus with the highest tolerance to osmotic stress were selected for further research.

2.4. Screening of Bacterial Strains for Plant-Growth-Promoting (PGP) Traits

The ability of bacterial strains to solubilize inorganic tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ (HiMedia, Mumbai, India) was determined on Pikovskaya medium, as described by Chen and Liu [32]. Plates were incubated at 28 ± 2 °C for 5 days, while the clear halo zone surrounding bacterial colonies was an indication of P solubilization. Indole-3-acetic acid (IAA) production was assessed using the method described by Glickman and Dessaux [33]. The bacterial cultures were inoculated in YEMB (*Bradyrhizobium* strains) and NB (*Bacillus* strains) supplemented with 300 µg mL⁻¹ of L-tryptophan (HiMedia, Mumbai, India) and incubated at 28 ± 2 °C and 150 rpm (Edmund Bühler SM-30 B, Bodelshausen, Germany) for 24 h. The cultures were centrifuged at 5000 rpm for 15 min (GYROZEN 1730R, Gyrozen Co. Ltd., Seoul, South Korea), and then 1 mL of supernatant was added to 2 mL of Salkowski reagent (1:2 *v/v*) (Sigma Aldrich, St. Louis, MO, USA). The optical density was recorded at 530 nm (UV/VIS Cary 60 E, Agilent, CA, USA) after 20 min, while IAA concentrations were determined using the standard curve of IAA (Sigma Aldrich, St. Louis, MO, USA). Screening of bacterial strains for 1-aminocyclopropane 1-carboxylate (ACC) deaminase activity was performed using the method described by Stamenov et al. [34]. Bacterial cultures grown in YEMA (*Bradyrhizobium* strains) and NA (*Bacillus* strains) were spot-inoculated onto DF minimal salts medium [35] supplemented with ACC (Sigma Aldrich, St. Louis, MO, USA) and then incubated at 28 ± 2 °C for 72 h. Growth of bacterial strains by utilizing ACC as a N source was compared to the negative control (DF supplemented with ammonium sulfate) and positive control (DF without ACC). Exopolysaccharide (EPS) production was assayed according to Liu et al. [36]. Bacteria were grown on YEMA (*Bradyrhizobium*

strains) and NA (*Bacillus* strains) media with the addition of 0.02% calcofluor dye (Sigma Aldrich, St. Louis, MO, USA) for 7 days in order to observe clear differences in fluorescence. The effect of calcofluor fluorescence was determined by Reed et al. [37]. The ability of bacteria to produce siderophores was tested on Chrome Azurol S (HiMedia, Mumbai, India) medium, as described by Milagres et al. [38]. The bacterial cultures were streaked on YEMA (*Bradyrhizobium* strains) and NA (*Bacillus* strains) near the borderline with CAS medium and incubated at 28 ± 2 °C for 5 days. The color of the CAS medium changing from blue to orange was considered a positive test result.

2.5. Inoculation Assay

Bradyrhizobium japonicum and *Bacillus* spp. strains were cultured in YEMB and NB with agitation at 150 rpm (Edmund Bühler SM-30 B, Bodelshausen, Germany) at 28 ± 2 °C for 96 h and 48 h, respectively. The inocula were adjusted to contain 2×10^9 CFU mL⁻¹. Soybean seeds were surface-sterilized in 1% NaOCl (Sigma Aldrich, St. Louis, MO, USA), rinsed with sterile water three times, and dried at room temperature. Seed inoculation was performed with 200 mL of inoculum at sowing, while the same inoculant amount was applied 5 days after emergence [25]. For co-inoculation treatments, two bacterial culture suspensions were mixed uniformly. Likewise, the non-inoculated control was treated with water.

The trial was carried out at Rimski Šancevi, Legume Department (IFVCNS), under semi-controlled conditions. The experiment was carried out in pots (10 dm³ capacity) containing a mixture of soil and sand (3:1). The soil used was collected at the experimental field of IFVCNS and classified as haplic chernozem, with the following basic properties: pH_(KCl)—7.54; CaCO₃—10.27%; humus—2.69%; total N—0.21%; AL-P₂O₅—151 mg kg⁻¹; and AL-K₂O—223 mg kg⁻¹. The plants were exposed to environmental factors but watered with tap water regularly to maintain about 80% field capacity. Plants were grown under mineral N-free conditions with a mean daily temperature of 21.5 °C. The effects of inoculation were observed in well-watered and water-stressed conditions. At the flowering stage (R3–R5) of the soybean, the pots were separated into two groups: plants that were watered daily and plants exposed to water stress treatment by withholding water for 7 days. After 7 days without irrigation, soil moisture content measured by the gravimetric method [39] was decreased to 55% field capacity.

Experimental Design

The experiment was conducted in three replications and included nine treatments, both in well-watered and stressful conditions: (1) non-inoculated control; (2) R1: inoculation with *Bradyrhizobium japonicum* Bj11; (3) R2: inoculation with *Bradyrhizobium japonicum* Bj33; (4) B1: inoculation with *Bacillus subtilis* B59; (5) B2: inoculation with *Bacillus subtilis* B63; (6) R1B1: inoculation with *Br. japonicum* Bj11 and *B. subtilis* B59; (7) R1B2: inoculation with *Br. japonicum* Bj11 and *B. subtilis* B63; (8) R2B1: inoculation with *Br. japonicum* Bj33 and *B. subtilis* B59; (9) R2B2: inoculation with *Br. japonicum* Bj33 and *B. subtilis* B63.

2.6. Plant Sampling and Analysis

All plants were harvested a week after inducing drought stress. Roots and nodules were carefully washed with water, and all nodules were collected from the roots. Leaves, roots, and nodules were frozen in liquid nitrogen and stored at −80 °C for subsequent analyses of antioxidant parameters. Shoots, roots, and nodules for determination of dry weight and N content were oven-dried at 50 °C for 72 h to a constant weight.

2.6.1. Antioxidant Parameters

Peroxidase (EC 1.11.1.7) activity was analyzed from fresh leaves, roots, and nodules according to Dos Santos et al. [40]. The activity of soluble and ionically cell-wall-bound peroxidase was assayed through the oxidation of guaiacol (Sigma Aldrich, St. Louis, MO, USA) to tetraguaiacol, followed for 5 min at 470 nm (SAFAS UV mc1 UV-Vis, Monaco, Monaco).

The activity was expressed as $\mu\text{mol tetraguaiacol min}^{-1} \text{ g}^{-1}$ fresh weight ($\text{U g}^{-1} \text{ FW}$), calculated through the extinction coefficient for tetraguaiacol ($\epsilon = 25.5 \text{ mM}^{-1} \text{ cm}^{-1}$). Determination of total antioxidant capacity in fresh leaves, roots, and nodules was performed by the ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) (Sigma Aldrich, St. Louis, MO, USA) radical cation assay. $\text{ABTS}^{+\cdot}$ free-radical scavenging activity was examined by the method of Miller et al. [41], modified by Bohm et al. [42]. The absorbance was read at 734 nm (SAFAS UV mc1 UV-Vis, Monaco, Monaco), and the results have been expressed as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma Aldrich, St. Louis, MO, USA) equivalent used as an antioxidant standard ($\mu\text{mol TE g}^{-1} \text{ FW}$) [43]. All biochemical assays were performed in triplicate.

2.6.2. Biomass Accumulation and Nitrogen Content

The dry shoot, root, and nodule weights were obtained using a laboratory balance (Radwag PS 200/2000/X, Radom, Poland). The content of nitrogen in the shoots, roots, and nodules was determined through elemental analysis using a CHNS VarioEL III analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany), according to the AOAC Official Method 972.43:2000. Nitrogen content was expressed as a percentage of the dry weight of the shoots, roots, and nodules. All analyses were performed in triplicate.

2.7. Statistical Analysis

All data were statistically processed using Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA). Differences in arithmetic means between each treatment were analyzed by analysis of variance (ANOVA), followed by a post hoc Tukey's test ($p \leq 0.05$). Correlation analysis included antioxidant and symbiotic efficiency parameters among bacterial strains and drought treatments expressed as Pearson's correlation coefficient.

3. Results

Bradyrhizobium and *Bacillus* strains were tested on osmotic stress tolerance induced by supplementing the YEMB and NB with PEG 6000. Lower osmotic potential significantly reduced growth of all tested bacterial strains (Table 1). However, both *Bradyrhizobium* and *Bacillus* strains varied in their response to PEG 6000 treatment. The PEG concentration of 36% had the smallest effect on the growth of strains *Br. japonicum* Bj11 and Bj33 and *Bacillus* spp. B59 and B63, causing a decrease of 20.6%, 22.1%, 29.4%, and 41% compared to the control, respectively (Table 1). Molecular identification based on a partial 16S rDNA sequence showed that the two most tolerant *Bacillus* strains belong to *B. subtilis*. Based on persistence in media supplemented with 36% PEG, the abovementioned strains were selected for further research.

Table 1. Growth of *Bradyrhizobium japonicum* and *Bacillus* spp. strains under different conditions of osmotic stress.

Bacterial Strain	<i>Bradyrhizobium japonicum</i>				Bacterial Strain	<i>Bacillus</i> spp.			
	0% PEG		36% PEG			0% PEG		36% PEG	
	log ₁₀ CFU mL ⁻¹	%	log ₁₀ CFU mL ⁻¹	%		log ₁₀ CFU mL ⁻¹	%	log ₁₀ CFU mL ⁻¹	%
Bj 11	12.34	100	9.80	79.4	B 59	19.43	100	13.72	70.6
Bj 33	11.43	100	8.91	77.9	B 63	12.55	100	7.41	59.0
Bj 18	12.16	100	8.32	68.4	B 44	17.23	100	9.38	54.4
Bj 30	14.21	100	9.71	68.3	B 47	13.39	100	6.82	50.9
Bj 3	10.77	100	5.76	53.5	B 53	16.27	100	6.06	37.2
Bj 22	13.15	100	4.46	33.9	B 62	15.54	100	4.81	30.9
Bj 26	11.29	100	2.08	18.4	B 51	20.65	100	5.72	27.7
Bj 12	14.55	100	2.34	16.1	B 45	12.18	100	3.20	26.3
Bj 7	12.19	100	1.41	11.6	B 68	14.23	100	2.26	15.9
Bj 27	12.18	100	1.08	8.9	B 39	14.03	100	1.65	11.8

Note: PEG, polyethylene glycol 6000.

Among four selected bacterial strains, only *B. subtilis* 59 demonstrated the phosphate-solubilizing ability (Table 2). Bacterial strains produced IAA in the range of 8.63–20.17 $\mu\text{g mL}^{-1}$. Higher amounts of IAA were produced by *B. subtilis* strains compared to *Br. japonicum* strains. All bacterial strains produced exopolysaccharides and grew on DF medium with ACC or ammonium sulfate, while bacterial growth was compared with DF medium without a N source. Siderophore production was detected only for *Bacillus* strains (Table 2).

Table 2. Plant-growth-promoting properties of selected bacterial strains.

Bacterial Strain	P Solubilization	Indole-3-Acetic Acid ($\mu\text{g mL}^{-1}$)	Exopolysaccharides	ACC Deaminase	Siderophores
<i>Br. japonicum</i> 11	–	8.63 $\pm$ 0.45 b	+	+	–
<i>Br. japonicum</i> 33	–	9.62 $\pm$ 0.61 b	+	+	–
<i>B. subtilis</i> 59	+	15.76 $\pm$ 1.50 a	+	+	+
<i>B. subtilis</i> 63	–	20.17 $\pm$ 1.68 a	+	+	+

Indole-3-acetic acid: mean $\pm$ standard error of the mean ($n = 3$) with different lowercase letters in the same column indicating significantly different values ($p \leq 0.05$, Tukey's HSD test); P solubilization, exopolysaccharides, ACC deaminase, and siderophores: (+) positive for the test, (–) negative for the test.

The antioxidant defense system of soybean plants under drought stress was evaluated through enzymatic guaiacol peroxidase (POX) and ionically cell-wall-bound peroxidase (POD) activities and non-enzymatic ABTS^{•+} radical scavenging capacity in leaves, roots, and nodules. The peroxidase enzymes activities and free-radical scavenging capacity were significantly affected by the water regime, bacterial inoculation, and their interactions (Tables 3–5).

Inoculation with selected bacterial strains significantly affected POX activity, both in well-watered and drought-stressed plants. In regularly watered plants, the lowest POX activity was obtained in leaves of plants inoculated with bacterial consortium R1B2, in roots with R1B1, and in nodules with single rhizobial R1 strain application. The highest levels of POX activity in leaves and roots were achieved when bacterial combinations were applied (R2B2 and R1B2) and in nodules with one rhizobial strain treatment (R2) (Table 3).

The effect of inoculation was more pronounced in plants that had been exposed to a water deficit. Under stress conditions, the lowest POX activities were recorded in leaves, roots, and nodules of non-inoculated plants, and the only POX activity that was in line with the control was recorded in nodules of R1 strain inoculation. In leaves, significantly higher levels of POX activity were noted in all inoculation treatments compared to non-inoculated, while significant differences in roots were not recorded only in R1B1 treatment. The highest POX values were determined in leaves with the R2B1, R1B2, and R1B1 inoculations; in roots with R2B1; and in nodules with R1B1 application (Table 3).

As a consequence of drought, in non-inoculated plants, the rise of POX activity was lower, i.e., 19%, 48%, and 85% in leaves, roots, and nodules, respectively. A significant increase in POX activity was noted due to inoculation. In leaves, the highest elevation was determined in R1B2 and R2B1 treatments (116% and 100%, respectively); in roots, in R2B1 and R1B2 treatments (169% and 140%, respectively); and in nodules, in R1B2 and R1B1 treatments (245% and 239%, respectively) (Table 3).

Likewise, the average POD activity in leaves, roots, and nodules in drought stress was significantly higher than activity in well-watered conditions (Table 4).

In well-watered plants, the most obvious influence of the bacterial strains was noted in nodules. A non-significant difference was recorded only between non-inoculated plants and co-inoculation with R1B2. Conversely, the inoculation showed negligible effect on POD activity in the leaves; thus, the only significant difference compared to the control was recorded with the R1 application. In roots, the lowest level of POD activity was obtained in non-inoculated plants, while the highest POD values were achieved with strains B1 and B2 (Table 4).

Table 3. Effects of drought stress and bacterial strains on guaiacol peroxidase (POX) activity in soybean leaves, roots, and nodules.

Inoculation Treatment	Drought Stress (Days)		Average	Increase (%)
	0	7		
	POX Activity in Leaves (U g ⁻¹ FW)			
R1	0.97 ± 0.05 cdGH	1.85 ± 0.02 abAB	1.41 DE	90
R2	1.42 ± 0.01 abDEF	1.58 ± 0.01 Ccd	1.50 BCD	11
B1	1.41 ± 0.06 abDEF	1.68 ± 0.04 bcBC	1.55 B	19
B2	1.47 ± 0.00 abdef	1.66 ± 0.02 cC	1.57 BC	13
R1B1	1.36 ± 0.02 bEF	1.86 ± 0.04 aA	1.61 AB	36
R1B2	0.87 ± 0.01 dH	1.88 ± 0.01 aA	1.38 E	116
R2B1	0.97 ± 0.00 cdGH	1.94 ± 0.08 aA	1.45 CDE	100
R2B2	1.52 ± 0.01 aCDE	1.85 ± 0.03 abAB	1.69 A	22
Control	1.11 ± 0.04 cG	1.32 ± 0.02 dF	1.22 F	19
Average	1.24 B	1.74 A	1.49	
POX Activity in Roots (U g ⁻¹ FW)		Average	Increase (%)	
R1	1.46 ± 0.02 abFGH	2.96 ± 0.01 cC	2.21 B	102
R2	1.34 ± 0.07 bcGH	1.93 ± 0.06 deDE	1.63 CD	44
B1	1.26 ± 0.03 cHI	2.15 ± 0.04 dD	1.71 C	70
B2	1.36 ± 0.01 bcGH	1.93 ± 0.09 deDE	1.65 CD	42
R1B1	0.87 ± 0.01 eJ	1.83 ± 0.05 efE	1.35 E	110
R1B2	1.55 ± 0.01 aFG	3.72 ± 0.06 bB	2.63 A	140
R2B1	1.49 ± 0.01 abA	4.01 ± 0.05 aFGH	2.75 A	169
R2B2	1.08 ± 0.05 dIJ	1.95 ± 0.04 deDE	1.52 D	81
Control	1.08 ± 0.04 dIJ	1.60 ± 0.01 fF	1.34 E	48
Average	1.28 B	2.45 A	1.86	
POX Activity in Nodules (U g ⁻¹ FW)		Average	Increase (%)	
R1	0.68 ± 0.01 fL	2.02 ± 0.07 fF	1.35 E	197
R2	1.80 ± 0.01 aFG	3.69 ± 0.03 cC	2.74 A	105
B1	1.71 ± 0.09 abGH	2.79 ± 0.06 eE	2.25 C	63
B2	1.51 ± 0.01 bGH	2.66 ± 0.02 eE	2.09 D	76
R1B1	1.30 ± 0.00 cIJ	4.41 ± 0.07 aA	2.85 A	239
R1B2	1.14 ± 0.01 cdeJK	3.93 ± 0.01 bB	2.54 B	245
R2B1	1.27 ± 0.00 cdBC	3.83 ± 0.06 bcJ	2.55 B	202
R2B2	1.09 ± 0.05 deJK	3.18 ± 0.04 dD	2.13 CD	192
Control	0.98 ± 0.06 eK	1.81 ± 0.03 fFG	1.40 E	85
Average	1.28 B	3.15 A	2.21	

Note: R1, *Br. japonicum* Bj11; R2, *Br. japonicum* Bj33; B1, *B. subtilis* B59; B2, *B. subtilis* B63; R1B1, *Br. japonicum* Bj11 and *B. subtilis* B59; R1B2, *Br. japonicum* Bj11 and *B. subtilis* B63; R2B1, *Br. japonicum* Bj33 and *B. subtilis* B59; R2B2, *Br. japonicum* Bj33 and *B. subtilis* B63. Data are represented as mean ± standard error of the mean ($n = 3$); means followed by the same lowercase/uppercase letters are not significantly different ($p \leq 0.05$, Tukey's HSD test).

Water deficit emphasized the role of bacterial inoculation in POD activity. The POD levels in roots and nodules of non-inoculated plants were significantly below the values recorded in all inoculated treatments. The POD activities in leaves were higher in bacterial treatments compared to the control, with the exception of B2 inoculation. The R1B1 bacterial consortium application resulted in the highest POD activity in the leaves, while R2B1 led to the highest POD activity in roots and nodules (Table 4).

The bacterial consortia also had the most pronounced influence on the increment of POD activity during drought. In leaves, R2B2 and R1B1 co-inoculation induced increases of 116% and 90%; in roots, R1B1 and R2B1 elevated activities by 102% and 94%; in nodules, the most influential combinations were R1B1 and R1B2 (159% and 103%, respectively). In leaves of non-inoculated plants, drought induced an increase of 21%, in roots 34%, and in nodules 44% (Table 4).

Table 4. Effects of drought stress and bacterial strains on ionically cell-wall-bound peroxidase (POD) activity in soybean leaves, roots, and nodules.

Inoculation Treatment	Drought Stress (Days)		Average	Increase (%)
	0	7		
	POD Activity in Leaves (U g ^{−1} FW)			
R1	1.20 ± 0.01 aD	1.45 ± 0.02 bC	1.33 B	21
R2	1.04 ± 0.03 bcdEF	1.49 ± 0.03 bC	1.27 B	43
B1	1.14 ± 0.02 abDE	1.84 ± 0.06 aB	1.49 A	61
B2	0.97 ± 0.01 dFG	1.24 ± 0.01 cD	1.11 C	28
R1B1	1.05 ± 0.03 bcdEF	1.99 ± 0.05 aA	1.52 A	90
R1B2	1.01 ± 0.04 cdEF	1.58 ± 0.03 bC	1.29 B	56
R2B1	1.10 ± 0.00 abcDEF	1.52 ± 0.01 bC	1.31 B	38
R2B2	0.85 ± 0.00 eG	1.84 ± 0.02 aAB	1.34 B	116
Control	1.03 ± 0.04 bcde	1.25 ± 0.01 c	1.14 C	21
Average	1.04 B	1.58 A	1.31	
POD Activity in Roots (U g ^{−1} FW)		Average	Increase (%)	
R1	1.69 ± 0.04 bGHI	2.30 ± 0.06 bcBCD	2.00 B	36
R2	1.82 ± 0.03 abFGH	2.08 ± 0.10 cDEF	1.95 B	14
B1	1.95 ± 0.00 aEFG	2.16 ± 0.09 cCDE	2.05 AB	11
B2	1.90 ± 0.09 aEFGH	2.15 ± 0.02 cCDE	2.03 AB	13
R1B1	1.27 ± 0.02 cdJ	2.57 ± 0.06 abAB	1.92 B	102
R1B2	1.30 ± 0.02 cdJ	2.52 ± 0.03 abAB	1.91 B	94
R2B1	1.74 ± 0.03 abGH	2.65 ± 0.08 aA	2.20 A	52
R2B2	1.45 ± 0.05 cIJ	2.40 ± 0.08 abcABC	1.92 B	66
Control	1.23 ± 0.02 dJ	1.65 ± 0.02 dHI	1.44 C	34
Average	1.59 B	2.28 A	1.94	
POD Activity in Nodules (U g ^{−1} FW)		Average	Increase (%)	
R1	2.99 ± 0.06 dG	5.12 ± 0.03 bB	4.05 C	71
R2	2.44 ± 0.03 eH	4.13 ± 0.04 dD	3.29 F	69
B1	3.33 ± 0.05 bF	4.50 ± 0.03 cC	3.91 CD	35
B2	3.11 ± 0.02 cdFG	3.95 ± 0.05 dDE	3.53 E	27
R1B1	2.54 ± 0.04 eH	5.15 ± 0.11 bB	3.85 D	103
R1B2	2.03 ± 0.04 fI	5.27 ± 0.05 bB	3.65 E	159
R2B1	3.81 ± 0.03 aE	6.11 ± 0.12 aA	4.96 A	60
R2B2	3.29 ± 0.05 bcF	5.97 ± 0.08 aA	4.63 B	81
Control	1.84 ± 0.04 fI	2.64 ± 0.06 eH	2.24 G	44
Average	2.82 B	4.76 A	3.79	

Note: R1, *Br. japonicum* Bj11; R2, *Br. japonicum* Bj33; B1, *B. subtilis* B59; B2, *B. subtilis* B63; R1B1, *Br. japonicum* Bj11 and *B. subtilis* B59; R1B2, *Br. japonicum* Bj11 and *B. subtilis* B63; R2B1, *Br. japonicum* Bj33 and *B. subtilis* B59; R2B2, *Br. japonicum* Bj33 and *B. subtilis* B63. Data are represented as mean ± standard error of the mean ($n = 3$); means followed by the same lowercase/uppercase letters are not significantly different ($p \leq 0.05$, Tukey's HSD test).

On average for all inoculation treatments, drought stress induced a significant increase in free-radical scavenging capacity in leaves, roots, and nodules in comparison with well-watered plants (Table 5).

The bacterial inoculants revealed the most pronounced impact on free-radical scavenging capacity in leaves, both constitutive and stress-induced. Significantly higher levels of ABTS^{•+} cation scavenging activity were recorded in all inoculated plants compared to non-inoculated plants, except for R1 well-watered treatment, which was in alignment with the control plants. The highest leaf free-radical scavenging capacity in well-watered conditions was achieved with R2B2 co-inoculation, and in drought stress with application of R1B2 and B2 strains (Table 5).

The least inoculation impact was demonstrated on non-enzymatic antioxidants in nodules in well-watered conditions. No significant differences were recorded between non-inoculated plants and plants inoculated with strains R1, R2, B2, R1B1, and R2B1. Moreover, significantly lower levels were recorded in treatments B1, R1B2, and R2B2. In conditions of

optimal water supply, free-radical scavenging capacity in roots was significantly higher due to R1B2 co-inoculation compared to all other treatments, while the significantly lowest level was achieved with R2B1 strains (Table 5).

Table 5. Effects of drought stress and bacterial strains on ABTS⁺ radical cation scavenging capacity in soybean leaves, roots, and nodules.

Inoculation Treatment	Drought Stress (Days)		Average	Increase (%)
	0	7		
	ABTS ^{•+} Radical Scavenging Capacity in Leaves (μmol TE g ⁻¹)			
R1	1.33 ± 0.05 dG	2.31 ± 0.0 abAB	1.82 D	74
R2	1.60 ± 0.0 bcDEF	2.41 ± 0.0 abA	2.00 BC	51
B1	1.60 ± 0.0 bcDEF	2.20 ± 0.0 bBC	1.90 CD	38
B2	1.55 ± 0.0 cEF	2.44 ± 0.0 aA	2.00 BC	58
R1B1	1.71 ± 0.0 bDE	2.29 ± 0.0 abAB	2.00 BC	34
R1B2	1.60 ± 0.0 bcDE	2.48 ± 0.0 aA	2.04 B	55
R2B1	1.61 ± 0.0 bcDE	2.43 ± 0.0 abA	2.02 BC	51
R2B2	2.09 ± 0.0 aC	2.29 ± 0.0 abAB	2.19 A	10
Control	1.40 ± 0.0 dFG	1.75 ± 0.0 cD	1.58 E	25
Average	1.61 B	2.29 A	2	
ABTS ^{•+} Radical Scavenging Capacity in Roots (μmol TE g ⁻¹)		Average	Increase (%)	
R1	1.48 ± 0.0 abCDEF	1.62 ± 0.0 cC	1.55 BC	10
R2	1.33 ± 0.0 dEFG	1.56 ± 0.0 cCD	1.44 CD	17
B1	1.36 ± 0.0 cdEFG	1.48 ± 0.0 cCDEF	1.42 DE	9
B2	1.43 ± 0.0 abcDEF	1.85 ± 0.0 bB	1.64 B	29
R1B1	1.31 ± 0.0 dFG	1.96 ± 0.0 bB	1.64 B	49
R1B2	1.49 ± 0.0 aCDE	2.37 ± 0.0 aA	1.93 A	59
R2B1	1.07 ± 0.0 fH	1.45 ± 0.0 cCDEF	1.26 F	36
R2B2	1.41 ± 0.0 bcDEF	2.48 ± 0.0 aA	1.95 A	76
Control	1.22 ± 0.0 eGH	1.42 ± 0.0 cDEF	1.32 EF	16
Average	1.34 B	1.80 A	1.57	
ABTS ^{•+} Radical Scavenging Capacity in Nodules (μmol TE g ⁻¹)		Average	Increase (%)	
R1	1.25 ± 0.0 aFGH	1.80 ± 0.0 abAB	1.53 A	44
R2	1.22 ± 0.0 aFGH	1.62 ± 0.0 bcdCD	1.42 ABC	40
B1	0.95 ± 0.0 dJ	1.31 ± 0.0 fEFG	1.13 E	38
B2	1.23 ± 0.0 aFGH	1.44 ± 0.0 defDE	1.34 CD	17
R1B1	1.15 ± 0.0 abGHI	1.55 ± 0.0 cdeD	1.35 BCD	35
R1B2	1.08 ± 0.0 bcHIJ	1.95 ± 0.0 aA	1.52 A	81
R2B1	1.15 ± 0.0 abGHI	1.74 ± 0.0 bcBC	1.45 AB	51
R2B2	1.01 ± 0.0 cdIJ	1.73 ± 0.0 bcIJ	1.37 BCD	71
Control	1.24 ± 0.0 aFGH	1.36 ± 0.0 efEF	1.30 D	10
Average	1.14 B	1.61 A	1.38	

Note: R1, *Br. japonicum* Bj11; R2, *Br. japonicum* Bj33; B1, *B. subtilis* B59; B2, *B. subtilis* B63; R1B1, *Br. japonicum* Bj11 and *B. subtilis* B59; R1B2, *Br. japonicum* Bj11 and *B. subtilis* B63; R2B1, *Br. japonicum* Bj33 and *B. subtilis* B59; R2B2, *Br. japonicum* Bj33 and *B. subtilis* B63. Data are represented as mean $\pm$ standard error of the mean ($n = 3$); means followed by the same lowercase/uppercase letters are not significantly different ($p \leq 0.05$, Tukey's HSD test).

In contrast to enzymatic activities (POX and POD), inoculation revealed a lower effect on the non-enzymatic antioxidative response in roots and nodules of drought-stressed plants. After 7 days of water deficit, the lowest ABTS⁺ radical cation scavenging capacity in roots was obtained in non-inoculated plants, as well as in plants inoculated with R1, R2, B1, and R2B1 strains. The highest ABTS⁺ values were recorded due to inoculation with R2B2 and R1B2 consortia. In nodules, significant differences between non-inoculated plants and B1, B2, and R1B1 treatments were not observed, while the highest level of ABTS⁺ radical cation scavenging capacity was recorded with R1B2 strain application (Table 5).

The highest increase in ABTS⁺ radical scavenging capacity under drought was observed due to R1 inoculation in leaves (74%), R2B2 in roots (76%), and R1B2 (81%) in nodules. Drought-induced enhancement was lower in leaves, roots, and nodules of non-inoculated plants and ranged 25%, 16%, and 10%, respectively (Table 5).

According to the obtained results, drought stress and inoculation treatments significantly affected soybean shoot and nodule dry weight but had no influence on root dry weight (Table 6). Reduced moisture induced a decline in both shoot and nodule dry weight.

Table 6. Effects of drought stress and bacterial strains on the dry weight of soybean shoots, roots, and nodules.

Inoculation Treatment	Drought Stress (Days)		Average	Increase (%)
	0	7		
	Shoot Dry Weight (g plant ^{−1})			
R1	3.68 ± 0.22 ab	3.29 ± 0.16 abBC	3.49 BC	−10
R2	3.95 ± 0.10 abAB	3.50 ± 0.24 abABC	3.72 AB	−11
B1	3.79 ± 0.25 abAB	3.76 ± 0.07 aAB	3.77 AB	−1
B2	3.66 ± 0.08 abABC	3.52 ± 0.11 abABC	3.59 AB	−4
R1B1	4.02 ± 0.28 aAB	3.28 ± 0.17 abBC	3.65 AB	−18
R1B2	3.62 ± 0.22 abABC	3.61 ± 0.10 abABC	3.62 AB	−3
R2B1	4.37 ± 0.08 aA	3.86 ± 0.34 aAB	4.11 A	−12
R2B2	3.98 ± 0.09 abAB	3.86 ± 0.12 aAB	3.92 AB	−3
Control	3.14 ± 0.03 bBC	2.81 ± 0.18 bC	2.97 C	−11
Average	3.80 A	3.50 B	3.65	
Root Dry Weight (g plant ^{−1})			Average	Increase (%)
R1	0.63 ± 0.09 aA	0.79 ± 0.07 aA	0.71 A	25
R2	0.63 ± 0.05 aA	0.75 ± 0.08 aA	0.69 A	19
B1	0.66 ± 0.05 aA	0.81 ± 0.06 aA	0.73 A	23
B2	0.74 ± 0.01 aA	0.84 ± 0.03 aA	0.79 A	14
R1B1	0.68 ± 0.03 aA	0.75 ± 0.10 aA	0.71 A	10
R1B2	0.64 ± 0.07 aA	0.74 ± 0.04 aA	0.69 A	16
R2B1	0.63 ± 0.03 aA	0.66 ± 0.05 aA	0.65 A	5
R2B2	0.65 ± 0.03 aA	0.78 ± 0.05 aA	0.72 A	20
Control	0.60 ± 0.03 aA	0.75 ± 0.12 aA	0.67 A	25
Average	0.65 A	0.76 A	0.71	
Nodule Dry Weight (g plant ^{−1})			Average	Increase (%)
R1	0.96 ± 0.15 abcAB	0.42 ± 0.04 abcBC	0.69 AB	−56
R2	0.94 ± 0.02 abcAB	0.48 ± 0.09 abBC	0.71 AB	−49
B1	0.39 ± 0.08 bcBC	0.31 ± 0.08 abcBC	0.35 BC	−21
B2	0.67 ± 0.30 abcABC	0.20 ± 0.04 bcC	0.43 BC	−70
R1B1	1.24 ± 0.29 aA	0.58 ± 0.15 abABC	0.91 A	−53
R1B2	1.19 ± 0.14 abA	0.63 ± 0.11 aABC	0.91 A	−47
R2B1	0.71 ± 0.01 abcABC	0.32 ± 0.04 abcBC	0.52 ABC	−55
R2B2	0.71 ± 0.12 abcABC	0.48 ± 0.05 abBC	0.60 AB	−32
Control	0.19 ± 0.06 cC	0.07 ± 0.03 cC	0.13 C	−63
Average	0.78 A	0.39 B	0.58	

Note: R1, *Br. japonicum* Bj11; R2, *Br. japonicum* Bj33; B1, *B. subtilis* B59; B2, *B. subtilis* B63; R1B1, *Br. japonicum* Bj11 and *B. subtilis* B59; R1B2, *Br. japonicum* Bj11 and *B. subtilis* B63; R2B1, *Br. japonicum* Bj33 and *B. subtilis* B59; R2B2, *Br. japonicum* Bj33 and *B. subtilis* B63. Data are represented as mean ± standard error of the mean ($n = 3$); means followed by the same lowercase/uppercase letters are not significantly different ($p \leq 0.05$, Tukey's HSD test).

Shoot dry weight did not vary significantly between single inoculations with *Bradyrhizobium* or *Bacillus* strains and non-inoculated plants, both in conditions of optimal and restrictive water supply. The highest dry weights in well-watered and drought-stressed plants were recorded with the R1B1 and R2B1 consortia, and additionally with R2B2 application in water deficit conditions. Compared to those treatments, the dry weight of non-inoculated plants was significantly lower in both optimal and water deficit conditions.

In well-watered plants, the highest nodule dry weight was noted with R1B1 inoculation, while in drought stress conditions, the highest nodule dry weight was observed with R1B2 consortium application. The inoculation effect on nodule dry weight was more pronounced during a water deficit. Significant differences were observed between nodule mass in non-inoculated plants and plants treated with R2, R1B1, R1B2, and R2B2 (Table 6).

Nitrogen content in soybean plants varied significantly depending on water stress and applied inoculation treatments (Table 7). On average, nitrogen content increased significantly under water deficit conditions in shoots, roots, and nodules (Table 7).

Table 7. Effects of drought stress and bacterial strains on N content (%) in soybean shoots, roots, and nodules.

Inoculation Treatment	Drought Stress (Days)		Average	Increase (%)
	0	7		
	N Content in Shoots (%)			
R1	1.62 ± 0.05 bD	1.90 ± 0.05 bD	1.76 BC	17
R2	2.90 ± 0.17 aA	2.94 ± 0.09 aA	2.92 A	1.3
B1	1.98 ± 0.15 bCD	2.11 ± 0.26 bCD	2.04 B	7
B2	2.02 ± 0.05 bCD	1.85 ± 0.05 bD	1.93 BC	−8
R1B1	1.97 ± 0.05 bCD	2.18 ± 0.14 bBCD	2.07 B	11
R1B2	1.51 ± 0.06 bD	1.65 ± 0.21 bD	1.58 C	9
R2B1	2.85 ± 0.10 aAB	2.95 ± 0.04 aA	2.90 A	4
R2B2	2.62 ± 0.15 aABC	3.01 ± 0.06 aA	2.81 A	15
Control	1.55 ± 0.17 bD	1.75 ± 0.15 bD	1.65 BC	13
Average	2.11 B	2.26 A	2.19	
N Content in Roots (%)			Average	Increase (%)
R1	1.53 ± 0.17 abcBCDEF	1.85 ± 0.09 abAB	1.69 ABC	21
R2	1.57 ± 0.06 abcABCDE	1.78 ± 0.02 abcABC	1.67 ABC	13
B1	1.29 ± 0.07 bcDEF	1.57 ± 0.07 bcdBCDE	1.43 CDE	22
B2	1.31 ± 0.20 abcCDEF	1.79 ± 0.08 abcAB	1.55 BCD	37
R1B1	1.08 ± 0.05 cF	1.47 ± 0.11 cdBCDEF	1.28 DE	36
R1B2	1.13 ± 0.06 cEF	1.50 ± 0.06 bcdBCDEF	1.31 DE	33
R2B1	1.79 ± 0.05 aAB	1.77 ± 0.03 abcABCD	1.78 AB	−1
R2B2	1.72 ± 0.04 abABCD	2.01 ± 0.02 aA	1.87 A	17
Control	1.10 ± 0.04 cEF	1.38 ± 0.12 dBCDEF	1.24 E	25
Average	1.39 B	1.53 A	1.54	
N Content in Nodules (%)			Average	Increase (%)
R1	4.48 ± 0.27 abAB	5.20 ± 0.22 aA	4.84 A	16
R2	4.27 ± 0.25 abAB	4.68 ± 0.20 aAB	4.48 AB	10
B1	4.04 ± 0.16 abAB	4.60 ± 0.26 aAB	4.32 AB	14
B2	4.07 ± 0.05 abAB	4.20 ± 0.36 aAB	4.14 AB	3
R1B1	4.48 ± 0.35 abAB	5.08 ± 0.18 aA	4.78 A	13
R1B2	4.77 ± 0.10 aA	4.95 ± 0.33 aA	4.86 A	4
R2B1	4.28 ± 0.17 abAB	4.75 ± 0.20 aA	4.52 AB	11
R2B2	4.71 ± 0.22 aA	5.02 ± 0.32 aA	4.87 A	7
Control	3.41 ± 0.35 bB	4.20 ± 0.08 aAB	3.81 B	23
Average	4.28 B	4.74 A	4.51	

Note: R1, *Br. japonicum* Bj11; R2, *Br. japonicum* Bj33; B1, *B. subtilis* B59; B2, *B. subtilis* B63; R1B1, *Br. japonicum* Bj11 and *B. subtilis* B59; R1B2, *Br. japonicum* Bj11 and *B. subtilis* B63; R2B1, *Br. japonicum* Bj33 and *B. subtilis* B59; R2B2, *Br. japonicum* Bj33 and *B. subtilis* B63. Data are represented as mean ± standard error of the mean ($n = 3$); means followed by the same lowercase/uppercase letters are not significantly different ($p \leq 0.05$, Tukey's HSD test).

The effect of inoculation on nitrogen content in shoots, both in optimal and water shortage conditions, was identical. A significantly higher shoot N content was recorded due to inoculation with R2, R2B1, and R2B2 compared to other treatments. In roots, a more notable inoculation impact was obtained under drought stress. Under optimal water conditions, the N content in control plants was significantly below the content in plants

inoculated with R2B1 and R2B2 consortia. The highest N content was observed with R2B2 under water deficit and with R2B1 in well-watered conditions. However, N content in non-inoculated plants during drought stress was significantly lower than in plants where strains R1, R2, B2, R2B1, and R2B2 were applied. Contrary to the roots, nitrogen content in nodules was influenced by inoculation only in regularly watered plants. Nodules of plants inoculated with strains R1B2 and R2B2 had the highest nitrogen content and the lowest in non-inoculated plants (Table 7).

Positive results from inoculation treatments were confirmed by correlation analysis (Table 8). Generally, a positive interrelationship was established between POX activity in leaves and nodules and other parameters, with the exception of shoot dry weight. POX activity in roots was significantly correlated with other parameters, except with shoot, root, and nodule dry weight, and N content in roots. Moreover, results revealed a strong correlation between POD activity in leaves, roots, and nodules and other parameters, with the exception of shoot dry weight. However, no significant correlation was established between POD activity in leaves and N content in shoots. A significant dependence was observed between ABTS⁺ radical cation scavenging capacity in leaves, roots, and nodules and other soybean parameters, except shoot dry weight. Additionally, ABTS⁺ radical cation scavenging capacity in roots and nodules was not significantly correlated with nitrogen content in shoots. Furthermore, ABTS⁺ radical cation scavenging capacity in roots was not significantly related to nodule dry weight. A strong dependence was established between shoot and nodule dry weight as well as shoot dry weight and N content in shoots. Also, N content in roots was significantly and positively correlated with N content in shoots (Table 8).

Table 8. Pearson’s correlation of the examined parameters in soybeans.

Variable	POX L	POX R	POX N	POD L	POD R	POD N	ABTS ⁺ L	ABTS ⁺ R	ABTS ⁺ N	SDW	RDW	NDW	NCS	NCR	NCN
POX L	1.00	0.65 ***	0.81 ***	0.67 ***	0.82 ***	0.82 ***	0.85 ***	0.63 ***	0.73 ***	−0.10 ns	0.41 **	−0.37 **	0.29 *	0.48 ***	0.41 **
POX R		1.00	0.68 ***	0.55 ***	0.76 ***	0.77 ***	0.69 ***	0.47 ***	0.82 ***	−0.07 ns	0.21 ns	−0.28 ns	0.10 ns	0.40 **	0.43 ***
POX N			1.00	0.83 ***	0.85 ***	0.80 ***	0.83 ***	0.65 ***	0.76 ***	−0.14 ns	0.33 *	−0.31 *	0.32 *	0.39 **	0.43 ***
POD L				1.00	0.78 ***	0.79 ***	0.67 ***	0.65 ***	0.69 ***	−0.18 ns	0.35 **	−0.31 *	0.20 ns	0.40 **	0.47 ***
POD R					1.00	0.90 ***	0.74 ***	0.62 ***	0.77 ***	−0.03 ns	0.39 **	−0.35 **	0.32 *	0.55 ***	0.41 **
POD N						1.00	0.81 ***	0.65 ***	0.77 ***	0.07 ns	0.32 *	−0.31 *	0.40 **	0.65 ***	0.53 ***
ABTS ⁺ L							1.00	0.65 ***	0.74 ***	−0.06 ns	0.40 **	−0.35 **	0.30 *	0.57 ***	0.53 ***
ABTS ⁺ R								1.00	0.69 ***	−0.13 ns	0.34 *	−0.12 ns	0.01 ns	0.34 *	0.48 ***
ABTS ⁺ N									1.00	−0.22 ns	0.37 **	−0.29 *	0.14 ns	0.47 ***	0.46 ***
SDW										1.00	0.06 ns	0.41 **	0.42 **	0.19 ns	0.09 ns
RDW											1.00	−0.10 ns	−0.06 ns	0.26 ns	0.05 ns
NDW												1.00	−0.02 ns	−0.32 *	0.15 ns
NCS													1.00	0.59 ***	0.19 ns
NCR														1.00	0.30 *
NCN															1.00

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ns—not significant. Note: POX L, POX in leaves; POX R, POX in roots; POX N, POX in nodules; POD L, POD in leaves; POD R, POD in roots; POD N, POD in nodules; ABTS⁺ L, ABTS⁺ in leaves; ABTS⁺ R, ABTS⁺ in roots; ABTS⁺ N, ABTS⁺ in nodules; SDW, shoot dry weight; RDW, root dry weight; NDW, nodule dry weight; NCS, N content in shoots; NCR, N content in roots; NCN, N content in nodules.

4. Discussion

Bradyrhizobium and *Bacillus* strains, locally isolated from soybean nodules and rhizosphere soil, respectively, displayed different abilities to grow under osmotic-stress conditions induced by 36% PEG-6000 (Table 1). Osmoadaptation potential varied from 8.9% to 79.4% in *Bradyrhizobium* strains and from 11.8% to 70.6% in *Bacillus* strains. The specific response of individual bacterial strains is determined by differences in their genetic potential for stress resistance. Higher osmotic stress tolerance could be related to higher production, accumulation, or release of different organic solutes (free amino acids, proline, betaine, glycerol, and soluble sugars), osmolytes, exopolysaccharides, stress proteins, and antioxidants that will preserve cells and provide stress resistance [44]. Previous studies also demonstrated specific adaptive potential to osmotic stress in different rhizobial and *Bacillus* strains [25,44–48].

In this study, all tested bacterial strains produced IAA, although higher concentrations of this plant hormone were detected in *Bacillus* strains (15.76–20.17 $\mu\text{g mL}^{-1}$) than in *Bradyrhizobium* strains (8.63–9.62 $\mu\text{g mL}^{-1}$) (Table 2). This is in agreement with the reports that IAA production can differ not only between bacterial species but also among the

strains [49,50]. Xing et al. [51] found that *Bacillus* was a superior IAA producer compared to *Bradyrhizobium*, while alternation in other PGP properties between these strains was also determined. Indole-3-acetic acid, as the auxin phytohormone, plays an irreplaceable role in plant growth and development. The IAA produced by PGPR increases lateral root formation, enhancing absorptive surface and water and nutrient acquisition [52]. In addition to IAA, these bacteria produce other plant hormones that promote growth and stress response, such as gibberellins, cytokinins, abscisic acid, brassinosteroids, etc. [53]. Moreover, PGPR produce ACC deaminase, which regulates ethylene production by cleaving ACC into α -ketobutyrate and ammonia, reducing ethylene production under stress conditions [54]. In this study, all examined bacterial strains were positive for ACC deaminase activity as well as EPS production. Exopolysaccharides protect microbial cell membrane structure against desiccation. Moreover, EPS excretion enhances soil microaggregate stability and maintains higher soil moisture, thus helping plants to withstand drought stress conditions [55]. EPS-producing PGPR strains can improve plant growth under stress and also change the levels of IAA and other hormones, increase production of osmolytes, and increase activity of antioxidant enzymes [50,56]. Our results are in agreement with previous findings, which also confirmed ACC deaminase and EPS production in rhizobial and *Bacillus* strains [57–60]. Only *Bacillus* strains tested in this study produced siderophores, while only the *Bacillus subtilis* B59 strain was capable of solubilizing phosphorus. Siderophores form complexes with Fe, thus improving their solubility and uptake. Previous studies showed that higher levels of siderophore production are associated with higher host plant resistance to water deficit [61]. Bacterial ability to solubilize inorganic phosphates can improve phosphorus availability, which is a possible mechanism for the plant growth promotion under nutrient-limited conditions [62].

Enhanced enzymatic and non-enzymatic antioxidant activities are important components of plant defense against negative drought impacts. In this study, both enzymatic and non-enzymatic antioxidant systems were elevated in leaves, roots, and nodules after 7 days of water deficiency compared with those in well-watered plants (Tables 3–5). The single inoculation and co-inoculation with selected *Bradyrhizobium* and *Bacillus* strains affected the POX and POD activities in leaves, roots, and nodules mainly under drought stress, while in well-watered plants, the effects of the inoculation were less evident (Tables 3 and 4). Peroxidases, as heme-containing oxidoreductases, catalyze the oxidation of different substrates using hydrogen peroxide as the electron donor and thus participate in various physiological and biochemical processes in plants [63]. They have an essential role in ROS scavenging and displayed about 1000-fold higher affinity for H_2O_2 compared to catalases [64]. Peroxidases are also involved in cell wall lignification. Higher concentrations of H_2O_2 under drought stress induce the activity of cell wall peroxidases and cross-linking of glycoproteins and phenolic compounds, thus limiting plant growth and dehydration [65,66]. The notable effects of inoculation during drought stress on the peroxidase activity in nodules and roots of legumes have also been demonstrated in previous research. For instance, *Bacillus* spp. strains from the soybean rhizosphere, which showed a variety of PGP traits, increased root and shoot length, proline, sugar, and protein, as well as catalase, superoxide dismutase, and peroxidase activities in drought-stressed plants [67]. Mansour et al. [68] found that inoculation of faba beans with *Rhizobium leguminosarum* and/or *Pseudomonas putida* significantly increased the POX activity under drought stress compared to non-inoculated plants, while the highest levels were achieved due to co-inoculation. Similarly, the highest POX and POD levels and the highest improvement of enzymatic activity compared to watered plants in this study were achieved due to co-inoculation with *Bradyrhizobium* and *Bacillus* strains (Tables 3 and 4). The results of the $ABTS^{\cdot+}$ radical cation scavenging capacity assay revealed that bacterial strains differently affected free-radical scavenging activity in soybean leaves, roots, and nodules under drought stress, while the highest effects were obtained by combining *Bradyrhizobium* and *Bacillus* strains (Table 5). $ABTS^{\cdot+}$ radical cation is a chemical compound that is often used to generate free radicals in a sample. The $ABTS^{\cdot+}$ radical cation neutralization capacity determines the ability of the sample to reduce the con-

centration of free radicals. Effective non-enzymatic antioxidants that “collect” free radicals and prevent or disrupt their chain reactions include metabolites like glutathione, ascorbate, flavonoids, phenolics, tocopherol, and carotenoids that maintain a redox state in cells during oxidative stress, acting as ROS scavengers [69]. A lower concentration of ABTS^{•+} radical cations in inoculated plants indicates a greater ability of these plants to produce non-enzymatic antioxidant compounds. Enhanced antioxidant capacity may be the result of a higher concentration of these compounds and a symbiotic association that neutralizes free radicals more efficiently, indicating a greater ability to alleviate the deleterious effects of drought stress. Singh et al. [70] observed enhanced values of ABTS^{•+} free-radical cation scavengers in inoculated and drought-stressed plants. Similarly, a significant influence of inoculation with *Bradyrhizobium japonicum* and *Bacillus megaterium* strains on constitutive and drought-induced antioxidant parameters in soybean roots and nodules was obtained in our previous research [31].

Plant biomass as well as plant nitrogen content reliably indicate the N-fixation efficiency of symbiotic relationships. A water deficit reduces plant biomass as a consequence of the disturbance in metabolic processes, which are crucial to sustain plant growth. The termination of growth usually happens earlier in above-ground plant parts than in the roots, enabling the stressed plants to direct growth to reach remaining soil water and nutrients [71]. Our results showed that in water deficit conditions, shoot and nodule dry weight significantly decreased, while root dry weight was not significantly altered (Table 6). Previous studies reported a reduction in shoot [72,73] and root dry biomass in drought-challenged soybean plants [74]. Other studies also showed that drought may affect root architecture, root/shoot ratio, and increase root biomass [75,76]. In this study, drought significantly increased nitrogen content in shoots, roots, and nodules, while more prominent results were recorded via co-inoculation (Table 7). Increased accumulation of free amino acids, especially proline, as well as N-containing compounds, contributes to osmotic adjustment in plants under drought stress. Nitrogen fixation is extremely sensitive to drought, which significantly reduces nitrogenase activity and eventually stops it completely. The higher N concentration in drought-stressed soybean plants could be a consequence of ureide accumulation, which might be connected to a feedback inhibition of nitrogenase activity [77]. The levels of enzymes such as nitrate reductase, glutamine synthase, and glutamate synthase can also affect the nitrogen metabolism capacity of soybean plants under drought stress [78]. Our results are in alignment with previous studies, which reported increased nitrogen content in leaves and nodules of drought-stressed soybean plants [77,79]. Furthermore, Jabborova et al. [80] showed that co-inoculation with rhizobia and PGPR improved N content both in plants and soil under drought conditions.

Significant correlations between both enzymatic and non-enzymatic antioxidant parameters indicate their simultaneous action within the antioxidant defense system. In most cases, a significant connection was observed between overall antioxidant capacity and root and nodule biomass, as well as between antioxidant parameters and nitrogen content in shoots, roots, and nodules, suggesting improved drought tolerance (Table 8). The present study showed that higher and more extensive antioxidant responses, plant biomass, and N-fixation efficiency were achieved by the introduction of *B. subtilis* strains in a mixture with *Br. japonicum*. Moreover, positive effects of *Bacillus* and *Bradyrhizobium* strains on investigated soybean parameters could be related to production of IAA, exopolysaccharides, and ACC deaminase activity. These findings are in agreement with Dubey et al. [67], who showed that ACC-deaminase-producing *Bacillus* spp. strains from the soybean rhizosphere increased root and shoot length, proline, sugar, and protein, as well as catalase, superoxide dismutase, and peroxidase activities in drought-stressed plants. Further, a drought-tolerant strain, *Bacillus pumilus*, with capacity for IAA, siderophore, and exopolysaccharide production as well as phosphate solubilization, improves plant growth and biomass even under high drought stress via modulation of plant hormones and antioxidant gene expression [81]. Previous findings also imply that combined inoculants might be useful for soybeans to withstand drought stress, especially during critical growth

and development stages. Seed biopriming with indigenous *Bacillus* strains enhanced the soybean germination parameters in optimal and stressful laboratory conditions, whereas a single or co-inoculation had a better effect than *Bradyrhizobium* sole application [82]. Co-inoculation with PGPR and *Bradyrhizobium* strains led to an increase in soybean yield of 44% and had better effects compared to the control or single inoculation [83]. Moreover, co-inoculation with *Bradyrhizobium japonicum* and *Pseudomonas putida* significantly enhanced soybean growth, nodulation, and nutrient uptake, as well as soil nutrients and soil enzymes under normal conditions and drought stress [80]. The inoculation with *Br. japonicum*, *B. subtilis*, and arbuscular mycorrhizal fungi (AMF) improved the growth, nitrogen fixation, and yield parameters, along with an increase in chlorophyll and nutrient content and a decrease in proline content of soybeans under drought stress [84].

5. Conclusions

This study revealed that newly isolated native strains of *Br. japonicum* and *B. subtilis*, which are more tolerant to osmotic stress, have great potential to enhance the antioxidant defense system and nitrogen fixation efficiency of soybeans under drought conditions. The variability in soybean parameters significantly depended on the applied bacterial strains, while a more pronounced inoculation outcome was observed in water deficit conditions. Combined inoculants led to the highest increase in POX and POD activities in leaves, roots, and nodules, as well as ABTS⁺ radical scavenging capacity in roots and nodules under drought stress, suggesting the synergy of their PGP functions. On average, co-inoculation had the highest effect on the dry weight of shoots and nodules and N content in roots compared to the control, while *Bradyrhizobium* strains and co-inoculants had the best effect on the N content in shoots and nodules. Seed inoculation with *Br. japonicum* and *B. subtilis* consortia can be recommended as a sustainable strategy for improving symbiotic performance and drought tolerance of the soybean, along with maintaining plant development in stressful conditions. Future research should be conducted to observe the long-term effects of drought stress and the benefits of selected bacterial strains over a full growing season and several experimental years. Firstly, the effectiveness of selected inoculants on the agronomic and production characteristics of the soybean will be evaluated through field planting of cultivars with different levels of drought tolerance under different environmental conditions. In addition to the soybean, the effect of selected *Bacillus* strains will be evaluated on multiple crops, field-grown under different soil types, climates, and agricultural practices. Simultaneously, nitrogenase activity responsible for nitrogen fixation, as well as IAA content in plants responsible for improving stress tolerance, will be measured in order to fully estimate the effectiveness of the inoculants under drought conditions.

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