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# Co-Inoculation with *Bradyrhizobium* and Humic Substances Combined with *Herbaspirillum seropedicae* Promotes Soybean Vegetative Growth and Nodulation

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**Abstract:** The effect of humic substances (HSs) in combination with or without plant-growth-promoting bacteria on soybean vegetative growth and root nodulation was examined in this study. Seeds were inoculated with *Bradyrhizobium japonicum* SEMIA 5079 in the presence of HSs from leonardite and *Herbaspirillum seropedicae* HRC54. Additional HSs and *H. seropedicae* application at the substrate surface was conducted at the V3 stage. The experiment was carried out in a greenhouse using pots filled with a top layer of an Oxisol soil, and plants were harvested at the R1 stage. The HS and *H. seropedicae* treatments significantly promoted plant shoot and root growth. The number and weight of soybean nodules were higher in the treated plants when compared to a control. The plant nodulation process was affected by the treatments that included activities of malate dehydrogenase (MDH), nitrate reductase (NR) and plasma membrane $H^+$-ATPase (MHA). At low concentrations, the HSs and *H. seropedicae* increased the nodule quantity, size and weight, favoring plant growth. Combining humic substances and plant-growth-promoting bacteria (PGPB) could be a promising approach to promoting soybean nodulation and increasing crop production.

**Keywords:** *Glycine max*; plant-growth-promoting bacteria; biostimulants; diazotrophs; biofertilizers; $N_2$-fixing bacteria



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## 1. Introduction

Agriculture faces mounting pressure due to its adverse environmental impacts and its inefficient utilization of resources. Innovative technologies have assumed increasing significance in mitigating the effects of abiotic stress, while the considerate use of chemical inputs remains pivotal in sustaining productivity [1]. Humic products (HPs) have received increasing attention in plant stimulation, and their effects are closely dependent on the concentration and source of the humic substances (HSs) contained in the HPs as well as on the plant species [2,3]. HSs promote both nutrient uptake and use efficiency due to their recognized ability to induce cell proton pump activity, thereby increasing the electrochemical gradient across the plasma membrane and enhancing nutrient transporters [2]. The primary and secondary plant metabolism can be stimulated by HSs [4,5], affecting plant growth and crop production.

Brazil is the world's largest producer and exporter of soybeans. The official estimate for the 2022–2023 production is around 150.36 million tons, extending across approximately 42.4 million hectares of planted area, with 3500 kg/ha of average productivity [6]. The rising frequency and severity of drought events, coupled with the high cost of fertilizers, have garnered the attention of growers in Brazil regarding the utilization of HPs. However, the effects of HPs on soybean yield have shown varying outcomes, ranging from no

response [7,8] up to positive and significant effects. Lenssen et al. [9] observed a 20% greater yield from treated plants than untreated control soybean plants. They concluded that applying HPs positively influenced yield and seed quality, particularly when plants were subjected to environmental stressors, such as rainfall deficits and air temperatures exceeding long-term average levels. The most frequently reported effect of HSs on plant morphology is the change in root architecture, resulting in enhanced surface area due to an increase in the number of lateral roots and root hairs [2,3], resembling typical auxin-like activity [10]. Earlier reports have also documented a substantial Increase in soybean root dry matter due to HSs, although there was no corresponding increase in the number of nodules [11]. However, more recent studies have shown that soybean plants treated with humic acids can promote plant growth and nodulation compared to control plants by stimulating the abundance of bacterial species in roots [12]. HS' modulation of the rhizosphere community was attributed to the increasing exudation of sugars [13] and other available organic compounds [14]. Also, the introduction of selected microorganisms can induce changes in the rhizosphere bacterial community.

The co-inoculation of soybean with *Bradyrhizobium* spp. and *Azospirillum brasilense*, a plant-growth-promoting bacterium (PGPB), showed a positive effect on soybean yield, including increased nodulation and root growth [15–20]. *Herbaspirillum seropedicae* is another PGPB with the recognized capability of symbiotic fixation of atmospheric nitrogen [21]. The process of endophytic colonization by *H. seropedicae* begins with the attachment of the bacteria to root surfaces. Furthermore, the colonization begins at the emergence points of lateral roots and penetration through discontinuities in the epidermis [22]. The bacterium triggers an auxin-like effect, activating the phytohormone signaling cascade to regulate the emission of lateral roots, thereby indirectly favoring the rhizobia. The root system and the number of bacteria in the tissues are favored by co-inoculation. The heterogeneity of plant surfaces covered by HSs and their bioactivity measured by the induction of lateral roots facilitates both anchorage and bacterial infection. In addition, the nodulation of common beans was increased by the co-inoculation of humic acids and *H. seropedicae* [23]. Therefore, this study aimed to assess the effects of sole inoculation with *B. japonicum* and co-inoculation with humic substances and *H. seropedicae* on soybean' vegetative growth and nodulation.

## 2. Materials and Methods

### *2.1. Humic Substances*

The humic product (HP) was provided by Monty's Plant Food, KY, USA, and was isolated from leonardite. The organic carbon content in the HP was 16 g $L^{-1}$ (wet combustion, Walkley–Black), and the humic and fulvic acid contents on a weight basis were 10% and 2%, respectively. Before treatment application, the HP was diluted in water at 100 mg C $L^{-1}$.

### *2.2. Microorganisms*

*Bradyrhizobium japonicum* strain SEMIA 5079 (with 1.2 × $10^9$ colony forming units (CFU) $mL^{-1}$) as an inoculant and *Herbaspirillum seropedicae* strain HRC54 as a co-inoculant were used in this study. The dose of the inoculant was calculated to deliver 1.2 × $10^6$ cells per seed. The co-inoculant was grown in vials with complete JNFb semisolid medium. The composition of the JNFb medium per liter consisted of the following components: malic acid (5.0 g), $K_2HPO_4$ (0.6 g), $KH_2PO_4$ (1.8 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.1 g), $CaCl_2$ (0.02 g), 0.5% bromothymol blue in 0.2N KOH (2 mL), vitamin solution (1 mL), micronutrient solution (2 mL), 1.64% Fe·EDTA solution (4 mL) and KOH (4.5 g). The vitamin solution in the amount of 100 mL contained biotin (10 mg) and pyridoxal-HCl (20 mg); 1 L of the micronutrient solution consisted of $CuSO_4$ (0.4 g), $ZnSO_4$ $7H_2O$ (0.12 g), $H_3BO_3$ (1.4 g), $Na_2MoO_4$ $2H_2O$ (1.0 g) and $MnSO_4$ $H_2O$ (1.5 g). The semisolid medium pH was adjusted to 5.8, and 1.9 $gL^{-1}$ of agar was added. The bacterial population was determined by the most-probable-number technique (MPN) with the help of pellicle formation using three replications and expressed as the log of the cell number $g^{-1}$ root fresh mass after growth

on JNFb N-free semisolid medium according to the method of Döbereiner et al. [24]. The bacterial suspension of *H. seropedicae* strain Z67 was grown on a JNFb liquid medium with added $NH_4Cl$ 1 g $L^{-1}$ at 34 °C for 16 h with shaking (150 rpm). Cells were pelleted by centrifugation (4000× *g* for 15 min) and resuspended in sterilized water at cell densities of $10^9$ colony-forming units (cfu) $mL^{-1}$.

### 2.3. Plant Growth Conditions

Soybean (*Glycine max* (L.) Merr.) BRs 55 seeds were used to carry out the study. The soybean seeds were washed three times with distilled water, and then three groups of one hundred seeds were randomly selected and soaked for six hours in a water solution containing (i) *B. japonicum* as the control, (ii) *B. japonicum* as the inoculant plus commercial humic substance (HS) from leonardite as a co-inoculant (1:400 *v*:*v*) or (iii) *B. japonicum* plus the humic substance, HS, plus *H. seropedicae* as co-inoculants. After the soaking time, five seeds were planted in a pot (2.5 L) filled with an Ultisol top layer (0–20 cm) with the following characteristics: pH = 4.6, C = 10.4 g/kg, N = 1.1 g/kg, C:N = 9.54, organic matter (OM) = 20.10 g $dm^{-3}$, P = 4.45 mg $dm^{-3}$, $Al^{+3}$ = 0.10 $cmol_c$ $dm^{-3}$, H + Al = 3.18 $cmol_c$ $dm^{-3}$, Ca = 0.80 $cmol_c$ $dm^{-3}$, Mg = 1.20 $cmol_c$ $dm^{-3}$, sum of bases (SB) = 2.11 $cmol_c$ $dm^{-3}$, saturation of bases (V)= 39.89 (%), saturation by $Al^{3+}$ (m) = 4.52% and cation exchange capacity (CEC) = 5.29 $cmol_c$ $dm^{-3}$. The Embrapa Soils Test Handbook (1997) describes the analysis methods. Soil was collected from the superficial layer (0–0.2 m) at Lagoa de Cima, Campos dos Goytacazes, State of Rio de Janeiro, Brazil, 21°44′24.6″ S 41°32′07.8″ W. Four replicates were used in a randomized statistical design. Plants were uprooted one week after germination, and one seedling was maintained per pot. Irrigation was supplied daily, and 200 mL of Hoogland nutrient solution was supplied weekly with a 25% concentration without N application. At the V3 stage, a new application of 200 mL per pot of humic and humic products plus H. seropedicae was carried out at the same concentration as the seed treatments. Four pots with one soybean seedling per pot were collected at the R1 stage (first flower). Roots and shoots were weighed and dried at low temperature (60 °C) until a constant weight was obtained. After the dry matter was weighed, the total N and C contents were analyzed by the dry combustion method on a CHN analyzer (Perkin-Elmer autoanalyzer series 2400, Norwalk, CT, USA), and the nutrients from one aliquot of solution digested using perchloric acid were analyzed in an inductively coupled plasma atomic emission spectrometer (ICP-AES).

### 2.4. Differential Transcription Level of Genes with RT-qPCR

Extraction of total RNA: A tissue sample of 100 mg of fresh leaves was homogenized with a mortar and pestle in liquid $N_2$. The homogenate was transferred to new RNAse-free microcentrifuge tubes (1.5 mL), and RNA was extracted using a mini-plant rNeasy Qiagen® kit (Germantown, MD, USA). Reverse transcription (RT) followed by polymerase chain reaction (PCR) and 1 µg of total RNA was used to produce cDNAs. The synthesis was performed using a high-capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA, USA). A PCR with a gradient temperature (59, 60 and 61 °C) was performed to confirm the specificity of the primers and the actual melting temperature. Electrophoresis in 2.0% agarose gel with TAE buffer was also performed to confirm the PCR products with the specific primers.

Primers for the genes GmNR, GmMDH and GmMHA were designed with the Primer3 program (https://primer3.org/manual.html (accessed on 28 August 2023)), and their characteristics were evaluated in the Oligothech program. After a rigorous analysis, they were synthesized using IDT technology. Confirmation of primer specificity was determined in a high-resolution gel, which gave single PCR products at the different temperatures tested and with the expected size. The melting curve obtained in the StepOne™ System (Thermo Fisher Scientific, Waltham, MA, USA) also confirmed the specificity. The real-time PCR (RT-qPCR) was conducted as follows: for statistical validation, two independent tests in the thermal cycler StepOne™ System software v2.3, with mRNA extracted from

the independent experiments, were performed. cDNAs of each experiment were used in quadruplicate for each condition evaluated. The medium for the PCR was prepared as follows (final concentrations): 5 ρM of the forward primer, 5 ρM of the reverse primer (1 µL), 7.5 µL of SYBR Green I component (Applied Biosystems®) and 0.5 µL of ultrapure water. A total of 10 µL of the medium was added to an ELISA plate, and 5 µL of cDNA was added. For the cDNA dilution curve, the following concentrations were used: 0.2, 2, 20 and 200 ng of cDNA template at the control condition. The whole procedure was performed in a laminar flow using sterile materials that were RNAse-free. After adding the reagents, the plate was sealed with adhesive and centrifuged gently. The protocol used for the experiment consisted of four steps: (i) denaturation program (10 min at 95 °C); (ii) amplification program and quantification repeated 45 times (10 s at 95 °C; 5 s at 61 °C for both genes (reference and target); 5 s at 72 °C with a single fluorescence acquisition mode); (iii) melting curve program (65–95 °C with a heating rate of 0.1 °C/s with continuous fluorescence acquisition) and (iv) cooling program to lower the temperature to 40 °C. Crossing points (CPs) were obtained and used in the subsequent calculations. CPs are the point at which the fluorescence achieves significantly higher levels than non-specific fluorescence. As previously described, the relative mRNA expression of the genes of interest and the endogenous control (ubiquitin (UBI)) were compared using a nonparametric pairwise fixed-reallocation randomization test.

### *2.5. Statistical Analysis*

The experiment was set up with a randomized design in pots in a greenhouse with four replicates (plants) per treatment. Data were analyzed by one-way ANOVA using R version 4.0.3 (Development Core Team 2020) and Fisher's LSD with separated means at $p < 0.05$.

## 3. Results

### *3.1. Vegetative Growth*

The plants co-inoculated with *Bradyrhizobium japonicum* plus HS and the plants co-inoculated with *B. japonicum* plus HS combined with *H. seropedicae* produced higher shoot and root fresh weights compared to the control plants inoculated with only *B. japonicum* as the inoculant (Figure 1A,B). The shoot fresh weights from the plants receiving HS and those receiving HS and *H. seropedicae* as co-inoculants were 145% and 180% higher, respectively, than the control plants (Figure 1A). The root fresh weights from the plants receiving HS and those receiving HS plus H. seropedicae were 321 and 393% higher, respectively, than the control plants (Figure 1B). The same trend was observed for dry matter accumulation (Figure 1C,D). The shoot dry weights from the plants receiving HS and HS plus *H. seropedicae* were 106% and 137% higher, respectively, than the control plants (Figure 1C), while the root dry weights from the plants receiving HS and from the plants receiving HS plus *H. seropedicae* were 261% and 311% higher, respectively, than the control plants. No significant differences were found between the plants receiving HS and HS plus *H. seropedicae*, considering the promotion of vegetative growth.

### *3.2. Plant Nodulation*

The treatments also affected the number and weight of the root nodules, as shown in Figure 2. The number of nodules in the control plants was lower, showing an average of nine nodules per root (9 ± 4) (mean followed by standard deviation). Meanwhile, the number of nodules from the plants receiving HS and *H. seropedicae* co-inoculation plus HS was enhanced by 38 (±10) and 28 (±8) nodules per root system, respectively, representing a significant increment of 322% and 311%, respectively, in the number of nodules per root (Figure 2A). Additionally, besides the significant increment in the number of nodules, this was followed by an increment in the weight of nodules (Figure 2B).

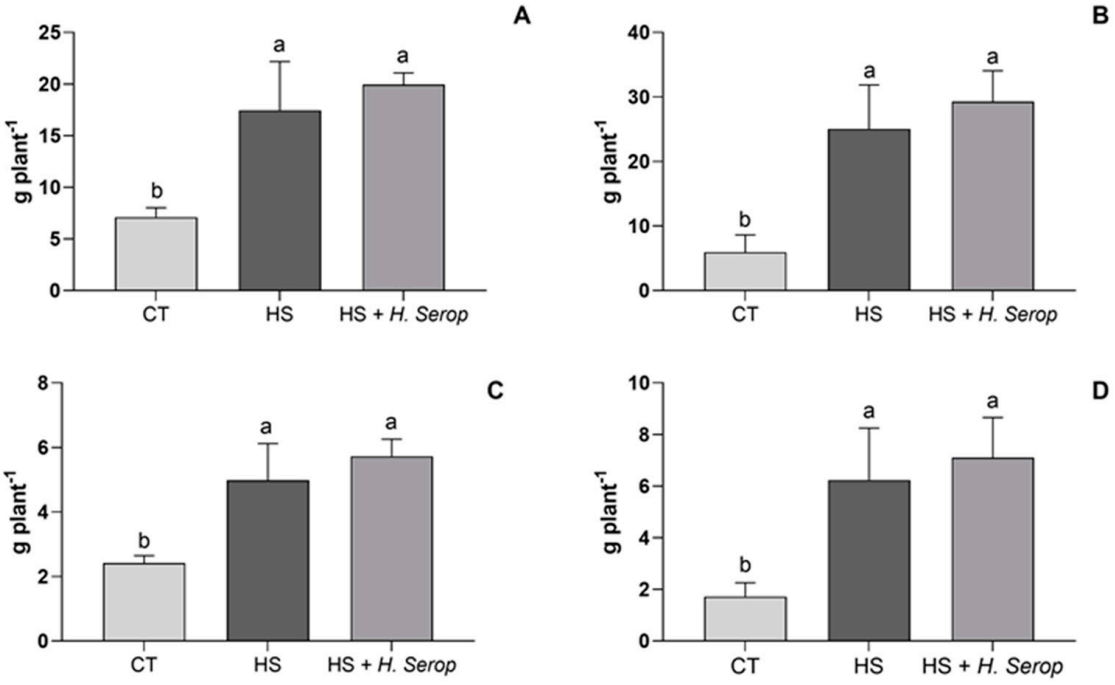


**Figure 1.** Shoot fresh weight (**A**) and root fresh weight (**B**) accumulation and shoot dry weight (**C**) and root dry weight (**D**) accumulation in soybean plants inoculated with *Bradyrhizobium japonicum* strain SEMIA 5079 (CT = control), co-inoculated with humic substances (HS) and co-inoculated with HS plus *Herbaspirillum seropedicae* strain HRC54 (HS + *H. Serop*). Means (n = 4) followed by different letters differ according to Fisher's LSD at $p < 0.05$.

The average weight of nodules in the control plants was 150 mg (±30) per nodule, while the nodule weights of the plants co-inoculated with HS and with HS + *H. Serop*. were 514 mg (±60) and 460 mg (±110), respectively, representing 3.4 to 3 times more mass in each nodule, respectively (Figure 2).

### *3.3. Plant Nutrient Content*

The nutrient content was slightly modified by the treatments (Table 1). A significant increase in the N and K content was observed in the shoot of the plants receiving HS and HS + *H. seropedicae*, which had 20 and 14% more N and K, respectively, than the control plants. No significant differences were observed in the nutrient contents in the roots except for the Fe content, which was higher in the roots, and the Mn content, which was higher in the shoots, from the plants treated with HS + *H. seropedicae* (Table 1). However, the extraction of macro- and micronutrients was significantly enhanced by the plants receiving HS and HS + *H. seropedicae* compared to the control plants (Figure 3).

**Table 1.** Nutrient content in soybean root and shoot subjected to different treatments.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | roots | | | | | | | | | | |
| Treatment | N | P | K | Ca | Mg | S | B | Cu | Fe | Mn | Zn |
| | g kg$^{-1}$ | | | | | | mg kg$^{-1}$ | | | | |
| Control | 12.0 (±1.5) | 0.9 (±0.1) | 4.1 (1.0) | 1.2 (0.2) | 1.2 (0.1) | 2.4 (0.2) | 3 (1) | 12 (1) | 6545 (60) | 184 (4) | 27 (5) |
| HS | 11.0 (±3.6) | 0.9 (0.2) | 2.7 (1.3) | 0.9 (0.1) | 1.3 (0.1) | 2.3 (0.3) | 2 (2) | 9 (1) | 5934 (435) | 173 (16) | 32 (6) |
| HS + *H. seropedicae* | 9.9 (1.1) | 0.9 (0.1) | 5.3 (1.3) | 1.1 (0.1) | 1.6 (0.2) | 2.8 (0.3) | 3 (1) | 10 (1) | 7006 * (492) | 192 (100) | 26 (3) |
| | shoots | | | | | | | | | | |
| | g kg$^{-1}$ | | | | | | | | mg kg$^{-1}$ | | |
| | N | P | K | Ca | Mg | S | B | Cu | Fe | Mn | Zn |
| Control | 29.6 (3.8) | 2.2 (0.1) | 14.0 (2.1) | 8.6 (0.7) | 3.9 (0.4) | 2.2 (0.2) | 29 (3) | 6 (0.4) | 251 (42) | 181 (20) | 85 (4) |
| HS | 35.6 * (1.3) | 2.4 (0.3) | 16.0 (5.4) | 8.4 (0.5) | 3.7 (0.1 | 2.2 (0.0) | 27 (3) | 7 (2) | 206 (6) | 197 (82) | 93 (18) |
| HS + *H. seropedicae* | 27.3 (3.5) | 2.3 (0.1) | 14.6 (0.8) | 7.8 (0.3) | 3.4 (0.2) | 2.3 (0.1) | 29 (1) | 6 (1) | 162 (18) | 266 * (68) | 92 (5.4) |

Values represent the mean followed by the standard deviation in brackets. * Represents a significant difference according to Fisher's LSD test ($p < 0.05$).

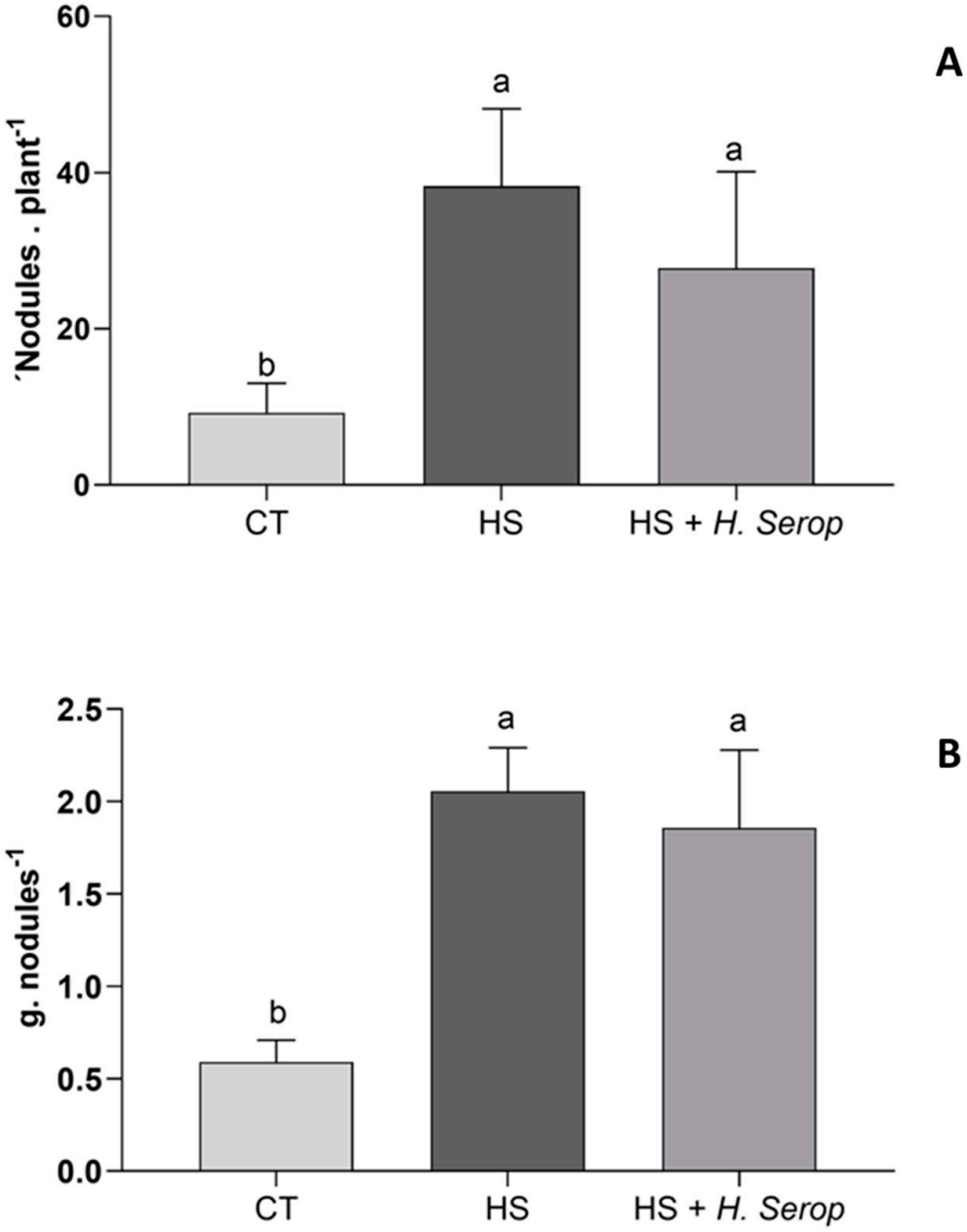


**Figure 2.** Number (**A**) and weight (**B**) of nodules in roots of soybean inoculated with *Bradyrhizobium japonicum* strain SEMIA 5079 (CT = control) and co-inoculated with humic substances (HS) and co-inoculated with humic substances and *Herbaspirillum seropedicae* (HS + *H. Serop*) strain HRC 54 plus HP. Means (n = 4) followed by different letters differ according to Fisher's LSD at $p < 0.05$.

### *3.4. Plant Metabolism was Affected by Co-Inoculation*

The growth enhancement, as shown by the large shoot and root weights (Figure 1), required an acceleration of the plant metabolism. The HS, in combination or not with *H. seropedicae*, significantly enhanced the transcription level of genes used as primary metabolism markers (Figure 4).

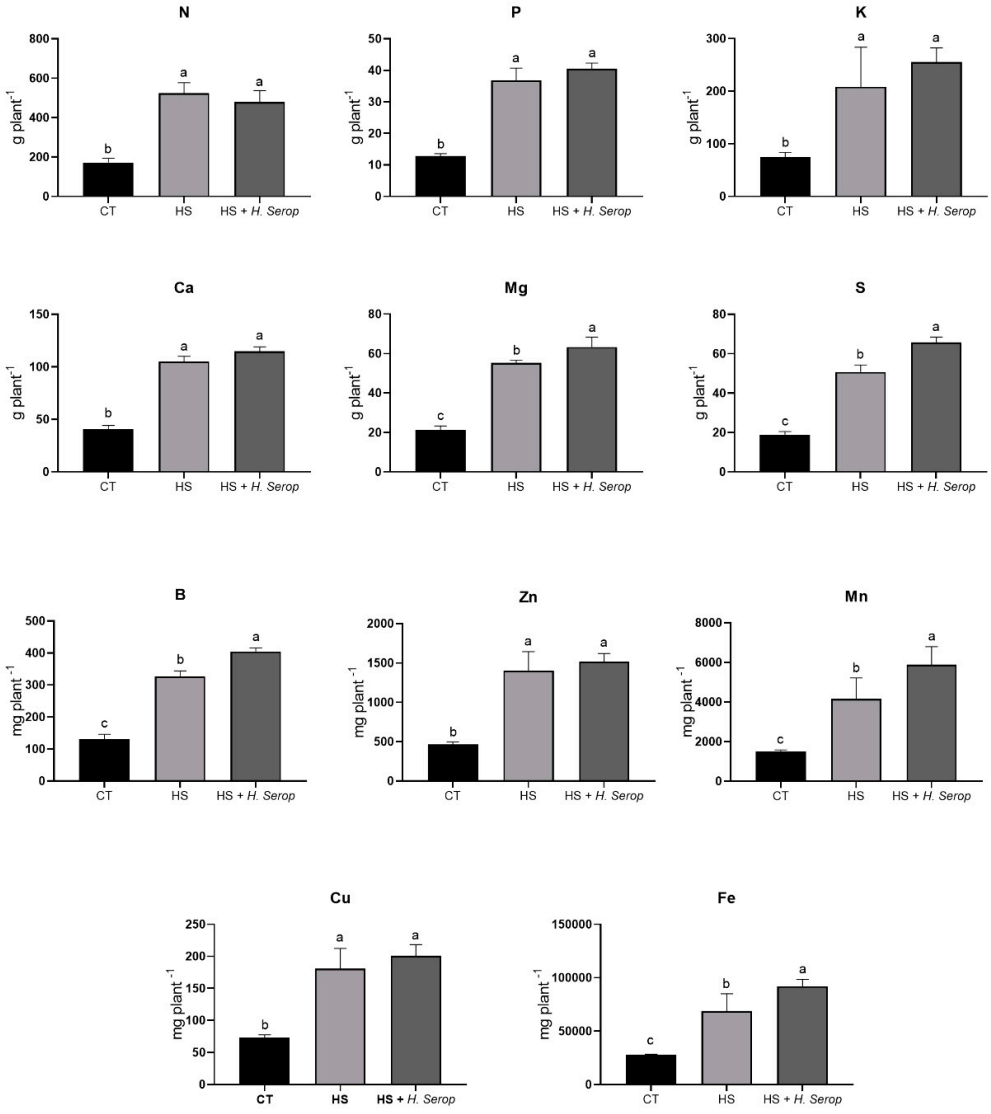


**Figure 3.** Total macro (N, P, K, Ca, Mg and S) and micronutrients (B, Zn, Mn, Cu and Fe) extracted per plant. Means (n = 4) followed by different letters indicate values that differ from one another (LSD $p < 0.05$).

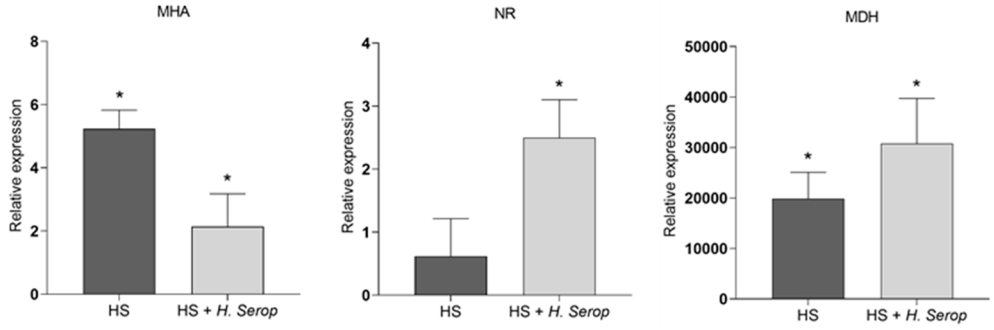


**Figure 4.** Differential transcription level from malate dehydrogenase (MDH), nitrate reductase (NR) and plasma membrane $H^+$-ATPase (MHA) genes measured by RT-qPCR concerning untreated plants (control = 0). *HS:* humic substances; *HS + H. Serop*: HS plus *Herbaspirillum seropedicae*. The expression was normalized concerning the control treatment. Data represent the mean and the standard deviation bars (n = 3) in three independent experiments. * Significant difference with respect to control (LSD $p < 0.05$).

## 4. Discussion

Soybean nodulation and biological nitrogen fixation play a central role in the sustainability and competitiveness of the crop [25], and plant biostimulants can be used as agronomic tools to amplify these benefits. However, more recent works concerning HS and *Glycine max* have emphasized the attenuation of different stresses [26–28] on the macro and micronutrient uptake of these plants [1,29–34], with a few investigating the promotion of vegetative growth [35] or root nodulation [36]. Here, we observed that the growth promotion induced by the co-inoculation of *B. japonicum* and HS or HS + *H. seropedicae* was more significant than the control (Figure 1). We used a superficial layer of low-natural-fertility soil as a substrate, which has not previously been tested for growing leguminous plants. To prevent multiple nutritional stresses, we added a dilute nutritive solution without N to highlight the role of biological N fixation. The seeds inoculated with *B. japonicum* promoted low root nodulation (Figure 2) in a range below that considered viable, i.e., 15 to 30 nodules, with a mass between 100 and 200 mg, according to Hungria et al. [37]. The co-inoculation significantly increased both the number and average mass of nodules (Figure 2), resulting in more efficient biological N fixation, as evidenced by the significant correlation between the nodule weight and N fixation [38]. Previous studies, including those conducted in a temperate climate, have not reported a significant effect of HSs on nodulation [7,8]. It is expected that nodulation is closely dependent on the cultivar [39] and HS concentration, as demonstrated by Reis et al. [40].

Da Silva et al. [12] showed that the application of humic acids could help soybean growth through its interference with the endophytic bacteria community, triggering the enrichment of microorganisms with the potential to act both on plant growth and defense against pathogens and abiotic stresses. The modulation of the endophytic and rhizosphere community by humic acids was previously described and can be depicted as a complex process that emerges from different interrelationships between components and functions. Anatomical changes in the root system makes the roots more extensive with a larger surface area, likely favoring their interaction with plant microorganisms. In addition, plants treated with HSs have shown a noticeable increase in root exudation due to a general metabolic acceleration [14–41]. HSs are believed to promote microbial activity through multiple processes, including chemical attraction, providing available C and N sources and electrochemical modifications to the soil–root interface [13]. Previous studies showed an increased microbial tissue colonization of different plants co-inoculated with HSs and plant-growth-promoting bacteria [42]. HSs enhance plant exudation of labile compounds such as sugars, amino acids and organic acids, which can be used as energy sources, favoring selected plant-growth-promoting bacteria.

The effects of co-inoculation on plant growth are significant concerning control plants due to multiple independent mechanisms. Olivares et al. [42] reviewed this issue, and their study included phosphate solubilization, alleviation of abiotic stress, siderophore production, rhizosphere engineering, production of 1-aminocyclopropane-1-carboxylate deaminase (ACC), quorum-sensing (QS) signal interference and the inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, the induction of systemic resistance, promoting beneficial plant–microbe symbioses and interference with pathogen toxin production in addition to biological N fixation.

Plant nodulation is subjected to many control factors that were grouped by Schwember et al. [43] such as primary metabolism (N and C), oxidative stress, phosphorous (P) levels and oxygen flow through nodules. Disregarding oxygen flow control, on which there has been no report about the involvement of HS, other mentioned factors are significantly affected. For example, malate is the primary fuel for $N_2$ reduction into ammonia in the bacteroid and is easily transformed into oxaloacetate (OAA) through malate dehydrogenase (MDH) activity. MDH activity was observed to be very high in legume nodules, leading C metabolism towards the production of malate [44]. Nardi et al. [4] showed that different fractions of HS could positively induce the activity of MDH and other enzymes of the tricarboxylic cycle. In this study, the level of MDH transcription in the roots of the plants

treated with HS and HS + *H. seropedicae* was noticeable (Figure 4). Furthermore, OAA, through the GS-GOGAT pathway, serves as a C skeleton for forming asparagine, which acts as the principal N export compound from nodules [45]. The transcription level of assimilatory N enzymes induced by HSs, including both GS and GOGAT and aspartate synthase, has already been reported [46]. This promotion was concomitant with the stimulation of the main TCA enzymes. MDH and NR were found in more significant transcriptional levels in the plants treated with HS and HS + *H. seropedicae* (Figure 4).

We only observed an enhancement of the N content in the plants treated with HS (Table 1). The effect of HS on plant nutrition may be mediated by the modulation of the synthesis and functionality of membrane proteins, especially the plasma membrane (PM) proton pumps (PM $H^+$-ATPase) that build up an electrochemical proton gradient across the PM, thereby modulating nutrient transport through the plant cell membrane [2]. This gradient energizes secondary active transport, which is accomplished by carrier proteins via a symport or antiport. The evidence of proton pump stimulation elicited by HSs was demonstrated in the late 1980s [2]. The stimulation of the PM $H^+$-ATPase activity takes place in association with an up-regulation of this enzyme [47]. The consequence of $H^+$ pumping stimulation is turning the outside of the PM more positively charged than the inside. Nitrate uptake is a typical 2:1 symport ($2H^+$:1 $NO_3^-$) that is favored by enhancing nitrate transporter activity and is promoted by HS [2]. The promotion of PM $H^+$-ATPase by co-inoculation with HS and HS + *H. seropedicae* was reported previously [48]. The inoculation activated the extracellular $H^+$ flux, thus changing maize root cell pH and membrane potential and altering the electrochemical potential generated by PM $H^+$-ATPase at the biochemical and molecular levels.

One crucial factor in nodulation control not mentioned in Schwember's review is the hormonal modulation of legume–rhizobial symbiosis [49]. Phytohormone signaling pathways have gradually emerged as essential regulators of root nodule symbiosis. Cytokinin, strigolactones and local auxin accumulation can promote nodule development, while ethylene, jasmonic, abscisic and gibberellic acid negatively regulate infection thread formation and nodule development. It is well known that the most reported effect of HSs in plants is their hormonal effect [10] and the induction of an auxin-like response by *H. seropedicae* [22]. Zhang et al. [50] found that water-soluble HS increased the number of nodules, thus changing the expression of genes codifying endogenous hormones in soybean plants. Furthermore, Souza et al. [51], using RNAseq in maize seedlings treated with humic acids isolated from vermicompost, showed a general mechanism for simultaneously regulating the activity of several hormones, where humic acids act as a key regulatory hub in plant responses integrating hormonal signaling and response pathways. Genes related to plant hormones (auxin, gibberellin, ethylene, cytokinin, abscisic acid, brassinosteroids, jasmonic and salicylic acids) were transcripted at differential levels in the maize root seedlings, as was the expression of hundreds of transcription factors modifying the signal transduction pathway via alterations to the subsequent gene response.

Finally, different aspects of the co-inoculation of *Bradyrhizobium japonicum* with plant-growth-promoting bacteria were studied [52], reflecting the increasing expansion of its use in the field. We showed that the co-inoculation of soybeans with HS and HS plus *H. seropedicae* enhanced the vegetative growth and the number and weight of root nodules. The metabolic biomarkers used (MDH, MHA and NR) demonstrated this plant growth, suggesting that soybean co-inoculation can be considered an additional tool to improve crop production at a low cost.

## 5. Conclusions

Humic substances applied to low concentrations, combined or not with *H. seropedicae* inoculation, enhanced the vegetative growth of soybeans in this study. The plant growth promotion was accompanied by increased nodulation in terms of the number and mean mass per nodule. More significant relative transcriptional levels of genes used as primary

metabolism markers were found in the treated plants, showing the positive effect of soybean co-inoculation.

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