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# $H_2S$ Crosstalk in Rhizobia Modulates Essential Nutrient Allocation and Transport in Soybean

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**Abstract:** Hydrogen sulfide ($H_2S$), a novel gas signaling molecule, plays a crucial role in plant growth and stress response. However, little attention has been devoted to the regulation of $H_2S$ on nutrient transport and utilization in legume–rhizobia symbiosis systems. Although we have previously proven that $H_2S$ synergized with rhizobia to considerably enhance nitrogen (N) metabolism and remobilization in N-deficient soybeans, it remains uncertain if changes in nutrient absorption, metabolism, and accumulation occur concurrently. Therefore, employing a synergistic treatment of $H_2S$ and rhizobia, we examined the dry matter biomass and carbon (C), N, phosphorous (P), and potassium (K) nutrient content in various organs of soybean from blooming to maturity. Firstly, $H_2S$ and rhizobia application obviously improved leaf and plant phenotypes and biomass accumulation in different organs during N-deficient soybean development. Second, from flowering to maturity, the contents and stoichiometric ratios of C, N, P, and K in various organs of soybean were changed to variable degrees by $H_2S$ and rhizobia. Furthermore, $H_2S$ collaborated with rhizobia to significantly affect grain nutrient harvest across soybean growth as well as overall plant nutrient accumulation. Consequently, $H_2S$ synergizes with rhizobia to optimize grain harvest quality and nutrient accumulation across the plant by managing the rational allocation and dynamic balance of nutrients in diverse organs, hence boosting soybean development and production.

**Keywords:** hydrogen sulfide; rhizobia; soybean (*Glycine max*); nutrient; allocation



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## 1. Introduction

Nitrogen (N), an essential phytonutrient, severely limits plant productivity and agricultural production globally due to scarcity and poor availability in soils. Excessive industrial N fertilizer is applied in the fields to maximize agricultural yields [1], which causes eutrophication of water and $N_2O$ emissions in soils, accelerating global warming [2]. Therefore, it is crucial to develop a natural and effective substitute for N fertilizer to boost crop yields. Symbiotic N fixation (SNF) is reported to be the largest natural source of N in agricultural systems [3]. Legumes have attracted much attention for their unique biological N fixation (BNF) potential. Under N-deficiency conditions, legumes interact with rhizobia to generate a new special plant organ, the nodule, where SNF occurs. This mutualistic relationship benefits both symbiotic partners: the host plant offers carbon (C) and energy to the rhizobia for development and function, while the rhizobia fix atmospheric $N_2$ to deliver reduced N to the hosts in the form of ammonium. Hence, this process provides an economically appealing and environmentally sound strategy for reducing external inputs while enhancing internal resources [4]. To improve knowledge of this critical process in sustainable agriculture, emphasis should be placed on the distribution and utilization of vital nutrients in symbiotic systems.

Plants require around 30 elements for development, and their roles and specific functions are well established [5,6]. C, N, phosphorus (P), and potassium (K), as common nutrient elements and basic chemical components of plant organisms, have a profound impact on photosynthetic and metabolic processes, as well as plant growth and production [7,8]. Therefore, in plants, tissue allocation of these vital elements and their functions in physiological activities are inextricably coupled [9,10]. Moreover, maintaining the correct nutrient balance in plants becomes crucial. In recent years, ecological stoichiometry has garnered growing attention in plants as a vital indication for assessing element composition and balance [11–15]. In a macroscopic study, Ågren and Weih [16] reported that element concentration patterns of plant stoichiometry at different scales were more reflective of the environment than genotypes. Furthermore, nutrient ratios are well recognized to have substantial impacts on physiological process regulation and resource acquisition in plants [17,18], accurately capturing the gradual dynamics of nutrient restriction and availability variations [19,20].

Soybean (*Glycine max* L.), as one of the most important food crops, is a rich source of high-quality protein and vegetable oil, as well as a high-quality animal feed. Although worldwide soybean output has increased considerably in recent years, it is still insufficient to meet rising global demand [21]. Plant nutrients, due to their scarcity and availability, perform an essential role in plant development and yield [22,23]. In addition, an imbalance between fertilizer nutrient input and plant nutrient requirements is a key factor restricting soybean seed yield [24]. Therefore, a better comprehension of nutrient requirements across soybean development is required to achieve sustained improvement in soybean yield. From ancient times to the present, many academics have studied the patterns of nutrient absorption, distribution, and remobilization in soybeans to better understand the physiology of nutrient accumulation [25–27]. It should not be ignored that soybean, as a legume, can rely on its unique BNF function to fix atmospheric $N_2$ for its own use. However, BNF cannot meet soybean N needs in high-yielding situations or when N fixation is impaired [28]. Since other essential nutrients, such as C, P, K, sulfur (S), and iron (Fe), play a crucial role in promoting the BNF process, N should be balanced with these elements to maximize seed yield [29,30]. Moreover, plants can not only remobilize resources collected in vegetative organs into mature reproductive organs but also re-transport and utilize nutrients in senescent tissues to other developing tissues [31,32]. Therefore, nutrients in various organs are constantly migrating and changing during soybean growth, severely impacting nutrient harvesting in grain production [33,34]. Meanwhile, some studies have demonstrated that knowing dry matter accumulation, nutrient uptake patterns, and nutrient mobility within plant tissues is crucial for improving crop yield, nutrient utilization efficiency, and seed nutrient composition [27,35].

Hydrogen sulfide ($H_2S$) has been extensively studied in plants as an endogenous gas transporter. Once synthesized, $H_2S$ may be involved in almost all plant life processes [36]. Among them, $H_2S$ signaling transduction properties are mainly observed during persulfidation, which is considered to underpin myriad cellular processes in plants related to growth, development, stress response, and phytohormone signaling [37,38]. Accumulating evidence suggests that exogenous $H_2S$ applied to plants appears to provide extra protection against stresses, such as salt, drought, extreme temperatures, and heavy metals, primarily by inducing antioxidant systems to mitigate oxidative cell damage [39,40]. Additionally, $H_2S$ modulates plant growth and physiological processes such as seed germination, stomatal movement, fruit ripening, and post-harvest quality maintenance [40,41]. However, little attention has been paid to $H_2S$ signaling regulation in legume–rhizobia symbiosis systems. In our preliminary work, $H_2S$, as an antioxidant, protected nodule cells and bacteroids from oxidative damage in soybean–rhizobia symbiosis systems, improving N-fixing capacity [42,43]. Therefore, N assimilation and remobilization in soybean symbiotic systems were evidently enhanced by $H_2S$ [44].

Critical constant elements such as C, N, P, and K are not only indispensable for plant development [8,10] but also play crucial parts in the SNF process of legume nod-

ules [29,30]. Although $H_2S$ encouraged N fixation and metabolism in the soybean symbiotic system [42–44], it is still unknown how essential nutrients circulate, distribute, and remobilize across various organs. Therefore, we explored whether $H_2S$ collaborates with rhizobia to govern nutrient distribution, transport, and accumulation during soybean growth.

## 2. Materials and Methods

### *2.1. Plant Culture and Treatment*

Soybean (*Glycine max*, Zhonghuang 13) seeds were surface sterilized for 30 s with 75% ethanol and then for 4 min with 50% sodium hypochlorite solution before being sown in sterilized polypropylene planting bags (17 cm × 33 cm × 0.05 mm, Xinglong brand, Baoding, China) containing 450 g growth substrate (river sand and perlite, v:v = 2:1) with a moderate N-free nutrient solution. The nutrition solution contained the following ingredients: 100 mg/L $CaCl_2{\cdot}2H_2O$, 100 mg/L $KH_2PO_4$, 5 mg/L $FeC_6H_5O_7$, 150 mg/L $NaH_2PO_4$, 120 mg/L $MgSO_4{\cdot}7H_2O$, 2.86 mg/L $H_3BO_3$, 2.03 mg/L $MnSO_4{\cdot}4H_2O$, 0.22 mg/L $ZnSO_4{\cdot}7H_2O$, 0.06 mg/L $Na_2MoO_4{\cdot}2H_2O$, and 0.08 mg/L $CuSO_4{\cdot}5H2O$. Plants were cultivated in a constant temperature incubator with a 12/12 h light/dark cycle, an 80% relative humidity, a temperature of 25/22 °C, and a photo-synthetically active radiation (PAR) of 190 μmol $m^{-2}$ $s^{-1}$.

NaHS was applied as an exogenous $H_2S$ donor [45]. After the first true leaves had fully developed, some plants were inoculated with rhizobia (*Sinorhizobium fredii* Q8 strain) by injecting a 10 mL rhizobia solution ($OD_{600}$ = 0.05) into each bag along the rhizome. Following that, some plants were treated by injecting 10 mL of 100-μM NaHS solution directly into the culture substrate every 3 days, while blank controls were treated with sterilized distilled water [42]. Therefore, $H_2S$ concentration in the culture environment was kept under control (around 3.226-μM) in order to maintain $H_2S$ content in soybean tissues between 10 and 80 nmol/g FW. Each bag received 30 mL of sterile N-free nutritional solution every 3 days to provide constant humidity and nutrition. Hence, the seedlings were divided into four groups: (1) Control, without rhizobia or NaHS; (2) NaHS, with 100-μM NaHS; (3) Q8, with rhizobia inoculation; and (4) Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS.

### *2.2. Sample Harvesting*

Seedlings from each treatment were collected on the 32nd (anthesis) and 47th (maturity) days after inoculation. Each soybean plant was removed from the substrate when harvested. Plant shoots were then removed from roots, and roots were cleaned with distilled water. Then, samples from different periods were separated by organ, which comprised roots, stems, old leaves, new leaves, pods, and nodules at flowering and roots, stems, leaves, outer pods, beans, and nodules at maturity. Old leaves usually refer to leaves with low-leaf position (L-LP), while new leaves represent leaves with medium-leaf position (M-LP) and high-leaf position (H-LP) (Figure 1C). Around 10 soybean plants were collected from each treatment. The results were derived from three experiments.

### *2.3. Measurement and Statistics of Chlorophyll Content, Plant Height, Root Length, Nodule Number, and Dry Biomass*

The basic growth phenotype was observed when the plant reached the flowering and pod-setting stages (32nd day). Among them, the chlorophyll content of leaves was identified using a chlorophyll meter (SPAD-502Plus, Konica Minolta, Tokyo, Japan). Plant height and root length were measured with a straightedge. The number of nodules in rhizobia-inoculated soybeans was counted. All organs collected from each plant at anthesis and maturity were put in separate envelopes and heated in a 65 °C oven. After drying to a constant weight, these organs were weighed individually to determine the dry biomass of each tissue during soybean growth.

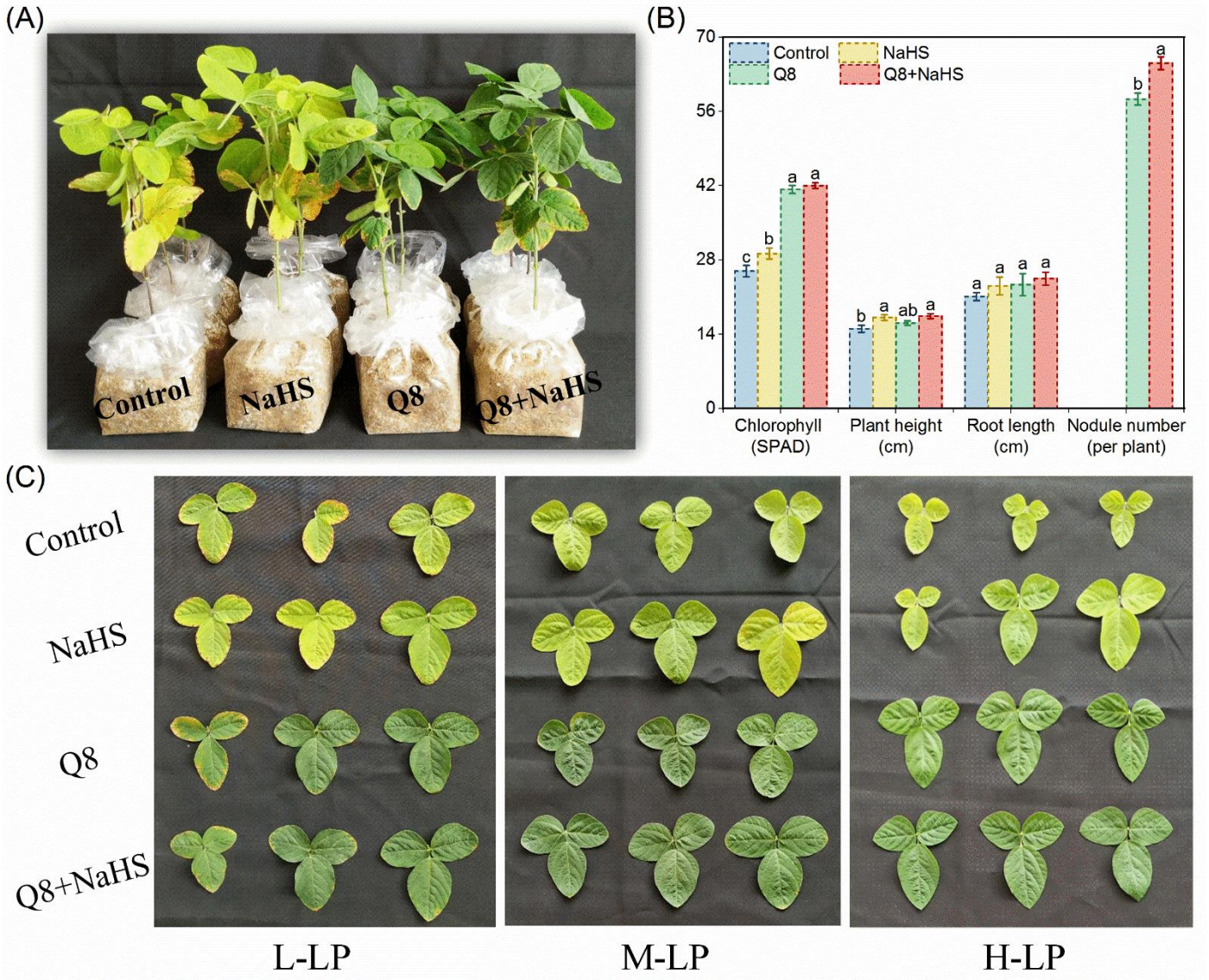


**Figure 1.** Plant phenotype (**A**), physiological characteristics (**B**), and leaf growth (**C**) of soybean under $H_2S$ and rhizobia application. The data are presented as the mean ± SE. Different letters indicate significant differences, $p < 0.05$. Control, without rhizobia or NaHS; NaHS, with 100-µM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-µM NaHS.

### *2.4. Determination of Plant Organic C, Total N, Total P, and Total K Content*

A conventional dichromate oxidation method used for soil organic matter estimation, known as the Walkley–Black procedure, was utilized to determine organic C content in plants [46]. The dried sample (0.02 g) passing through a 100-mesh sieve was weighed into a dry hard test tube. Following that, 5 mL of 1.2 mol/L (1/6 $K_2Cr_2O_7$) standard solution was accurately added to the tube, followed by 4 mL of concentrated $H_2SO_4$, and the tube was shaken well. The tubes were put in wire cages (each with 1 or 2 blank tubes), which were then placed in a paraffin oil bath at a temperature of 185–190 °C. Afterwards, the temperature in the oil bath was maintained at 170–180 °C, and the test tube was removed after 5 min of boiling and bubbling. After cooling, the contents of the test tube were poured into a 250 mL triangular flask, and distilled water was added to make a total volume of 60–70 mL of solution, keeping the concentration of mixture (1/2 $H_2SO_4$) at 2–3 mol/L. Next, 2–3 drops of phenanthroline indicator were added until the solution changed from orange-yellow to blue-green to brick red, which is the end point, and the $FeSO_4$ titration

mL was recorded. The $FeSO_4$ titration volume in the blank tube was recorded at the same time. Then, the organic C content was calculated.

Initially, 0.1 g of dry powdered material was put into a digestive tube. Next, 5 mL of concentrated $H_2SO_4$ was added, which was shaken and mixed. The mixture was then digested at 365 °C in a microwave digestion system (LabtecTM Line, FOSS, Nordberg, Denmark), with 7–8 drops of 30% $H_2O_2$ added every 30 min. This was performed 3–4 times until the digesting solution became clear from black. The resultant digestion solution was diluted to a constant volume with distilled water and would be utilized to determine the N, P, and K contents, respectively. According to the Kjeldahl method [47], the N content was determined using an automatic Kjeldahl apparatus (Kjeltec$^{TM}$ 8400, FOSS, Denmark). Moreover, the total P content was directly determined using a UV spectrophotometer (UV-1900, Shimadzu, Kyoto, Japan) relying on the molybdenum blue colorimetric method [48]. The total K concentration was analyzed by an atomic absorption spectrophotometer (PinAAcle900, PerkinElmer, Massachusetts, USA) [49]. The C:N, C:P, C:K, N:P, N:K, and P:K ratios for various growth organs of soybeans at anthesis and maturity were calculated using the C, N, P, and K values from the corresponding samples. Each experimental index was reproduced at least three times.

### *2.5. Calculation and Evaluation of Nutrient Harvest and Accumulation*

The nutrient harvest and accumulation in plants at various developmental stages were calculated using C, N, P, and K values from the corresponding samples. During harvest, the soybean was divided into dry remains (DR; roots, stems, and leaves) and total pods (PODS). The dry weight (DW) of each tissue corresponds to the dry biomass. The harvest index (HI) was calculated as the $(DW_{PODS})/(DW_{DR} + DW_{PODS})$ ratio, which indicates the yield. The DW and nutrient content (Nutrient%) were combined to determine the nutrient HI (Nutrient HI), a critical indicator of grain filling with nutrients, as $(Nutrient\%_{PODS}\ DW_{PODS})/(Nutrient\%_{DR}\ DW_{DR} + Nutrient\%_{PODS}\ DW_{PODS})$. Therefore, the HIs for C, N, P, and K were defined as CHI, NHI, PHI, and KHI. Moreover, nutrient accumulation in plants was described as $(Nutrient\%_{DR}\ DW_{DR} + Nutrient\%_{PODS}\ DW_{PODS})$ [50].

### *2.6. Statistical Analysis*

At least three replications were conducted for element analysis. One-way analysis of variance (ANOVA) was performed to detect significant differences in histograms and boxplots, and results were given as mean ± SE. Tukey's test was applied to analyze post-hoc comparisons at a significance level of $p < 0.05$ (SPSS 22.0).

## 3. Results

### *3.1. $H_2S$ Synergizes with Rhizobia to Improve the Growth Phenotype of N-Deficient Soybeans*

During the blooming stage, $H_2S$ and rhizobia obviously improved the growth phenotype of soybean under N deficiency conditions (Figure 1A). Although NaHS considerably increased chlorophyll content in leaves, the role of rhizobia was more prominent. In addition, $H_2S$ and rhizobia boosted plant height but not root length. The effective symbiosis between soybean and rhizobia resulted in the formation of nodules, with $H_2S$ addition contributing to an apparent increase in nodule number (Figure 1B). Meanwhile, $H_2S$ and rhizobia not only greatly alleviated senescence in L-LP leaves but also encouraged healthy development in M-LP and H-LP leaves (Figure 1C).

### *3.2. $H_2S$ and Rhizobia Jointly Affect Biomass Changes during Soybean Growth*

At anthesis, the biomass in roots and old leaves did not change with treatment. Rhizobia had the ability to diminish biomass in stems. However, $H_2S$ and rhizobia induced a considerable rise in biomass in new leaves and pods. The difference was that $H_2S$ was more conducive to elevating biomass in new leaves, which was at its peak under Q8 + NaHS. Biomass in pods was boosted more markedly by rhizobia. Furthermore, $H_2S$ clearly enhanced biomass in symbiotic nodules (Figure 2A).

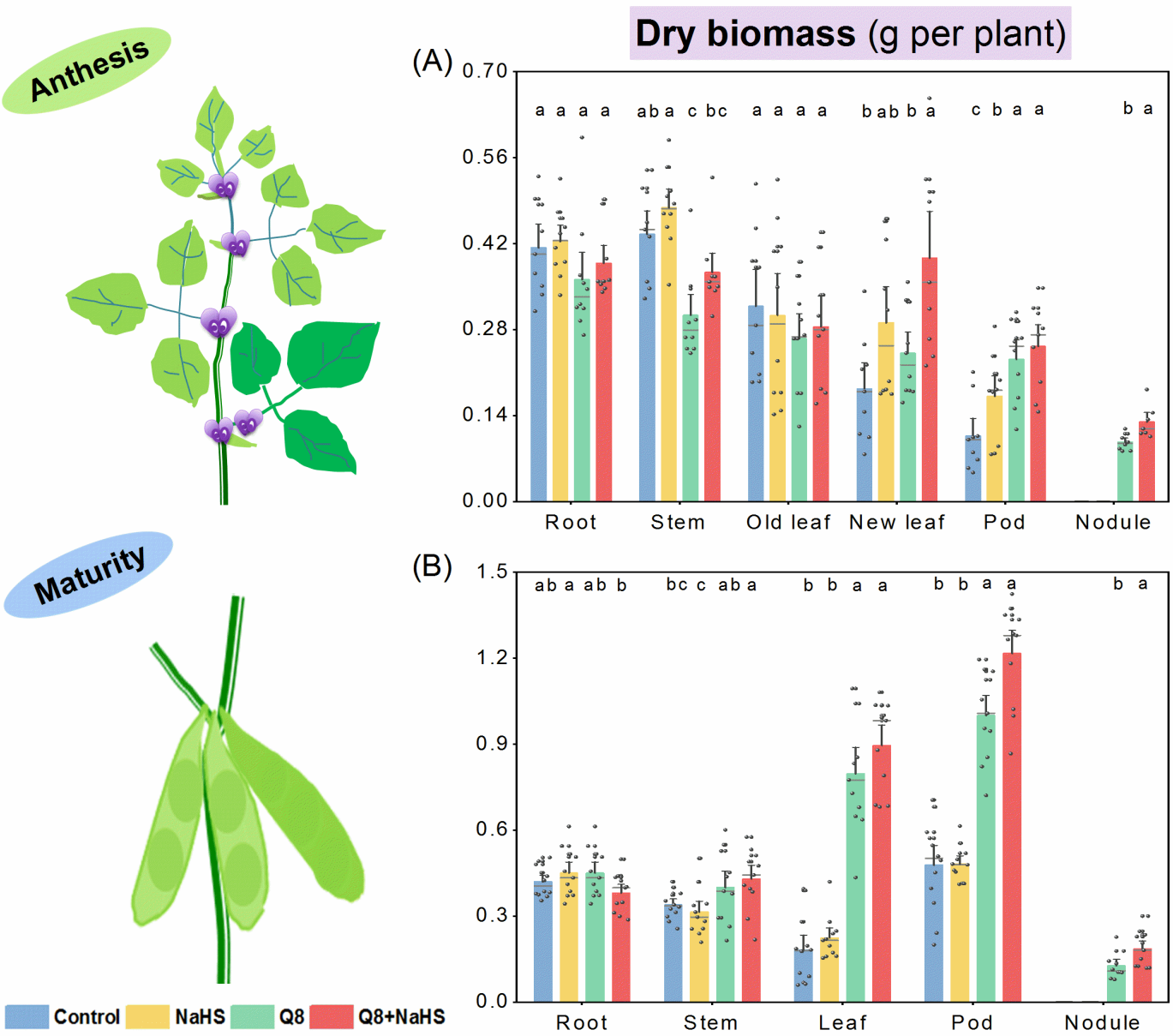


**Figure 2.** $H_2S$ collaborates with rhizobia to affect biomass changes in different organs of soybean at anthesis (**A**) and maturity (**B**). Each value is the mean ± SE. Dots indicate the measured values of each plant. The line reflects the median of each measurement. Different letters show significant differences, $p < 0.05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS.

When soybeans reached maturity, root biomass still did not change significantly. However, rhizobia increased stem biomass, with the synergistic effect of $H_2S$ and rhizobia being more prominent. Interestingly, symbiosis increased biomass in leaves and pods considerably. Moreover, nodule biomass in symbiotic systems was definitely enhanced by $H_2S$ (Figure 2B).

### *3.3. $H_2S$ and Rhizobia Synergistically Modulate the Distribution and Transport of Essential Nutrient Elements in Different Organs during Soybean Growth*

At anthesis, $H_2S$ and rhizobia reduced the organic C content in stems. However, organic C content in roots and new leaves was observably enhanced in symbiotic systems. Strikingly, $H_2S$ synergized with rhizobia to generate the greatest increase in organic C accumulation in roots. Moreover, $H_2S$ and rhizobia markedly promoted organic C storage in newborn organ pods. Organic C accumulation in nodules was not changed by $H_2S$ (Figure 3A). At maturity, the organic C level in stems remained low due to rhizobia. $H_2S$ and rhizobia were beneficial to the accumulation of more organic C in roots and leaves. In mature pods, outer pods and beans stored more organic C due to rhizobia. Furthermore, $H_2S$ enhanced organic C content in nodules (Figure 3B). N storage in different developing organs, from flowering to maturity, exhibited a consistent trend of considerable increase in response to $H_2S$ and rhizobia (Figure 3C,D). $H_2S$ cooperated with rhizobia to maintain the highest levels of total N in stems, old leaves, new leaves, and pods during blooming (Figure 3C). In addition, $H_2S$ promoted nodule N fixation in symbiotic systems at anthesis, but the opposite happened at maturity (Figure 3C,D).

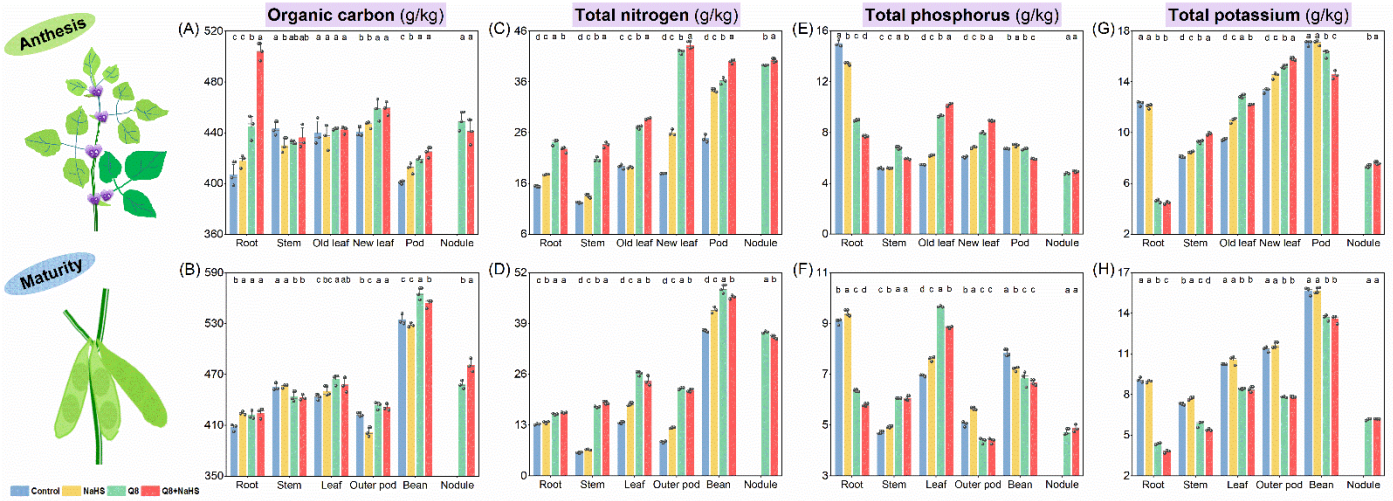


**Figure 3.** $H_2S$ and rhizobia are involved in regulating the distribution and transport of organic carbon (**A**,**B**), total nitrogen (**C**,**D**), total phosphorus (**E**,**F**), and total potassium (**G**,**H**) in different organs during soybean development. The data are presented as the mean ± SE. Dots indicate the measured values for each sample. The line reflects the median of each measurement. Different letters indicate significant differences, $p < 0.05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS.

$H_2S$ and rhizobia reduced P accumulation in roots and pods during flowering while increasing P storage in stems, old leaves, and new leaves, and this tendency was most obvious when the two worked together (Figure 3E). At maturity, symbiosis reduced the P level in roots and outer pods. $H_2S$ and rhizobia inhibited P accumulation in beans. However, $H_2S$ and rhizobia were beneficial to P storage in stems and leaves (Figure 3F). At blooming, whereas rhizobia inoculation significantly reduced K content in roots and pods, $H_2S$ and rhizobia enhanced K accumulation in stems, old leaves, and new leaves. Moreover, $H_2S$ improved K distribution in nodules (Figure 3G). However, symbiosis inhibited K accumulation in all organs at maturity (Figure 3H).

### *3.4. $H_2S$ Collaborates with Rhizobia to Regulate Stoichiometric Ratios of Vital Nutrients in Various Organs during Soybean Growth*

$H_2S$ and rhizobia clearly reduced the C:N ratio in all organs throughout flowering and maturity, with rhizobia having a greater influence (Figure 4A,B). During flowering, although $H_2S$ and rhizobia obviously lowered the C:P ratio in stems, old leaves, and new

leaves during flowering, rhizobia elevated it in roots and pods, reaching the highest level under Q8 + NaHS. Furthermore, the C:P ratio in symbiotic nodules was reduced by $H_2S$ (Figure 4C). Similarly, when soybeans matured, $H_2S$ and rhizobia substantially lowered the C:P ratio in stems and leaves, but it maintained higher in roots, outer pods, and beans due to symbiosis (Figure 4D). However, except for the fact that rhizobia significantly enhanced the C:K ratio in roots, it did not appear to change with treatment in other organs during flowering (Figure 4E). Interestingly, at maturity, symbiosis kept the C:K ratio in all organs at an extremely high level (Figure 4F).

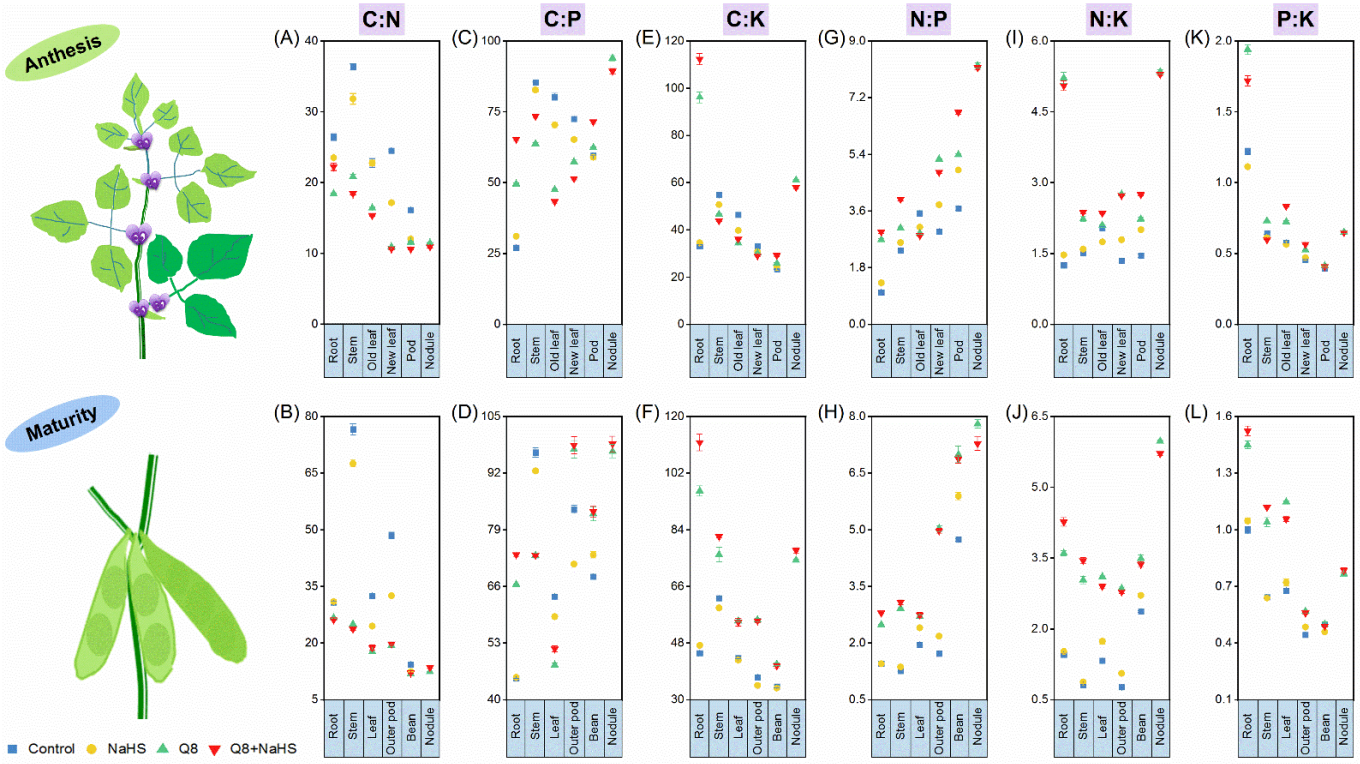


**Figure 4.** Effects of $H_2S$ and rhizobia on C:N (**A**,**B**), C:P (**C**,**D**), C:K (**E**,**F**), N:P (**G**,**H**), N:K (**I**,**J**), and P:K (**K**,**L**) ratios in soybean organs from anthesis to maturity. Each value is the mean ± SE. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS.

At flowering, $H_2S$ and rhizobia collaborated to maintain a higher N:P ratio in all organs except old leaves (Figure 4G). When soybeans matured, the N:P ratio in roots and stems remained higher because of rhizobia. $H_2S$ and rhizobia significantly increased the N:P ratio in leaves, outer pods, and beans. However, $H_2S$ reduced the N:P ratio in nodules (Figure 4H). Furthermore, rhizobia inoculation greatly enhanced the N:K ratio in roots and stems during flowering. In response to $H_2S$ and rhizobia, new leaves and pods exhibited much greater N:K ratios (Figure 4I). Similarly, at maturity, symbiosis caused a substantial rise in the N:K ratio in roots and stems, a process facilitated by $H_2S$. Moreover, $H_2S$ in concert with rhizobia proved particularly beneficial for raising the N:K ratio in leaves, outer pods, and beans (Figure 4J). However, only rhizobia elevated the P:K ratio in roots and old and new leaves of soybean during flowering (Figure 4K). Due to symbiosis, all organs except beans maintained a higher P:K ratio at maturity (Figure 4L).

### *3.5. $H_2S$ Works Together with Rhizobia to Affect Grain Nutrient Harvest during Soybean Development*

HI refers to the ratio of economic yield to biological yield at crop harvest, which may be used to measure agricultural yield levels. Therefore, we evaluated the HI and nutrient HI of soybean grains throughout development. HI in grains exhibited a higher state in symbiotic systems during flowering and maturity (Figure 5A,B). $H_2S$ and rhizobia increased all nutrient HI in grains during blooming, with rhizobia exerting a significant role (Figure 5C). However, only symbiosis greatly boosted CHI and KHI in grains at maturity (Figure 5D).

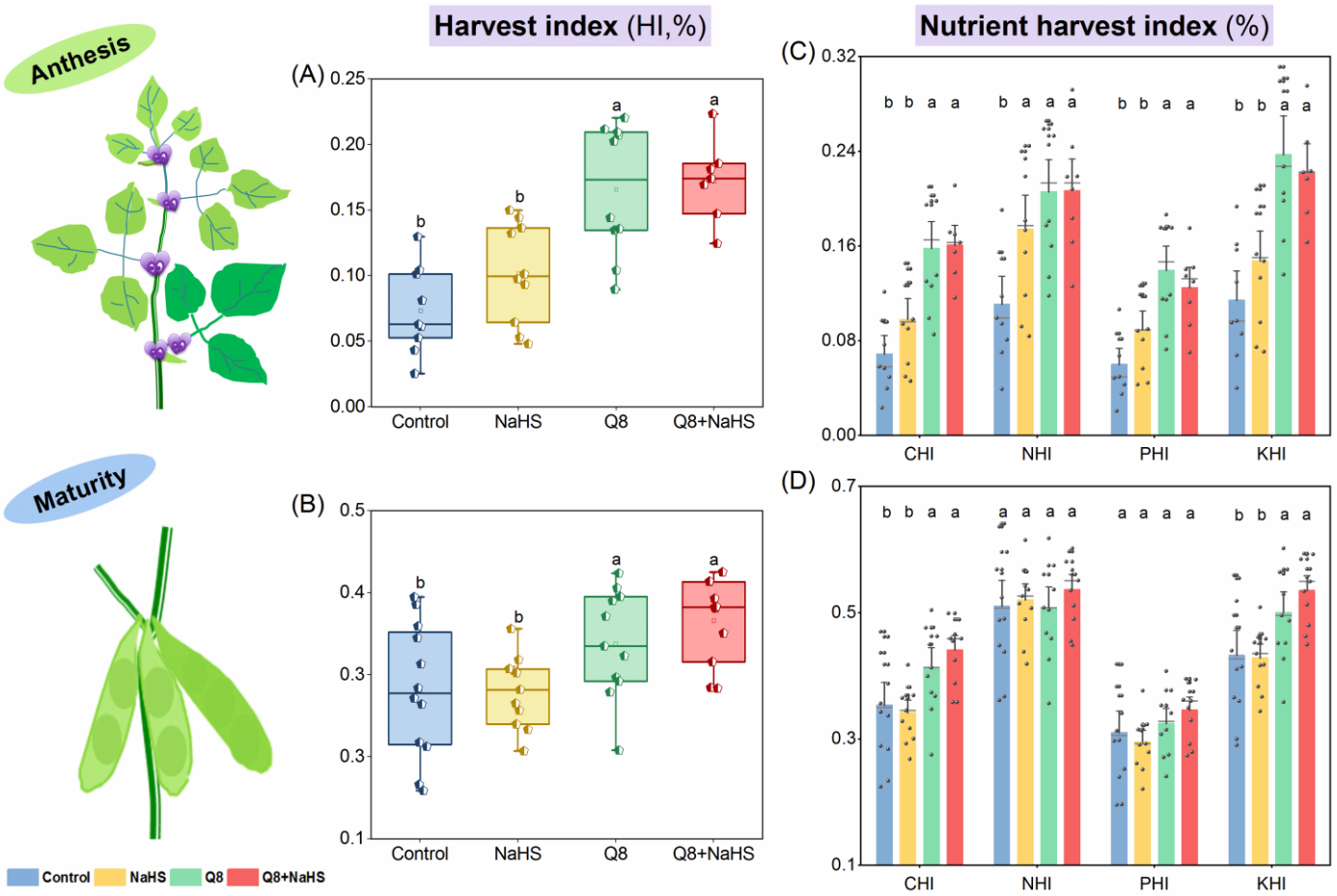


**Figure 5.** $H_2S$ crosstalk in rhizobia controls harvest index (**A**,**B**) and nutrient harvest index (**C**,**D**) of grains during soybean growth. The data are presented as the mean ± SE. Dots represent the calculated values for each plant. The line reflects the median of each calculation. Different letters indicate significant differences, $p < 0.05$. Control, without rhizobia or NaHS; NaHS, with 100-μM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-μM NaHS.

### *3.6. $H_2S$ and Rhizobia Jointly Manage Whole-Plant Nutrient Accumulation during Soybean Growth*

$H_2S$ and rhizobia were advantageous to total N accumulation in soybeans at anthesis and maturity, with the synergistic effect of the two being the most prominent (Figure 6C,D). Additionally, $H_2S$ significantly increased C, P, and K accumulation in soybeans during blooming (Figure 6A,E,G). When soybeans reached maturity, symbiosis had a decisive role in promoting C, P, and K accumulation. More crucially, $H_2S$ addition clearly aided this propensity (Figure 6B,F,H).

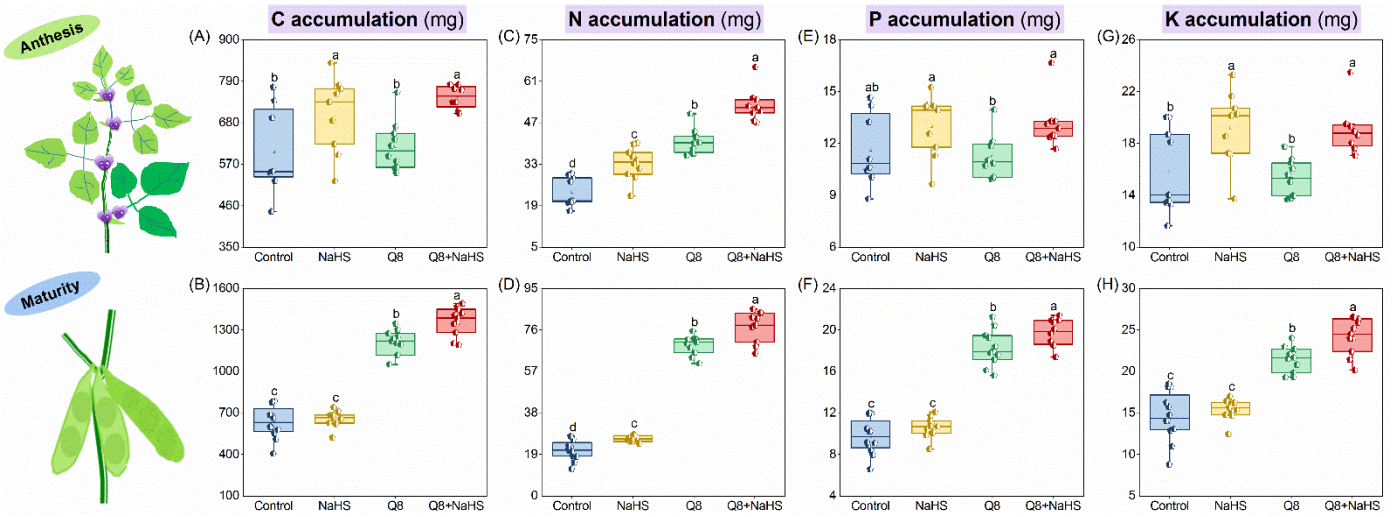


**Figure 6.** $H_2S$ combines with rhizobia to modulate C (**A**,**B**), N (**C**,**D**), P (**E**,**F**), and K (**G**,**H**) accumulation throughout the plant during soybean growth. Each value is the mean ± SE. Dots represent the calculated values for each plant. The line reflects the median of each calculation. Different letters show significant differences, $p < 0.05$. Control, without rhizobia or NaHS; NaHS, with 100-µM NaHS; Q8, with rhizobia inoculation; and Q8 + NaHS, with rhizobia inoculation and 100-µM NaHS.

## 4. Discussion

### *4.1. $H_2S$ and Rhizobia Synergistically Improve Growth and Development in N-Deficient Soybeans*

In our study, $H_2S$ and rhizobia significantly enhanced soybean leaves and growth phenotypes under N deprivation by elevating leaf chlorophyll content and plant height (Figure 1). Indeed, it has previously been demonstrated that $H_2S$ can extend life duration by reducing chlorophyll loss and increasing leaf and root biomass, thus shielding soybean seedlings against drought-induced oxidative stress [51]. Following that, we discovered that $H_2S$ totally inhibited the interveinal chlorosis of soybean leaves under iron-deficient conditions while enhancing seedling biomass [52]. Furthermore, SNF generated by interactions with rhizobia becomes the primary N source for legumes [29]. N abundance and deficiency directly determine leaf chlorophyll concentration and agricultural productivity. Therefore, rhizobia symbiosis mitigated old leaf (L-LP) senescence and new leaf (M-LP and H-LP) yellowing caused by N deficiency in soybeans by considerably boosting leaf chlorophyll content, ultimately encouraging healthy plant development overall (Figure 1). Moreover, when using substrates for soybean culture early on, $H_2S$ and rhizobia increased shoot biomass rather than root biomass during development, as well as grain yield at maturity [44]. However, after extensive screening and cultivation, we found that river sand culture is ideal for researching soybean-symbiosis systems since it allows plants to grow more vigorously. Therefore, in the current study of cultivating soybeans with river sand, it was further confirmed that $H_2S$ and rhizobia were highly beneficial to new leaf biomass and primary pod output during blooming, whereas rhizobia greatly boosted shoot biomass and full pod yield at maturity (Figure 2). Furthermore, our previous study indicated that $H_2S$ promoted plant growth by enhancing nodulation and N fixation in soybean-rhizobia symbiotic systems [42]. This supported the substantial increase in $H_2S$ on nodule number and biomass in symbiotic systems (Figures 1B and 2). Therefore, in symbiotic systems, $H_2S$ may enhance chlorophyll content by boosting N fixation performance, thereby affecting photosynthetic rate and promoting soybean growth and production.

### *4.2. $H_2S$ and Rhizobia Are Involved in Managing the Allocation and Recycling of Essential Nutrients during Soybean Growth*

Organic carbohydrates, as a C-skeleton and energy source, are essential for plant growth and development [53,54]. Especially in legumes, nodules due to symbiosis are likewise inseparable from the host's C supply. Photosynthesis provides the C-skeleton

for all organic materials generated by plants throughout their life activities. In soybeans, leaves, including old and new leaves, are crucial source organs for photosynthesis to produce assimilated organic products, whereas roots, stems, pods, and nodules are all sink organs. $H_2S$ stimulates photosynthetic C uptake and metabolism, supplying energy and a C skeleton to plants developing under various stresses [55–57]. Moreover, in legumes, N nutrition given by active symbiosis will directly lead to higher photosynthesis and C fixation [58,59]. Of course, the same was true for soybeans [60,61]. Here, during flowering, $H_2S$ and rhizobia were more inclined to promote organic C transport and storage from stems and old leaves to vigorously developing organs such as new leaves, pods, and roots (Figure 3A). At maturity, rhizobia symbiosis dominated in regulating the rapid utilization and accumulation of organic C in roots, leaves, pods, and nodules (Figure 3B). Furthermore, as we previously demonstrated, $H_2S$ promoted nodulation and N fixation in soybean symbiotic systems [42,44], which explains the higher organic C storage in nodules driven by $H_2S$ (Figure 3B).

N is well known for its involvement in directing C assimilation and primary production as a building block of chlorophyll and numerous compounds in plants [62,63]. Increasingly, studies suggest that $H_2S$ increases amino acids and total N content in organs by activating N metabolism, hence improving plant tolerance to diverse environmental challenges [64–68]. The symbiosis between legumes and rhizobia is undoubtedly connected to an increase in host N content [29]. In this study, from blooming to maturity, $H_2S$ and rhizobia significantly boosted N accumulation in practically all growth organs (Figure 3C,D). Moreover, we observed an intriguing phenomenon in which, during blooming with robust metabolic activities, $H_2S$ in collaboration with rhizobia may play the most important function in preserving high N in various tissues by efficiently enhancing nodule N fixation (Figure 3C).

P is not only a component of plant cell structure and various essential compounds, but it also participates in a variety of plant life activities such as energy metabolism, carbohydrate metabolism, and metabolic pathway regulation [69,70]. P in soybeans is mostly derived from phosphate absorbed by roots from the environment in our experiment. At both blooming and maturity, $H_2S$ and rhizobia tended to stimulate soybeans to more efficiently transfer and store P acquired from roots to stems, leaves, and nodules rather than pods (Figure 3E,F). The possible explanation is that in soybeans, $H_2S$ and rhizobia are quite helpful at avoiding excessive accumulation of total P in pods, resulting in respiratory consumption, thereby promoting high storage of P in stems and leaves for recycling during organ development and transport of P to nodules for better N fixation. Indeed, $H_2S$ exhibits the ability to modulate P distribution in various soybean tissues [71,72]. Furthermore, the continuous N supply generated by symbiosis meets the host's N demand. Therefore, if N is no longer the most restricted resource, host resource allocation and acquisition will adjust to accommodate preferentially assimilating other scarcer nutrient resources such as P [60,73,74]. Moreover, in legume–rhizobia symbiosis systems, nodule N fixation and organ development are inextricably linked to an adequate P supply [75,76]. This is also why symbiosis benefitted soybeans by preferentially transporting P absorbed in roots to stems, leaves, and nodules (Figure 3E,F).

K, as an activator of over 60 enzymes, is linked to several metabolic activities throughout plant growth, including photosynthesis, assimilation product transport, carbohydrate metabolism, and protein synthesis [77–79]. Similar to the change in P, $H_2S$ and rhizobia lowered the distribution of K in roots and pods while increasing its accumulation in stems, old leaves, new leaves, and nodules during flowering (Figure 3G). Therefore, $H_2S$ and rhizobia also promoted efficient K transfer from roots to stems and leaves for more active metabolic activities while inhibiting respiratory consumption caused by excessive K accumulation in pods. Similarly, previous research found that $H_2S$ protected plants from stress by increasing K uptake and distribution [72,80]. Through enhanced N metabolism, SNF promotes fast transit and allocation of K ions [71,81]. Accordingly, the synergistic effect of $H_2S$ and rhizobia triggered the highest K accumulation in stems and new leaves for

metabolism and recycling (Figure 3G). Furthermore, K is required for nodule growth and function [30,82], implying that $H_2S$ might improve N fixation by increasing K distribution in nodules (Figure 3G). However, at slow-growing maturity, rhizobia tended to suppress K levels in all organs to avoid active metabolic activities after achieving soybean growth needs (Figure 3H).

To sustain growth and development, the supply of various elements in plants must reach a certain equilibrium. Growth rate theory states that organisms must modify their C:N:P:K ratios in real time to accommodate fluctuations in growth rates [83,84]. Therefore, we evaluated the C:N:P:K ratio in several organs across soybean growth to explore element changes and partitioning caused by $H_2S$ and rhizobia. It was obvious that during flowering possessing vigorous metabolic activity, $H_2S$ and rhizobia supported a significant rise in N content in diverse tissues. Subsequently, growth acceleration caused by increased N levels clearly stimulated the quick transfer and utilization of P and K in stems, old leaves, and new leaves and promoted the storage of more C in roots and pods (Figure 4A,C,E,G,I,K). However, at maturity with stable metabolism, symbiosis had a more dominant role in N accumulation in various organs. Similarly, the resulting accelerated plant growth spurred greater P and C storage in stems and leaves, as well as increased C accumulation in roots, outer pods, and beans (Figure 4B,D,F,H,J,L). Therefore, we speculate that $H_2S$ and rhizobia may cooperatively modulate nutrient assimilation, distribution, and metabolism in various organs during soybean growth by influencing critical enzyme activities, protein abundance, and gene expression involved in C, N, P, and K metabolism.

### *4.3. $H_2S$ Synergizes with Rhizobia to Regulate Grain Nutrient Harvest and Overall Plant Nutrient Accumulation during Soybean Development*

The physiological nature of HI indicates the fraction of crop assimilates in grains and vegetative organs, which is widely used as an evaluation criterion for maximizing crop production and is highly influenced by photosynthetic properties and N levels [85]. Appropriate crop management is expected to promote HI for improved crop output, primarily by increasing source activity, sink intensity, and remobilization of pre-stored C from vegetative tissues to grains [86]. In legumes, symbiosis not only stimulates sink strength through nodule C cost but also promotes photosynthesis by increasing leaf N nutrition [61]. In our study, soybean–rhizobia symbiotic systems consistently maintained higher HI during development, resulting in high grain production (Figures 2 and 5A,B). There is evidence that rhizobia symbiosis improves bean output by increasing HI [87,88]. Further, NHI denotes the ratio of grain N accumulation to total plant N accumulation, which quantifies the transfer and distribution of N from vegetative parts to grains [85,89]. Here, we evaluated the HI of C, N, P, and K in turn. Facts proved that during blooming with vigorous metabolic activity, the HI of these four critical elements was all boosted by $H_2S$ and rhizobia, indicating a greater distribution of nutrients in grains. Of course, symbiosis played a particularly important role in this process by fixing N and encouraging development (Figure 5C). However, at steady maturity, NHI and PHI stability may be attributable to the fundamental completion of protein synthesis in grains. Elevated CHI and KHI levels in symbiotic systems suggest that rhizobia aid in the appropriate transfer and utilization of C and K to grains (Figure 5D).

In plants, N accumulation in each organ is equal to the product of each organ's dry matter mass and N content. The overall plant N accumulation is the sum of all organ accumulations. Actually, N accumulation in legumes has been extensively studied [90,91]. We focused on critical nutrient buildup during soybean development here. $H_2S$ and rhizobia considerably increased N accumulation in soybeans by modulating N fixation and metabolism, with their synergistic impact being the most obvious (Figure 6C,D). This was also confirmed in our previous study [44]. Furthermore, $H_2S$ enhanced C, P, and K accumulation in soybeans during blooming via its development-promoting function (Figure 6A,E,G). However, at maturity, symbiosis performed an absolutely dominant role in soybean nutrient accumulation by virtue of its superior N-fixing ability (Figure 6B,F,H). This

is because rhizobia affect host resource allocation and access through N fixation, pushing the host to modify root development and structure in order to effectively absorb and utilize more required nutrients [60,73,74]. Concurrently, symbiosis stimulates metabolism and signaling in hosts and symbionts, resulting in a greater demand for other resources to sustain further development [92]. Interestingly, $H_2S$, in collaboration with rhizobia, enabled soybean to finally achieve maximal nutrient accumulation (Figure 6B,F,H), most likely due to $H_2S$'s efficient enhancement of N fixation and metabolic capability in symbiotic systems [42,44]. Therefore, $H_2S$ collaborates with rhizobia to promote photosynthesis and stimulate growth and metabolism by improving source activity and sink intensity, thereby effectively controlling and managing nutrient harvest and accumulation during soybean development.

## 5. Conclusions

After entering plants, C, N, P, and K nutrients received in various ways are continually transported and recycled among numerous organs in soybean–rhizobia symbiotic systems. Among them, C is mostly obtained by photosynthesis of old and new leaves. N is primarily dependent on nodule N fixation, whereas P and K are absorbed from the environment via roots (Figure 7). However, does $H_2S$ serve an important regulatory role in the acquisition, transport, and accumulation of these vital nutrients? Sufficient and strong evidence suggests that $H_2S$ collaborated with rhizobia to manage the rational distribution and dynamic balance of nutrients in varied organs of soybean, impacting grain nutrient harvest and plant nutrient accumulation and, ultimately, boosting plant growth and productivity. Therefore, our research provides a sound theoretical platform for future control of optimal nutrient transport and utilization in legume–rhizobia symbiotic systems.

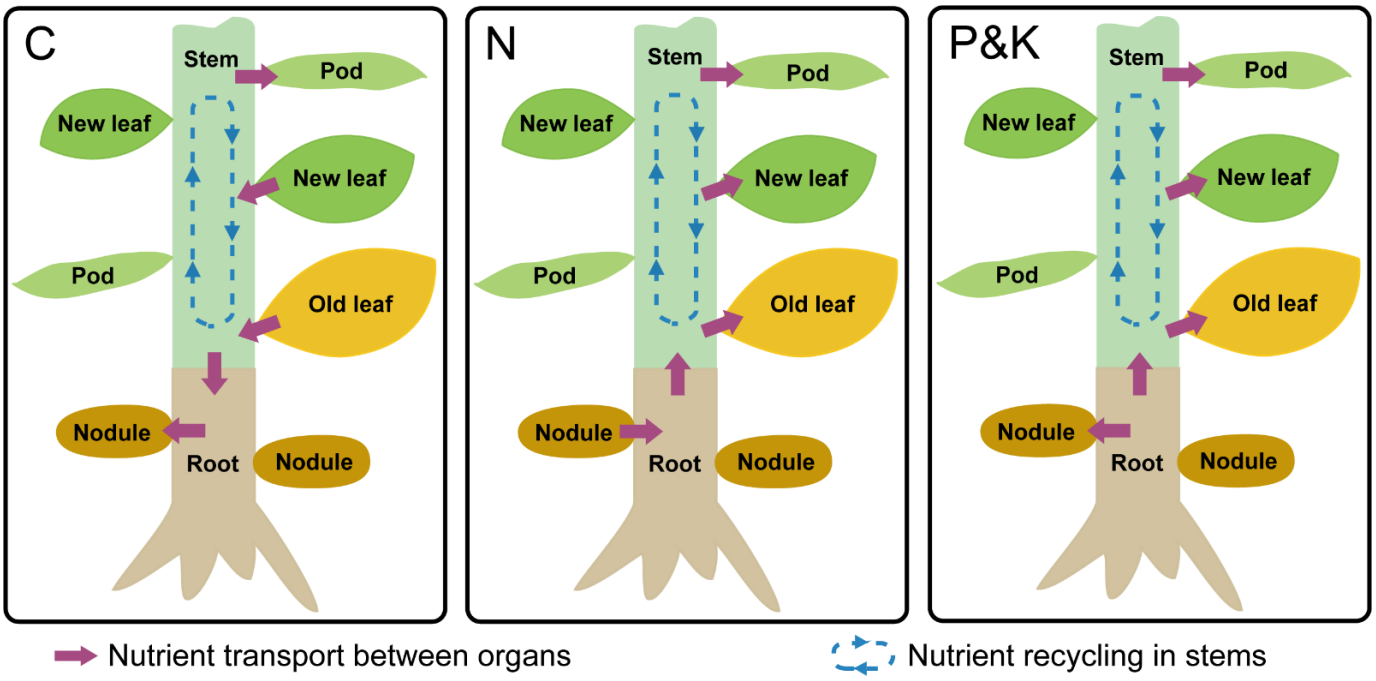


**Figure 7.** Model diagram of nutrient transport and recycling among various organs in soybean-rhizobia symbiotic systems.

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