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Effect of Foliar Treatment with Aqueous Dispersions of Silver Nanoparticles on Legume-*Rhizobium* Symbiosis and Yield of Soybean (*Glycine max* L. Merr.)

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Abstract: Interest in the use of silver as a component of plant protection products and growth regulators appeared relatively recently with the development of methods for the effective stabilization of colloidal systems containing nanoparticles of this metal. In the present work, we studied the effect of foliar treatments with aqueous dispersions of silver nanoparticles stabilized by polyhexamethylene biguanide hydrochloride with an average diameter of 6 ± 1 nm and a zeta-potential of $+47.4 \pm 1.3$ mV on legume-*Rhizobium* symbiosis, which largely determines the efficiency of soil nitrogen assimilation and the yield of soybean (*Glycine max* L. Merr.). Based on the results of a two-year field experiment, it was shown that treatments with low doses of silver nanoparticles lead to a significant increase in the number of root nodules and an increase in soybean yield. The observed biological effectiveness of silver nanoparticles dispersions is explained by an increase in the enzymatic activity of peroxidases and polyphenol oxidases in the terrestrial part of plants. It is very likely that the treatment with silver nanoparticles and the increase in peroxidase activity in non-infected parts of the plant lead to a more effective prevention of the penetration of rhizobacteria into the aboveground soybean organs, which, in turn, may be the reason for the observed decrease in the activity of peroxidase and polyphenol oxidase in parts of plant roots susceptible to rhizobia. The latter, as is known, contributes to an easier flow of the nodulation process and the development of legume–*Rhizobium* symbiosis.

Keywords: silver nanoparticles; polyhexamethylene biguanide; soybean; foliar application; peroxidase; polyphenol oxidase; legume–*Rhizobium* symbiosis

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1. Introduction

Soybean is one of the most significant crops and interest in its cultivation technologies is increasing every year due to the growing demand for high-protein raw materials of plant origin [1,2]. World soybean production in the 2020–2021 agricultural year amounted to 364 million metric tons. In addition to general factors, such as soil and climatic conditions and the use of rational agrotechnologies for the application of agrochemicals and plant protection products, the value of soybean yield is largely determined by the efficiency of legume–*Rhizobium* symbiosis [3]. Legume–*Rhizobium* symbiosis is of particular importance for leguminous plants (although not strictly necessary) and considered as a source of available nitrogen synthesized as a result of biological fixation of atmospheric N_2 by prokaryotic organisms—diazotrophs (rhizobacteria or rhizobia in the case of legumes).

Symbiotic associations are very specific, and, as a rule, only one specific type of rhizobia is capable of infecting a plant of a particular species [4]. In particular, legumes (of the Fabaceae family) are capable of forming symbiotic associations only with Gram-negative bacteria of the genus *Rhizobium*, while soybean is capable of forming symbiotic associations with rhizobacteria of the species *Bradyrhizobium diazoefficiens*/*Bradyrhizobium japonicum* [5].

These soil bacteria can function and multiply independently of the plant but acquire the ability to fix nitrogen only in symbiosis with the plant organism, transforming into a bacteroid form and losing the ability to divide [6]. With legume–*Rhizobium* symbiosis, the so-called rhizobia are formed in the parenchymal tissue of the root nodules in which rhizobia, being in the form of bacteroid, fix molecular nitrogen under microaerobic conditions [6]. Such associations are of extremely significant economic importance; therefore, the task of finding growth regulators that affect not only the formation of reproductive organs and create the potential not only for the intensification of photosynthesis processes, but especially symbiotic processes, is relevant in modern chemical and agricultural science [7,8].

The widespread use of silver and its compounds in medicine has always been determined by the excess of the expected benefits over the risks of its use for therapeutic purposes [9]. For more than 30 years, there has been a steady increase in the use of silver nanoparticles (Ag NPs) in many other areas such as cosmetics, catalysis, chemical sensing, optoelectronics, and mainly in the health industry [10–13]. In the agriculture field, Ag NPs were also applied as fungicides, plant-growth stimulators, fruit ripening enhancement or seed treatments [14,15]. Interest in the practical use of Ag NPs as an active component of plant protection products (pesticides and growth regulators) appeared recently with the development of methods for the effective stabilization of the aqueous dispersions of Ag NPs and the recognition of the decisive role of stabilizer's chemical structure in the formation of dispersed systems with the required colloidal and biological properties [16].

Among different nanomaterials, producing metal nanoparticles with a precise control of surface properties and homogeneity of the particle size without agglomeration has consistently been a problem, which could be solved by modifying surface properties of nanoparticles using a proper stabilizer agent. Commonly used stabilizers for the synthesis of metal nanoparticles are polyvinyl alcohol (PVA), polyvinyl chloride (PVC), cetyltrimethylammonium ammonium bromide (CTAB) and polyvinylpyrrolidone (PVP) [17]. The use of chemical compounds from the class of polymeric guanidines for the effective stabilization of aqueous dispersions of silver NPs, which is applied in our experiment, was first proposed in 2009 [18].

Silver nanoparticles display unique biological effects and could be considered a novel stimulator for plant growth. Some of the positive activities of Ag NPs are such as enhanced biomass, stimulation of germination, or increased pigment content, and development of growth and postharvest life of the fruit [19]. Moreover, Ag NPs may enhance the amount of glutamine and asparagine, and improve the activity of catalase, superoxide dismutase (SOD) and peroxidase in roots and shoots [20]. Nowadays, there are many reports on phyto-stimulating effect of colloidal Ag NPs on various plants indicating species-specific features [21]. However, in most studies, when assessing the effect of NPs on ontogeny and biochemical parameters of plants, the structure of NPs surface and its effect on the biological activity of the applied nanomaterials are not considered [22,23]. Indeed, the results of the studies concerning the biological effects of Ag NPs often are very contradictory [18,24] due to the use of various stabilizers that affect the biological activity of NPs in different ways. In most of these works, there are no comprehensive data on colloidal properties of the studied materials. In this research, we studied the biological effect of aqueous dispersions of Ag NPs stabilized with positively charged macromolecules of polyhexamethylene biguanide hydrochloride (PHMB) with known colloidal stability, zeta potential, size distribution of NPs, etc., on the symbiotic apparatus (legume-*Rhizobium* symbiosis), plant development and yield of soybean.

2. Materials and Methods

2.1. Reagents and Materials

Silver nitrate (Sigma-Aldrich, St. Louis, MO, USA, 99+%), sodium borohydride (Acros Organics, Geel, Belgium, 99%), 20% aqueous solution of polyhexamethylene biguanide hydrochloride with an average molar mass of 3300 g/mol and an average degree of polymerization n = 15 (98%, Shanghai Terppon Chemical, Shanghai, China) were used without further purification. Distilled water was used in all experiments where needed.

2.2. Preparation of Silver NPs Dispersions

Aqueous dispersions of NPs containing 500 µg/mL of silver and 100 µg/mL of PHMB were obtained according to our previously developed procedure [25]. Briefly, 50 mL of a 0.046 mol/L aqueous solution of silver nitrate was added dropwise to 300 mL of 0.0167 wt.% aqueous solution of PHMB (M = 3300) with continuous stirring. The resulting milky-white suspension was kept for 15 min, and then an aqueous solution of sodium borohydride (150 mL, 0.062 mol/L) was added dropwise to this suspension with constant vigorous stirring. After adding the reducing agent, the mixture was kept under stirring for 1 h, while the color of the dispersion changed to dark brown. A portion of the obtained suspension washed several times with double distilled water and centrifuged at 25,000 rpm and finally dried with air for 24 h at 50 °C.

2.3. UV-Visible Spectrophotometry

A Shimadzu UV-1800 double-beam spectrophotometer (Shimadzu Corp., Kyoto, Japan) applied to record absorption spectra in the visible and near UV wavelengths.

2.4. Dynamic Light Scattering (DLS)

The size distribution of silver NPs was determined using a Zetasizer Nano ZS high-performance two-angle particle and molecular size analyzer (Malvern Instruments Ltd., Malvern, UK) equipped with a He–Ne laser (λ = 633 nm) with a power of 4 mW. The zeta-potential was measured by determining the particle velocity in an electric field using the laser Doppler anemometry method on the NanoBrook Omni attachment.

2.5. Transmission Electron Microscopy (TEM)

Microphotographic images of silver NPs samples were obtained using a LEO 912 AB OMEGA transmission electron microscope (Carl Zeiss, Wetzlar, Germany) with an operating accelerating voltage of 100 kV. Samples were prepared by applying 1–2 µL of the dispersion onto a formvar-coated copper grid, followed by air drying. Particle size distribution from microscopy data was calculated using the Femtoscan Online v. software. 2.2.91. (Center for Advanced Technologies, Moscow, Russia).

2.6. Powder X-ray Diffraction

X-ray images were recorded by a Bruker D8 Advance X-ray diffractometer (in the Bragg-Brentano geometry) using CuKα radiation from the anode. Diffraction peaks were identified using the JCPDS database. The sizes of coherent scattering regions (CSRs) of nanocrystalline silver samples were calculated using the Scherrer formula:

$$D_{hkl} = \frac{K \cdot \lambda}{[\beta_{hkl}(2\theta) - s]\cos(\theta)} \quad (1)$$

where θ is the position of the peak maximum, λ is the CuK_{α} X-ray wavelength (0.154056 nm), β_{hkl} (2θ) is the total physical broadening of the diffraction maximum, and s is the instrumental broadening (0.1°). The value of the Scherrer constant (K) was taken equal to 1. To determine the value of β, after subtracting the background, the profile of the (111) silver X-ray peak was mathematically described by the pseudo-Voigt function.

2.7. Field Experiment on the Biological Effectiveness of Silver NPs Dispersions

The study of the biological effectiveness of silver dispersions was carried out under the conditions of a field experiment in 2019–2020 on the farm of JSC "Anastasievskoye" of the Slavyansky district of the Krasnodar Territory under the control of the Kuban State Agrarian University. In the experiment, an aqueous dispersion of colloidal silver (500 mg/L) stabilized with polyhexamethylene biguanide hydrochloride (100 mg/L) (Ag-PHMB) was used. The soil of the experimental site is leached chernozem. Agrochemical analyses of the soil were carried out in the Krasnodar Center of Agrochemical Service. The humus content averaged 2.8% over three years. The reaction of leached chernozems was neutral, and the exchangeable acidity of the soil was pH_{KCl} = 7.1. The leached chernozem contained P_2O_5—96 mg/kg of soil; K_2O—430 mg/kg of soil; hydrolytic acidity—0.63 mmol/100 g; the sum of the absorbed bases was 20.8 mq per 100 g. The experiment scheme was presented in six variants:

1. Control—N0P0 (without additional application of fertilizers—mineral nitrogen and phosphorus), no treatment with Ag-PHMB;
2. Background—N30P40 (additional application of fertilizers—30 kg/ha of mineral nitrogen and 40 kg/ha of mineral P_2O_5; the sources of mineral nitrogen and P_2O_5 were NH_4NO_3 and $NH_4H_2PO_4$), no treatment with Ag-PHMB;
3. Background + Ag-PHMB—seed treatment (25 mL/ton, working solution consumption 10 L/ton) + plant spraying: 1st—in the phase of 3rd trifoliate (V3 phase), 2nd—in the phase of beginning bloom (R1 phase) (40 mL/ha, working solution consumption 300 L/ha);
4. Background + Ag-PHMB—seed treatment (50 mL/ton, working solution consumption 10 L/ton) + plant spraying: 1st—in the phase of 3rd trifoliate (V3 phase), 2nd—in the phase of beginning bloom (R1 phase) (80 mL/ha, working solution consumption 300 L/ha);
5. Background + Ag-PHMB—seed treatment (75 mL/ton, working solution consumption 10 L/ton) + plant spraying: 1st—in the phase of 3rd trifoliate (V3 phase), 2nd—in the phase of beginning bloom (R1 phase) (120 mL/ha, working solution consumption 300 L/ha);
6. Background + PHMB (pure stabilizer without silver NPs)—seed treatment (75 mL/ton, working solution consumption 10 L/ton) + plant spraying: 1st—in the phase of 3rd trifoliate (V3 phase), 2nd—in the phase of beginning bloom (R1 phase) (120 mL/ha, working solution consumption 300 L/ha).

The experiment was performed in four repetitions, and the area of the plot in each case was 20 m^2. Soybean cultivation technology is generally accepted for this agro-climatic zone with a temperate continental climate. To optimize mineral nutrition, nitrogen and phosphorus fertilizers were applied in spring for pre-sowing tillage at a dose of N30P40 (mineral fertilizers were applied at 30 and 40 kg/ha, respectively, in terms of nitrogen and phosphorus). For the mineral nutrition of plants, ammonium nitrate (N—34 wt.%) and ammonium dihydrogen phosphate (N—11 wt.%, P—50 wt.%) were used.

Soybeans of the mid-season cultivar Vilana were sown (selection and seed production—V.S. Pustovoit All-Russian Research Institute of Oil Crops) with a vegetation period of 115–118 days. Before sowing, soybean seeds, in accordance with the scheme of the experiment, were manually processed using a sprayer (moistening 2.0%) with an aqueous solution of the Ag-PHMB dispersion. In the control and background variants, the seeds were treated with distilled water. In the 6th variant, the seeds were treated with an aqueous solution of the stabilizer at a dosage corresponding to its content in the Ag-PHMB dispersions. Spraying of vegetative plants was carried out twice—in the 3rd trifoliate (V3) phase and the beginning bloom (R1) phase. Distilled water was used for dilutions. The determination of indicators of photosynthetic activity of crops was carried out according to the method of A.A. Nichiporovich [25,26]. The soybean yield was recorded when the phase of full maturity (R8) was reached.

During the entire period of the field trials, no inoculants containing industrial strains of rhizobacteria were introduced into the soil. All rhizobacteria that were the cause of the formation of nodules were part of the natural rhizosphere of the soil of the place of the experiment.

Tissues selected in the field from different parts of plants (leaves, stem, root) were immediately frozen after separation at a temperature of −196 °C and transported in

Dewar vessels containing liquid nitrogen directly to the place where enzymatic activity was determined.

2.8. Determination of the Specific Activity of Peroxidase in Soybean Biomass

Peroxidase activity (EC 1.11.1.7) was determined using the classical spectrophotometric technique [27], which is based on measuring the rate of oxidation of benzidine (4,4′-diaminodiphenyl) with hydrogen peroxide in the presence of peroxidase. The frozen plant material was homogenized in acetate buffer (pH 5.4). The resulting homogenate was subjected to centrifugation, and the precipitate was discarded. Then, the kinetics of the oxidation of benzidine with hydrogen peroxide in the presence of peroxidase contained in the supernatant was studied. Peroxidase activity was determined from the increase in the optical density of the benzidine oxidation product (diaminodiphenyl quinone), measured at a wavelength of 590 nm, and was calculated in arbitrary units per 1 g of raw plant material per second. The determination of peroxidase activity in biomass was carried out in triplicate. Biomass samples were taken from five different plants.

2.9. Determination of the Specific Activity of Polyphenol Oxidase in Soybean Biomass

PPO activity (EC 1.10.3.1) was determined using a spectrophotometric technique [27] based on measuring the kinetics of pyrocatechol oxidation with atmospheric oxygen in the presence of PPO by recording an increase in the optical density of pyrocatechol oxidation products. For this, the plant material was homogenized in phosphate buffer (pH 7.4) with the addition of polyamide, the mixture was subjected to centrifugation, and the precipitate was discarded. The supernatant solution containing PPO was mixed in a cuvette with a solution of catechol and phosphate buffer, and the change in optical density was recorded at a wavelength of 420 nm every 2 s for 120 s. PPO activity was calculated in arbitrary units per 1 g of raw plant tissue per minute. The determination of PPO activity in biomass was carried out in triplicate. Biomass samples were taken from five different plants.

2.10. Determination of Residual Amounts of Silver in Biomass

The quantitative content of silver in soybean samples was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES) on an Agilent 720-ES spectrometer (Agilent Technologies, Santa Clara, CA, USA) with a Cetac 5000 UT+ ultrasonic nebulizer. Leaves were separated from soybean plant samples (randomly from different parts of the sample), or small pieces of stems were cut (randomly from different parts of the sample) in the amount of 10 g. Samples taken from one plant were averaged. A sample of stems or leaves weighing about 2.5 g, taken with an accuracy of 0.1 g, was placed in a beaker, 10 mL of concentrated HNO_3 (Panreac) and 10 mL of concentrated $HClO_4$ (Panreac) were added, and heated until complete decomposition, stirring occasionally, avoiding boiling. Upon completion of decomposition, the solution was evaporated to intense white vapors of $HClO_4$, cooled, transferred to a 25 mL volumetric flask, and diluted with deionized water (Milli-Q, 18.2 MΩ cm) to the mark, and finally, the obtained solutions were analyzed.

The determination of silver was carried out in five repetitions among samples taken from plots with the maximum application rate of the Ag-PHMB preparation.

2.11. Statistical Processing of Results

Standard deviations were calculated for the obtained data (n = 3 or 4). Analysis of statistically significant deviations among treatment options was determined using a one-way analysis of variance (ANOVA) test. SPSS 20.0 (IBM, Endicott, NY, USA), followed by Tukey’s mean analysis at a significance level of 0.05. In some cases, the least significant difference (multiple t-test without alpha correction) is also given.

The statistical processing of data from the field experiment was carried out according to B.A. Dospekhov [28]. The experimental values were considered to be significantly different if the difference between them exceeded the calculated least significant difference HSR05 for the 5% significance level.

3. Results and Discussion

3.1. Synthesis of Aqueous Dispersions of Silver NPs Stabilized with PHMB

The chemical reduction of ionic silver with sodium borohydride is one of the most frequently used methods for the preparation of silver NPs in both homogeneous and heterogeneous systems [25]. The reduction of silver with sodium borohydride in the presence of PHMB leads to the formation of a brown-red aqueous dispersion, which is able to maintain colloidal stability for 3 years. The average diameter of as obtained Ag NPs is 6 ± 1 nm. The UV-vis absorption spectrum of aqueous Ag NPs dispersion shows a characteristic absorption band of silver NPs with a maximum at the wavelength of 400–410 nm due to the surface plasmon resonance (Figure 1a,c). The intensity of this band was used to assess the colloidal stability of Ag NP dispersions.

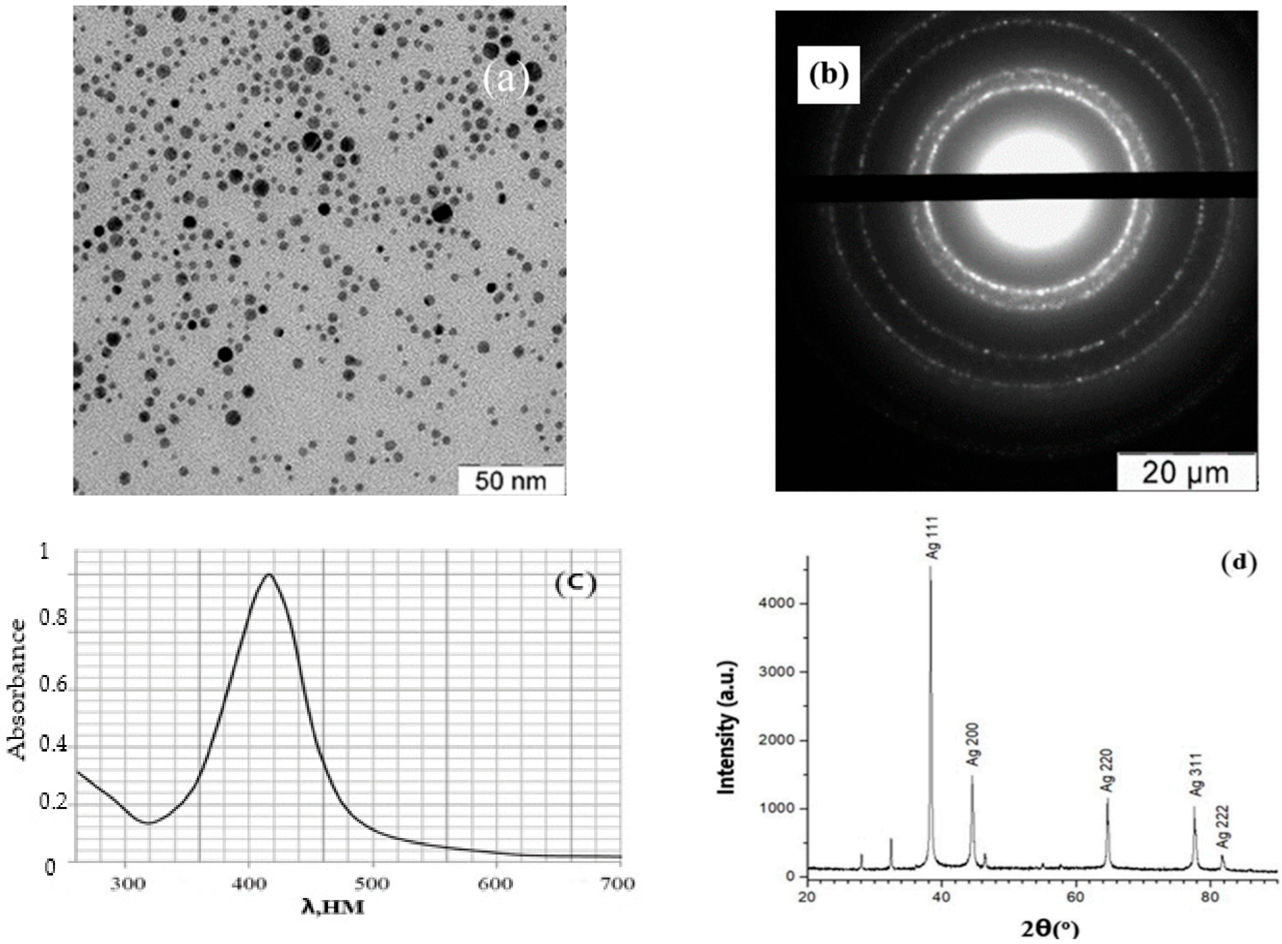

Figure 1. Electron micrographs (**a**), electron microdiffraction (**b**), absorption spectrum in the visible region (**c**), and data of X-ray phase analysis (**d**) of silver NPs stabilized by PHMB used during the field trials.

The data of electron microdiffraction (Figure 1b,d) on the crystalline phase of the samples were in full agreement with the results of X-ray phase analysis. The distances between the atomic planes of the crystal lattice (d-spacing) were determined by measuring the diameters of the first four rings in the diffraction pattern. The identity of the values of the first four d-distances in the diffraction pattern obtained from a sample of PHMB-stabilized Ag NPs (2.34, 2.01, 1.43, 1.22 Å), and the standard (metallic silver) (2.36, 2.04, 1.44, 1.23 Å) confirmed that Ag NPs had a crystalline structure of metallic silver [29]. In the diffraction pattern obtained from the passage of an electron beam with an energy of 100 keV through the selected region of the sample of NPs stabilized by PHMB, lines of the amorphous phase (2–2.3 Å) were also recorded, which also confirms the presence of disordered polymer molecules of the stabilizer on the surface of Ag NPs.

Since PHMB, as was mentioned above, is a polycationic polymer, the silver NPs stabilized by it carry a positive electrokinetic potential at the slipping plane. The determination of the zeta-potential of PHMB-stabilized Ag NPs gave a value in the range of +47 $\pm$ 1 mV, which indicates that the electrostatic component plays a key role in the colloidal stability of such dispersions. Indeed, we have previously shown that a decrease in the zeta-potential below the critical value of +30 mV for such systems by reaching the critical concentration of chloride anions acting as a coagulation agent leads to a complete loss of the colloidal stability of PHMB stabilized Ag NPs [25].

3.2. Field Experiments

3.2.1. Weather Conditions

Weather conditions in 2019 were favorable for the growth and development of soybeans, especially in the first half of the growing season. Precipitation in May and June contributed to the active growth and development of plants. Dry winds during the growing season were not observed, the wind speed was insignificant, and the relative humidity of the air in its parameters fitted into the optimal values. In May–August, 277.3 mm of precipitation fell, while the climatic norm for this period was 232.0 mm. Less than normal precipitation fell only in August—by 23.3%. The average daily air temperature exceeded the norm by 1.9 °C, and the relative humidity was close to the norm.

The weather conditions in 2020 differed significantly from the long-term average and were not favorable enough for the growth and development of plants. There was a shortage of precipitation in June and August at average daily temperatures exceeding the climatic norm. The high temperature and the established long-term drought had a negative impact on the formation of reproductive organs, soybean yields and the quality of the products obtained. However, due to the rains in May and July, the amount of precipitation during the soybean growing season was close to the climatic norm: during the period May–August, 223.0 mm of precipitation fell against a norm of 232.0 mm. The average daily air temperature exceeded the long-term average values by 2.1–3.1 °C. The relative air humidity was close to the climatic norm.

3.2.2. Effect of Different Seed Treatments

Treatment with a stabilizer solution (the sixth version of the experimental scheme) did not lead to significant differences with the background variant (the second version of the experimental scheme) in terms of such parameters as the number of nodules, morphometric parameters, enzyme activity and yield; therefore, the experimental data obtained in this variant were excluded from further research discussion.

Seed treatment with Ag-PHMB dispersion and subsequent foliar treatment of plants with Ag-PHMB dispersion against the background of nitrogen–phosphorus nutrition statistically significantly increased the number of root nodules compared to the variants without treatments with colloidal silver dispersions. The number of nodules was counted in the phase R1 of beginning bloom (this phase also called a "budding phase"—a stage just before flowering) and in the R6 phase of full seed. In R6 phase pod containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf. In the R1 phase, an increase in the number of nodules in the parenchymal tissue of the plant root was noted, which was directly dependent on the dose of the applied preparation. At the same time, the average (for 2019–2020) number of root nodules in the R1 phase in the control variant was 4.8 pcs/plant (with an average weight of 23.7 mg/plant), and in the background variant, it was 4.3 pcs/plant (with an average weight of 23.3 mg/plant); the average (for 2019–2020) number of nodules in the pod formation phase in the control variant was 14.8 pcs/plant (with an average weight of

218.2 mg/plant); in the background variant, it was 21.5 pcs/plant (with an average weight of 359.3 mg/plant).

With an increase in the dosages of the Ag-PHMB dispersion during seed treatment and foliar application in the phases of 3rd trifoliate (V3) and bloom beginning (R1), the average number of nodules in the bloom beginning (R1) phase increased from 5.2 (options 3 and 4) to 5.7 (option 5) pcs/plant. The average weight of nodules in the R1 phase was 33.8 mg, 36.5 mg, and 40.1 mg for the 3rd, 4th, and 5th variants, respectively.

Similarly, with an increase in the dosages of the Ag-PHMB dispersion during seed treatment and foliar application in the phases V3 and R1, the average (for 2019–2020) number of nodules in the bean formation phase increased from 29.7 nodules/plant (option 3) to 33.0 pcs/plant (option 5). The average weight of nodules increased from 568.2 mg/plant to 731.7 mg/plant for the third and fifth variants, respectively. In variant 4, the average number of nodules was 30.5 pieces/plant with an average weight of 620.8 mg/plant. Generalized measurement results are given in Table 1. Thus, there is a dependence between the development of the symbiotic organs of plants and the dose (seed treatment and double foliar treatment) of applied Ag-PHMB dispersion. With an increase in the dose of the applied dispersion, both the average number of nodules and their average weight per plant increased statistically significantly. The difference between the control variant and the variant with the maximum dose of application of the Ag-PHMB dispersion was especially clear during the R6 phase, where the average weight of nodules in the 5th variant exceeded the mass of nodules in the control and background variant by 235 and 104%, respectively.

Table 1. Effect of seed and foliar treatments during phases V3 and R1 with aqueous dispersions of silver NPs stabilized with PHMB (Ag-PHMB) on the number and average weight of root nodules (averaged values for 2019–2020), confidence probability, $p = 0.95$.

Treatments	Average Number of Root Nodules per Plant		Average Weight of Root Nodules, mg per Plant	
	Phase R1	Phase R6	Phase R1	Phase R6
Control-N_0P_0, no treatment with Ag-PHMB dispersion	4.8 ± 0.1	15 ± 1	24 ± 2	218 ± 5
Background ($N_{30}P_{40}$), no treatment with Ag-PHMB dispersion	4.3 ± 0.2	22 ± 1	23 ± 2	259 ± 6
Background, Ag-PHMB dispersion—seed treatment 25 mL/t + double plant spraying −40 mL/ha	5.2 ± 0.1	30 ± 2	34 ± 3	568 ± 4
Background, Ag-PHMB dispersion—seed treatment −50 mL/t + double plant spraying −80 mL/ha	5.2 ± 0.2	31 ± 1	37 ± 2	621 ± 6
Background, Ag-PHMB dispersion—seed treatment −75 mL/t + double plant spraying −120 mL/ha	5.7 ± 0.2	33 ± 2	40 ± 3	732 ± 6
LSD_{05}	0.31	0.16	9.02	6.95

The development of legume–*Rhizobium* symbiosis is influenced by many factors of temporal, genetic, biochemical, physiological, and ecological nature [30,31]. Thus, at the initial stage of nodule formation, legumes secrete special chemotactic compounds (polyphenols, flavonoids), which are recognized by rhizobia [32]. The main flavonoid that is endogenously produced by soybean plants to attract *Bradyrhizobium diazoefficiens*/*Bradyrhizobium japonicum* is daidzein (1), a biologically active substance from the isoflavone class with multiple signaling and other physiological functions [33].

The concentration of this polyphenol, like all other antioxidant compounds, depends on the activity of PPO and other enzymes of the oxidase class [33]. To elucidate the relationship between the intensity of the development of legume–*Rhizobium* symbiosis (one of the indicators is the number and weight of nodules) and the activity of enzymes, we carried out an experimental assessment of the level of activity of PPO and peroxidase in the roots and aboveground parts of soybean plants on the second day after treatment with

Ag-PHMB dispersion in R1 phase. The study of enzyme activity was carried out in the growing season of 2020.

The high protein content in soybean seeds and plants determines the high need of the culture for nitrogen, which is largely satisfied because of the endogenous biosynthesis of ammonium nitrogen from molecular nitrogen by nodule bacteria. The effectiveness of legume–*Rhizobium* symbiosis depends on the size and activity of the symbiotic apparatus. The dynamics of the formation of the number of nodules in all variants of the experiment was different (Figure 2).

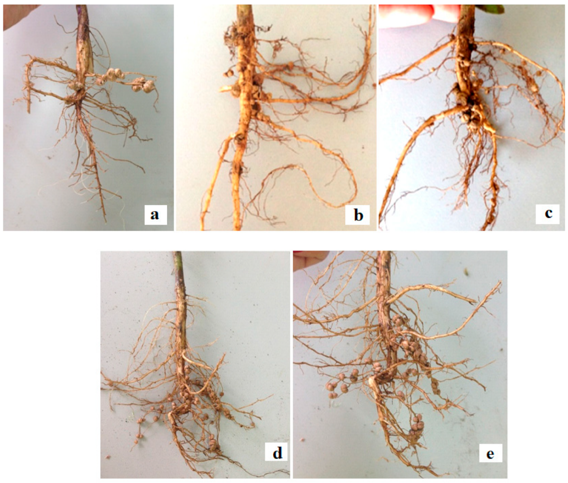

Figure 2. Development of the root system and symbiotic apparatus of soybean plants upon treatment with Ag NPs dispersions: (**a**) Control, N_0P_0, no treatment with Ag-PHMB dispersion; (**b**) background ($N_{30}P_{40}$), no treatment with Ag-PHMB dispersion; (**c**) background, Ag-PHMB dispersion—seed treatment 25 mL/t + double plant spraying (V3 and R1 phases) 40 mL/ha; (**d**) background, Ag-PHMB dispersion—seed treatment 50 mL/t + double plant spraying (V3 and R1 phases) 80 mL/ha; (**e**) background, Ag-PHMB dispersion—seed treatment 75 mL/t + double plant spraying (V3 and R1 phases) 120 mL/ha.

At the same time, the mass of raw nodules and their size changed significantly depending on the treatment with Ag-PHMB dispersion in various doses, thereby leading to stimulation of the development of a powerful root system and active (with a high content of leghemoglobin) nodules on soybean roots, increasing the nitrogen-fixing ability of legumes plants. In the control and background variants, one can notice a smaller number of them, while in the variant where the seeds were treated with Ag-PHMB dispersion at a dose of 75 mL/t and foliar treatment of plants was carried out at a dose of 120 mL/ha, there was a maximum number of nodules in comparison with other options (Table 2).

As a result, it was found that the activity of PPO in the aerial parts of plants increased upon treatment with dispersions of Ag NPs, while the activity of this enzyme decreased in the roots. The pattern of changes in peroxidase activity was the same: a significant increase in activity in the aerial part and a slight decrease in activity in the root part. A decrease in the activity of these two key antioxidant enzymes can lead to an increase in the content of various flavonoids, including the signaling substance daidzein, which promotes nodulation at the initial stages. However, this factor (a possible increase in the endogenous synthesis of daidzein by the roots), which affects the infection of root hairs only at the very initial stage, in our opinion, cannot be decisive. Indeed, it is well known that only 1–5% of the total number of all root hairs are infected with rhizobia, and only 10–20% of such cases lead to the formation of nodules [34]. The rest of the infection threads stop their growth and die because of the hypersensitive response (HR) of the plant to invasion of rhizobia and cells apoptosis. HR is a well-known process that occurs with the participation and interaction of ROS and reactive nitrogen species (RNS) [35]. Despite the fact that the process of reduction of molecular nitrogen to ammonium nitrogen in the presence of the nitrogenase enzyme occurs under strictly anaerobic conditions, plants need to maintain a strictly controlled level of ROS, which are necessary for the growth and development of the root system and the regulation of various responses to the impact of biotic and abiotic factors (change soil moisture and chemical composition, pathogen invasion, temperature fluctuations, and many others). Numerous studies have shown that the concentration of ROS and ethylene increases in the infection threads of legumes, but only in the space surrounding the bacteria and not in the bacteria themselves [6]. To reduce the destructive effect of ROS on infectious pathways while maintaining the concentration of ROS at the level necessary for the implementation of important signaling functions, nodules contain an impressive set of antioxidant systems, only a few of which, for example, glutathione peroxidase [36], have been characterized in detail to date. An important component of such systems, in addition to plant homologues of respiratory burst oxidases (Rboh, NADPH oxidases) [37,38], is undoubtedly PPO and peroxidase, which control the concentration of ROS and perform two opposite functions, i.e., the regulation of mechanisms that ensure the development of a symbiotic interaction between rhizobia and soybean plant (PPO), and the regulation of mechanisms preventing the development of legume-*Rhizobium* symbiosis (peroxidase). Indeed, there is evidence that the activity of soluble peroxidases in pea roots decreases upon infection but increases in aboveground areas that are immune to rhizobia [39].

Table 2. Influence of treatments with aqueous dispersion of PHMB-stabilized silver NPs (Ag-PHMB) on the activity of PPO and peroxidase enzymes in the roots and aboveground parts of soybean plants (average values for 2020), confidence probability, p = 0.95.

Treatments	Polyphenol Oxidase Activity, [Arbitrary Units (AU) per 1 g of Raw Plant Tissue per Minute]		Peroxidase Activity, [Arbitrary Units (AU) per 1 g of Raw Plant Tissue per Second]	
	Roots	Leaves	Roots	Leaves
Control—N_0P_0, no treatment with Ag-PHMB dispersion	8.7 ± 0.3	5.8 ± 0.1	291 ± 9	169 ± 3
Background ($N_{30}P_{40}$), no treatment with Ag-PHMB dispersion	9.1 ± 0.2	5.4 ± 0.2	276 ± 7	155 ± 2
Background, Ag-PHMB dispersion—seed treatment −25 mL/t + double plant spraying −40 mL/ha	8.1 ± 0.3	6.1 ± 0.1	249 ± 8	180 ± 5
Background, Ag-PHMB dispersion—seed treatment −50 mL/t + double plant spraying −80 mL/ha	7.8 ± 0.1	6.3 ± 0.3	245 ± 8	191 ± 7
Background, Ag-PHMB dispersion—seed treatment −75 mL/t + double plant spraying −120 mL/ha	7.5 ± 0.2	6.3 ± 0.1	249 ± 5	187 ± 7

These data are in correlation with the results obtained in our work. Previously, using tomato as an example, we showed that the treatment of plants infected with phytopathogenic strains of fungi with Ag NPs dispersions led to a significant increase in peroxidase activity in the aerial part of the plant [40]. A higher dynamic of peroxidase activity's growth in the ground part of soybean plants infected with rhizobia, which were treated with a dispersion of Ag NPs stabilized with PHMB, indicates an increase in the activity of one of the key nonspecific defense reactions and, consequently, the nonspecific resistance of the treated plants. It is very likely that treatment with Ag NPs and the increase in peroxidase activity in uninfected parts of the plant lead to a more effective prevention of the penetration of rhizobia into the soybean aerial organs. On the other hand, the decrease in the activity of peroxidase and PPO in parts of plant roots that are susceptible to rhizobia may be the reason that facilitates the process of nodulation and the development of legume–*Rhizobium* symbiosis. The multidirectional changes in enzyme activity in the root and aboveground parts of the plant shown in our work agree with the well-known concept of the spatiotemporal scheme of symbiosis regulation with the participation of ROS and RNS [41]. It can be said that the treatment with silver NP dispersions has a complex effect on the immunity of the leguminous plant—it enhances the development of induced systemic resistance (ISR) of the aerial parts of the plant to external biotic and abiotic factors, including infection of various parts of the plant with rhizobia. All the described processes occurring under the influence of treatments with Ag NPs together can lead to an increase in the number and total mass of root nodules and, as a result, the intensification of other metabolic processes that depend on the efficiency of nitrogen assimilation.

Photosynthesis is another and perhaps the most important physiological process, which is dependent on nitrogen uptake [42]. The use of Ag-PHMB for seed treatment in combination with spraying of vegetative plants contributed to the active growth of the leaf surface. In the pod formation phase, the difference between the average leaf area in the background variant and the experiment with the maximum dosage of Ag NPs dispersion was 30.7%.

It is known that about 95% of dry organic matter is formed in the process of photosynthesis; therefore, the formation of the maximum soybean yield is impossible without the creation of an optimal leaf surface and photosynthetic activity [42]. The maximum average leaf surface of a soybean plant was formed in the R6 phase; it reached, depending on the weather conditions of the growing season and experimental options, 535.3–671.8 cm^2 in the R1 phase and 584.5–842.9 cm^2 in the R6 phase. Seed treatment and double plant spraying during V3 and R1 phases with Ag-PHMB dispersion contributed to an increase in the assimilation surface during the R1 phase and the R6 phase observed in a two-year study. The maximum increase in the leaf surface area was noted in the R6 phase when using Ag-PHMB dispersion at a dose of 75 mL/t + 120 mL/ha (Table 3).

Table 3. Effect of the treatment with aqueous dispersion of Ag-PHMB on the morphological parameters of soybean plants (Vilana cultivar) during the R1 and R6 phases (average data for 2019–2020), confidence probability, $p = 0.95$.

Treatments	Plant Height, cm		Leaf Area, cm^2	
	Phase R1	Phase R6	Phase R1	Phase R6
Control—N_0P_0, no treatment with Ag-PHMB dispersion	68 ± 2	83 ± 3	535 ± 15	585 ± 31
Background ($N_{30}P_{40}$), no treatment with Ag-PHMB dispersion	72 ± 3	88 ± 2	559 ± 18	645 ± 37
Background, Ag-PHMB dispersion—seed treatment −25 mL/t + double plant spraying −40 mL/ha	78 ± 3	92 ± 3	600 ± 16	712 ± 39
Background, Ag-PHMB dispersion—seed treatment −50 mL/t + double plant spraying −80 mL/ha	81 ± 3	101 ± 2	638 ± 22	781 ± 41
Background, Ag-PHMB dispersion—seed treatment −75 mL/t + double plant spraying −120 mL/ha	85 ± 3	105 ± 3	672 ± 24	843 ± 42
$LSD_{0.5}$	3.4	4.2	28.9	46.5

The main factors determining the growth and development of plants and, ultimately, the yield of soybeans are the biological characteristics of the variety and the soil and climatic conditions of the growing region. However, the rational use of modern agricultural technologies, including the use of plant growth regulators, contributes to obtaining a quality crop in an amount that is as close as possible to the genetic potential of the variety. Indeed, field studies have shown that the use of Ag NPs dispersion had an additional positive effect on the growth of soybean plants. In the phases of R1 and R6 in all variants with the use of an aqueous dispersion of Ag-PHMB, soybean plants had a significantly higher height than in the variants without treatment. Moreover, the maximum values were noted in the variant with the maximum concentration of the Ag-PHMB aqueous dispersion, where the average height of soybean plants reached 92.4 and 105.2 cm, which is higher than the control variant by 17.1 and 22.4 cm and the background variant by 13.4 and 17.4 cm.

In comparison with the background and control variant, the highest degree of increase in leaf area and plant height during seed treatment and double spraying with Ag-PHMB dispersion was noted in 2020, which was unfavorable in terms of climatic conditions. This was especially noticeable in the early stages of plant growth and development. The formation of the largest leaf surface area in the variants with the use of Ag-PHMB dispersion at a dose of 75 mL/t + 120 mL/ha also provided higher soybean productivity. Even though different weather conditions in 2019–2020 had a significant impact on the formation of the crop, in general, the use of the Ag-PHMB dispersion in the V3 phase and beginning bloom (R1) phase significantly increased the yield of soybean grain both in 2019 and 2020.

The positive trend in the application of Ag NPs dispersions with maximum dosages persisted throughout the entire period of research. Two-year trials have shown that with an increase in the dose of the Ag NPs, the soybean yield increased. In 2019, the increase in soybean seed yield was 27.3–33.1% with a yield in control of 2050 kg/ha and 13.9–19.2% against the background, with a yield in the background of 2290 kg/ha. With insufficient moisture supply in 2020, a positive effect of the Ag-PHMB dispersion on soybean yield was also recorded. The increase in yield increased by 9.6–31.4% with a yield in the control variant of 19,700 kg/ha and by 13.4–19.9% compared with the background, with a yield against the background (21,600 kg/ha) (Table 4).

Table 4. Effect of the treatment of soybean plants with Ag-PHMB dispersion on soybean yield in 2019–2020, confidence level, $p = 0.95$.

Treatment	**Yield, kg/ha**		**Increase to Control, kg/ha**		**Increase in the Background, kg/ha**	
	2019	**2020**	**2019**	**2020**	**2019**	**2020**
Control—N_0P_0, no treatment with Ag-PHMB dispersion	2100 ± 100	2000 ± 100	-	-	-	-
Background ($N_{30}P_{40}$), no treatment with Ag-PHMB dispersion	2300 ± 100	2200 ± 100	200 ± 200	200 ± 200	-	-
Background, Ag-PHMB dispersion—seed treatment −25 mL/t + double plant spraying −40 mL/ha	2600 ± 100	2500 ± 100	600 ± 200	500 ± 200	300 ± 200	300 ± 200
Background, Ag-PHMB dispersion—seed treatment −50 mL/t + double plant spraying −80 mL/ha	2700 ± 100	2500 ± 100	600 ± 200	600 ± 20	400 ± 200	400 ± 200
Background, Ag-PHMB dispersion—seed treatment −75 mL/t + double plant spraying −120 mL/ha	2700 ± 100	2600 ± 100	700 ± 200	600 ± 200	400 ± 200	400 ± 200
$LSD_{0 \cdot 5}$	1.46	1.18	-	-	-	-

At the macroscopic level, the increase in soybean yield under the action of small doses of Ag NPs, in addition to the effect on the processes of legume–*Rhizobium* symbiosis, may be the result of a superposition: effects associated with blocking the ethylene-dependent signaling system [43,44] and consisting mainly of stimulation vegetation, root and shoot growth, biomass accumulation and slowing down of maturation and wilting processes; effects associated with oxidative stress, consisting in disruption of normal cell activity, on the one hand, and stimulation of resistance to adverse external influences, including infection with phytopathogens, on the other hand. That is, the effect of small concentrations of Ag NPs in our case should be stimulating in relation to the growth, development, and resistance of plants, while higher concentrations (from 50 mg/L) should have the opposite phytotoxic effect [45]. In this regard, it is interesting to note the work [46], where the effect of high concentrations of Ag NPs stabilized by polyvinylpyrrolidone (PVP) in soil (up to 800 µg/kg) on the germination and development of garden bean (*Vicia faba* L.) plants was studied. Such high doses of Ag NPs inhibited germination by 40%, significantly slowed down the processes of nodulation and arbuscular-mycorrhizal interaction, and nitrogenase activity.

The results of field studies of the biological effect of colloidal silver on soybeans are in good agreement with the previously described mechanisms of action of silver at the molecular level [47,48]. Over the past few years, several works have appeared devoted to the experimental study of the molecular mechanisms of the effect of low doses of Ag NPs (15 nm, 2–5 mg/L) on the development of soybean seedlings under stressful conditions of wetting (excessive moisture) [49,50]. It is known that soaking has a very negative effect on soybean development, especially at the initial stages, and is accompanied by hypoxia, deficiency of CO_2, ethylene, and other signaling compounds, which leads to disruption of normal metabolism and accumulation of phytotoxic metabolites in cells [51–53]. These works demonstrated the positive effect of Ag NPs on plants under abiotic stress. The study of changes in the transcriptomic and proteomic profiles showed a decrease in the activity of glyoxalase II 3, a decrease in the expression of alcohol dehydrogenase 1 and pyruvate decarboxylase 3 genes, which may indicate a levelling of the effect of hypoxia and a decrease in the formation of concomitant cytotoxic metabolites of glycolysis (glyoxal, etc.). In general, changes in the proteomic profile of soybean seedlings under the action of Ag NPs concerned proteins responsible for the adaptive response to stress, signaling functions, and cell metabolism. Such changes, according to the authors, were the cause of metabolic shifts characteristic of the functioning of plant cells under normal conditions, which led to a positive effect on the growth and development of soybean plants under stress. The above regularities can be an indirect confirmation of the significant increase (compared to 2019) in the leaf surface area and plant height (compared to the background and control variants) observed by us in the field experiment at the stages of growth and development in June 2020 under conditions of abiotic stress (drought).

3.3. Determination of Residual Amounts of Silver in Plant Biomass

In parallel with the study of the effect of the Ag-PHMB dispersion on legume–*Rhizobium* symbiosis and soybean yield, studies were carried out to determine the silver content in the dry mass of plants after seed treatment with Ag-PHMB dispersion of 75 mL/t and double spraying of vegetative plants with Ag-PHMB dispersion at a dosage of 120 mL/ha. According to the results of the development of an analytical method for determining the silver content in liquid samples obtained by decomposing dry plants with a mixture of nitric and perchloric acids, it was found that the lower limit of the determined silver content under these conditions is 1 μg/L. In the solutions of the sample with the addition of silver, 10 and 11 μg/L for leaves and stems, respectively, were found with an additive of 10 μg/L, which confirmed the correctness of the analysis results (according to the "introduced-found" method).

In all the studied samples, after decomposition, the silver content was less than 1 μg/L, which corresponded to the content in dry matter of less than 0.1 μg/kg. Thus, the silver content in the studied samples was below the limits of analytical detection.

4. Conclusions

The effect of foliar treatments with aqueous dispersions stabilized by PHMB silver NPs on the legume–*Rhizobium* symbiosis and the yield of soybean was studied. The dispersions were previously characterized in detail from the colloid and chemical points of view: synthesized Ag NPs had an average diameter of 6 $\pm$ 1 nm, and their zeta-potential was +47.4 $\pm$ 1.3 mV. Based on the results of a two-year field experiment in 2019–2020, it was shown that treatments with low doses of Ag NPs lead to the intensification of the process of legume–*Rhizobium* symbiosis, which is expressed in a significant increase in the number of root nodules and an increase in soybean yield. After the treatments, a significant increase in the enzymatic activity of peroxidases and polyphenol oxidases in the terrestrial parts of plants and a decrease in activity in the roots were recorded. The increase in activity may be a consequence of the intensification of several key nonspecific defense reactions induced by exogenous forms of ROS formed during the interaction of Ag NPs with components of plant cell walls. It is very likely that the treatment with Ag NPs and an increase in peroxidase activity in non-infected plant parts led to a more effective prevention of the penetration of rhizobia into the aboveground soybean organs, which, in turn, may be the reason for the observed decrease in peroxidase and PPO activity in the parts of plant roots susceptible to rhizobia. The latter, as is known, contributes to an easier flow of the nodulation process and the development of legume–*Rhizobium* symbiosis.

The increase in soybean yield under the action of small doses of Ag NPs, in addition to the effect on the processes of legume–*Rhizobium* symbiosis, may be the result of a superposition:

(a) Effects associated with blocking the ethylene-dependent signaling system, and consisting mainly in stimulating vegetation, root and shoot growth, biomass accumulation and slowing down maturation and wilting processes;
(b) Effects associated with oxidative stress, consisting in disruption of normal cell activity, on the one hand, and stimulation of resistance to adverse external influences, including infection with phytopathogens, on the other hand.

Thus, the action of low doses of Ag NP dispersions stabilized with PHMB leads to the intensification of legume–*Rhizobium* symbiosis processes and an increase in soybean yield, which may be associated with a moderate activation of the plant's defense signaling systems and the processes of formation of nonspecific resistance (both systemic acquired resistance (SAR) and induced systemic resistance) in response to the impact of exogenous abiotic elicitors.

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