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Rhizobium Symbiosis Leads to Increased Drought Tolerance in Chinese Milk Vetch (*Astragalus sinicus* L.)

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Abstract: Chinese milk vetch (*Astragalus sinicus* L.) is an important leguminous green manure that frequently suffers from seasonal drought. To improve its drought tolerance, the effects of rhizobia inoculation on drought tolerance and the underlying physiological mechanism were investigated. Drought tolerance in combination with nitrogen assimilation, free amino acids, and polyamines was investigated in milk vetch with active nodules (AN), with inactive nodules (LN), or without nodules (NN). AN plants had increased drought tolerance compared to LN and NN plants. Glutamine synthetase (GS), glutamine 2-oxoglutarate amino transferase (GOGAT), and glutamate dehydrogenase (GDH) activities were decreased after drought, with higher levels in AN plants than in LN and NN plants under both control and drought stress conditions. Higher levels of proline (Pro), arginine (Ala), alanine (Ala), and glutamate (Glu) were observed in AN plants compared with LN and NN plants. Putrescine (Put), spermidine (Spd), and spermine (Spm) levels were increased in response to drought, and higher levels of Put and Spd were maintained in AN plants. It is suggested that active nodulation leads to increased drought tolerance in milk vetch, which is associated with improved nitrogen fixation and ammonium assimilation, which in turn lead to the promotion of free amino-acid and polyamine synthesis.

Keywords: Chinese milk vetch; drought stress; free amino acids; nitrogen fixation; polyamines; symbiosis

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1. Introduction

Legume–rhizobia symbiosis is the mutual interaction in which rhizobia colonize plant roots and form a new root organ, i.e., a symbiotic nitrogen-fixing nodule [1]. The atmospheric nitrogen is fixed via nitrogenase in the nodule to provide leguminous plants with ammonium using the carbon sources from plants as energy supply. Ammonium is assimilated to produce glutamine and glutamate catalyzed by glutamine synthetase (GS) and glutamine 2-oxoglutarate amino transferase (GOGAT) [2,3]. In addition, GDH catalyzes the reversible conversion of α-ketoglutarate to glutamate via a reductive amination [4,5]. Rhizobia, legume–rhizobia symbiosis, and nodule function are greatly influenced by soil drought [6,7]. Bacterial growth and persistence are reduced under drought conditions [8,9]. The symbiosis nitrogen fixation process is negatively influenced; for example, the development of new plant–bacterium interactions and infection threads at the early infection stage is greatly reduced under drought stress [10–12]. Nodule number, leghemoglobin content, and nitrogenase activity are reduced, and nodule senescence is accelerated by drought stress [13–15].

Although rhizobium symbiosis is inhibited by abiotic stresses, the host plants with symbiotic nitrogen fixation survive better than non-inoculated ones under stressful conditions such as drought, salt, and alkali stress [15–18]. Alfalfa plants with active nodules (AN) show enhanced drought tolerance with decreased lipid peroxidation and increased catalase activity, as well as reduced glutathione (GSH) level [16]. Compared to the non-nodulated (NN) plants, nodulated plants in *M. truncatula* showed delayed leaf senescence after drought stress and improved recovery after rewatering [19]. Compared with nitrate-fed plants, nodulated pea (*Pisum sativa*) plants had increased drought tolerance, associated with improved photorespiratory flux and decreased stomatal conductance [20]. Enhanced drought tolerance was also obtained in nodulated common bean (*Phaseolus vulgaris*) and *Lotus corniculatus* with increased pod dry weight and proline accumulation compared with NN and nitrate-fed plants [21,22]. However, nodulation-improved drought tolerance has not been widely investigated in leguminous plants, and our understanding of enhanced drought tolerance by nodulation is still limited.

Chinese milk vetch (*A. sinicus* L.) is commonly cultivated as a green manure legume in paddy fields in rice-cropping systems in southern China [23]. It improves soil fertility as a result of symbiotic nitrogen fixation, which leads to reduced chemical fertilizer application for rice growth and production [24–26]. Its incorporation into the soil in combination with rice-straw return produces large amounts of soil dissolved organic matter to maintain soil productivity and improve rice yields [25,27]. In addition, the cultivation of milk vetch and its incorporation into soil lead to changes in the bacterial community structure and the functional groups related to nutrient transformation in soil [28]. A seasonal drought in autumn occurs frequently in southern China, which largely affects milk vetch growth during the seedling stage. However, the responses of milk vetch to drought and whether nodulation affects drought tolerance in milk vetch plants are still unknown. The objective of this study was to investigate the effect of nodulation on drought tolerance in milk vetch. Drought tolerance in combination with nitrogen utilization, free amino acids, and polyamines, as affected by nodulation, was examined. The results suggest that nodulation increases drought tolerance in milk vetch.

2. Materials and Methods

2.1. Plant Growth and Treatments

The seeds of milk vetch were sterilized by 10 min of treatment with 10% NaC_lO (*v*/*v*) solution, followed by washing three times with water. The sterilized seeds were placed on a wet paper towel in Petri dishes for germination under room temperature. The germinated seeds were planted in plastic pots filled with sterilized gravelly soil/perlite mixture (1:1, *v*/*v*), and 10 seedlings were maintained in each pot for growth in a greenhouse with temperatures from 20 °C to 30 °C under natural light. Four-day-old seedlings were divided into three groups. Two groups were inoculated with 5 mL of *Mesorhizobium huakuii* strain 7653R. The rhizobia were cultured to OD_{600} = 0.6–0.8, and then diluted to OD_{600} = 0.05 with sterile water. Then, 5 mL of rhizobium liquid was inoculated per plant. The first group was irrigated with full Hoagland nutrient [29], while the second group was irrigated with low-N (3 mM nitrogen) Hoagland nutrient solution. The third group was not inoculated with *M. huakuii* and irrigated with 5 mL of Hoagland nutrient solution as a control. Plants were irrigated with the above nutrient solution once every 5 days and allowed to grow for 90 days. For the drought treatment, plants were entirely irrigated, followed by withholding irrigation for 11 days in the greenhouse. Three pots were used as replications for each group of plants, and the leaves and roots were harvested to determine the concentrations of free amino acid and polyamine, as well as enzyme activity.

2.2. Determination of Nitrogenase Activity

Nitrogenase activity was determined by the acetylene reduction assay (ARA) using gas chromatography [30]. Nodules were harvested 90 days after inoculation. Nodules from each plant were placed into 20 mL glass vials sealed with a rubber cap, and 2 mL of acetylene was immediately injected into the vials so that nodules were incubated in 10% (*v*/*v*) acetylene for 2 h at 28 °C in darkness. One milliliter samples were injected into the gas chromatography (Thermo Scientific, Dreieich, Germany) to determine the amount of produced ethylene. The nitrogenase activity was calculated on the basis of the standard curve of ethylene, and one unit was defined as 1 μmol ethylene per h per plant. Each assay was carried out with three biological replicates.

2.3. Evaluation on Drought Tolerance

Relative water content (RWC) and ion leakage were determined from the leaves of pot plants after 9 or 11 days of withholding irrigation as previously described [31]. For measurement of RWC, fresh leaves were weighed (W_f) and immersed in water until the weight was constant (W_s). The water-saturated leaves were then dried at 80 °C for weighing the dry weight (W_d). RWC was calculated according to $(W_f - W_d)/(W_s - W_d) \times 100$. For measurement of ion leakage, leaf samples were immersed in 15 mL of distilled water with slight shaking for 3 h. The conductivity of the solution (C_1) was measured. After the samples were heated in boiling water bath for 20 min and cooled to room temperature, the conductivity of killed tissues (C_2) was measured. Ion leakage was calculated according to $(C_1/C_2) \times 100$. Survival rate was measured by counting the survival plants after 2 days of re-irrigation following 14 days of withholding irrigation. The surviving plants were counted for calculating the survival rates [32].

2.4. Determination of Free Amino Acids

Free amino acids were determined as previously described [33]. Fresh leaves (1 g) were homogenized in 3 mL of 6 % (*w*/*v*) 5-sulfosalicylic acid at 4 °C for extraction of free amino acids, followed by centrifugation at 4 °C at 15,000 rpm for 20 min. The supernatant was subjected to derivatization by phenyl isothiocyanate, followed by filtration (0.22 μm). Then, 20 μL of the filtrate was injected into the amino-acid analyzer (L-8800, Hitachi Co. Ltd., Tokyo, Japan), supplied with a Hitachi chromatographic column 855−350, for measurement of amino acids.

2.5. Determination of Polyamines

Free polyamines were extracted from leaves (0.1 g) in 3 mL of 5% (*v*/*v*) perchloric acid and incubated for 1 h at 4 °C, followed by centrifugation at 15,000× *g* for 30 min. The supernatant was used for the detection of free polyamines using ultra-performance liquid chromatography (UPLC) as previously described [34]. Aliquots (0.5 mL) of the supernatants were benzoylated [35], while the benzoyl polyamines were resuspended in 1 mL of mobile phase solution for filtration (0.45 μm filter). Then, 2 μL of the filtrate was injected into the HPLC system (Agilent infinity 1290, Agilent Technologies, Waldbroon, Germany), supplied with an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm). Identification and quantification of putrescine (Put), spermidine (Spd), and spermine (Spm) in each sample were achieved by comparing each peak retention time and peak area with the standard Put, Spd, and Spm, being detected at 230 nm. Three individual samples from different plants were used for measurement as replications.

2.6. Determinations of Enzyme Activity

For measurements of glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) activities, fresh leaves (0.2 g) were ground in 1.5 mL of extraction buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 0.5% Triton X-100, and 0.1% β-mercaptoethanol. After centrifugation as above, the supernatants were collected for measurements of enzyme activity. For measurement of GS activity, 100 μL of enzyme extract was added into 250 μL of the reaction buffer containing 50 mM imidazole (pH 7.2), 18 mM ATP, 28 mM $MgCl_2$, 25 mM hydroxylamine, and 92 mM L-glutamate. After the vortex, the mixture was incubated for 20 min at 30 °C, followed by adding 1 mL of stop solution consisting of 370 mM $FeCl_3$, 200 mM TCA, and 700 mM HCl. After centrifugation, the supernatant was collected to measure absorbance at 540 nm. One unit of GS activity was defined as the amount of enzyme required for the formation of 1 μmol of γ-glutamyl monohydroxamate (GMH) [36]. For measurement of GOGAT activity, the reaction solution (3 mL) contained 25 mM sodium phosphate (pH 7.5), 0.4 mL of 5 mM 2-oxoglutarate, 1 mM EDTA, 0.1 mL of NADH, and 0.5 mL of extract enzyme, while 0.4 mL of 20 mM L-glutamine was added to initiate reaction. The decreased absorbance within 3 min at 340 nm was recorded [37]. The assay mixture of GDH consisted of 1.7 mL of 100 mM sodium phosphate (pH 8.1), 0.3 mL of 200 mM 2-oxoglutarate, 0.3 mL of 1.5 M ammonium sulfate, 0.6 mL of 1 mM NADH, and 0.1 mL of enzyme. The decreased absorbance within 3 min at 340 nm was recorded [37]. One unit of GOGAT or GDH activity corresponded to the amount of enzyme that converted 1 μmol of the substrate within 1 min at 25 °C.

2.7. Statistical Analysis

All data were subjected to analyses of variance according to the model for a completely randomized design using the SPSS program (SPSS Inc., Chicago, IL, USA). Differences among means of treatments were evaluated by Duncan's test at the 0.05 probability level.

3. Results

3.1. Nodulation in Milk Vetch under Different Levels of Nitrogen

The effects of rhizobia inoculation on the morphological and physiological characteristics of milk vetch plants were analyzed. No nodule (NN) was observed in roots when rhizobia were not inoculated (Figure 1A). Active nodules (AN) pink in color were observed in roots of the plants inoculated with rhizobia and cultured in low-N nutrient solution (Figure 1C), while inactive nodules (LN) smaller and white in color were observed in roots of the plants inoculated with rhizobia and cultured in Hoagland nutrient solution (Figure 1B). The nodule weight per plant was twofold higher in AN plants than in LN plants (Figure 1D). The pink nodules in AN plants had higher nitrogenase activity than those in LN plants (Figure 1E).

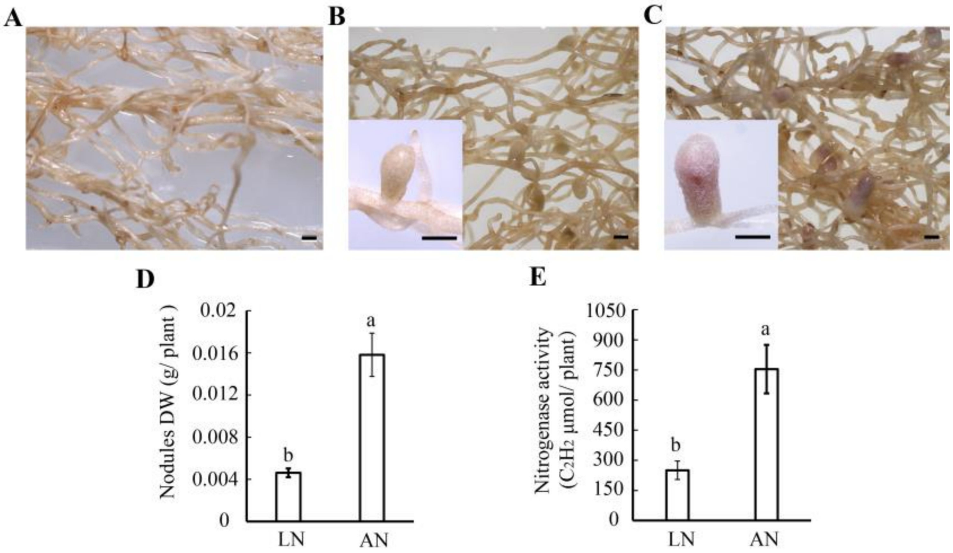

Figure 1. Analysis of rhizobia inoculation on nodule formation and nitrogenase activity in Chinese milk vetch. Roots with no nodule (NN) (**A**) were observed in 3 month old plants without inoculation and cultured in Hoagland nutrient solution. Low-activity nodules (LN) (**B**) and active nodules (AN) (**C**) were observed in 3 month old plants inoculated with rhizobia and cultured in Hoagland nutrient solution or in the low-N nutrient solution, respectively. Dry weight (**D**) and nitrogenase activity (**E**) of the nodules in 3 month old LN and AN plants were measured. Scale bars with 1 mm are presented in the figures. The data presented are means ± standard errors from 10 replicates. The same letter above the column indicates no significant difference at $p < 0.05$.

3.2. Nodulation Led to Increased Drought Tolerance in Milk Vetch

Relative water content (RWC) and ion leakage in leaves were measured during drought treatment and after recovery of rewatering. RWC was decreased in all plants upon withholding irrigation. The highest level was maintained in AN plants, and higher levels were observed in NN than in LN plants after 9 or 11 days of withholding irrigation. RWC was increased and reached a similar level for all three types of plants after 2 days of rewatering

(Figure 2A). Ion leakage was increased after drought treatment, and the lowest level was observed in AN plants, whereas NN plants had a lower level than LN plants after 9 and 11 days. Ion leakage was decreased after rewatering, and AN plants had the lowest level (Figure 2B). Whereas LN plants showed serious wilting and most of them died after 14 days of withholding irrigation, some of the AN and NN plants still remained green (Figure 3A). After 2 days of re-irrigation for recovery, AN and NN plants had survival rates of 97.5% and 42.5%, respectively, compared to the 5% survival rate in LN plants (Figure 3A,B). The results indicated that AN plants had increased drought tolerance compared with NN and LN plants, while LN plants had the lowest drought tolerance.

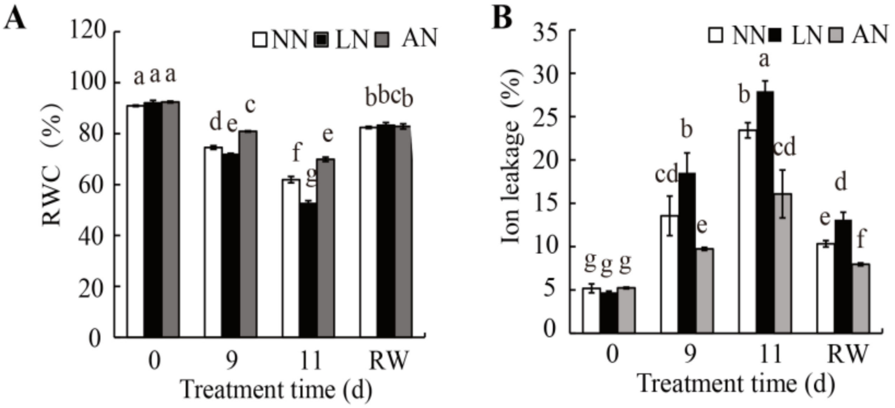

Figure 2. The effects of rhizobia inoculation on relative water content (RWC) and ion leakage in response to drought stress. Ninety-day-old *A. sinicus* plants were withheld irrigation for drought stress treatment. RWC (**A**) and ion leakage (**B**) were measured at 0, 9, and 11 days after withholding irrigation and 2 days after rewatering (RW). AN, LN, and NN indicate the plants with active nodules, low-activity nodules, and no nodules, respectively. The data presented are means and standard errors of three replicates, and the same letter above the column indicates no significant difference at $p < 0.05$.

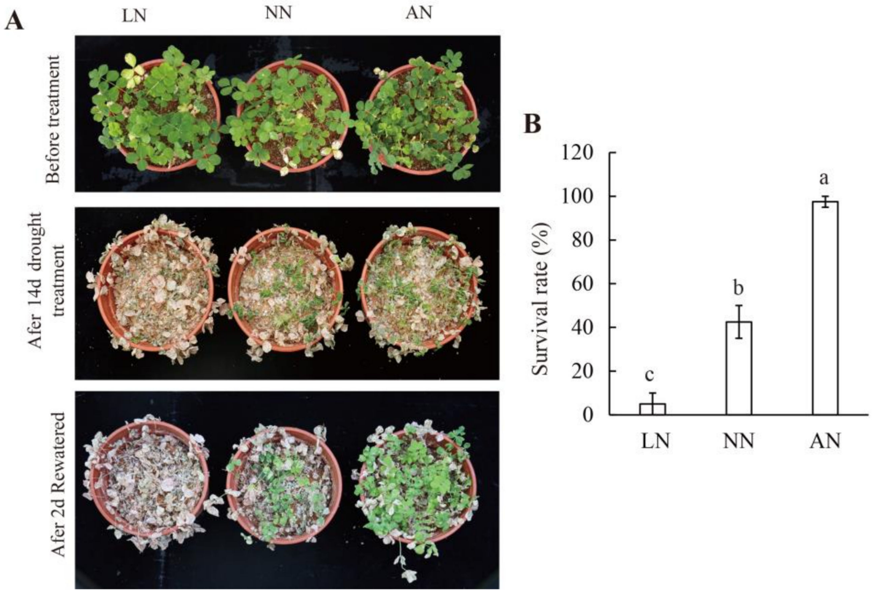

Figure 3. Plant performance in Chinese milk vetch as affected by rhizobia inoculation and drought stress. Ninety-day-old plants were exposed to 14 days of withholding irrigation, followed by 2 days of rewatering. Photographs were taken at the times indicated in figure (**A**), while the survival rate was recorded after 2 days of rewatering (**B**). AN, LN, and NN indicate the plants with active nodules, low-activity nodules, and no nodules, respectively. The data presented are means and standard errors of three replicates, and the same letter above the column indicates no significant difference at $p < 0.05$.

3.3. The Nodulated Milk Vetch Had Increased GOGAT, GS, and GDH Activities under Drought Conditions

Glutamine synthetase activity was higher in AN plants than in LN and NN plants, while it showed no difference between LN and NN plants under control conditions (Figure 4A). Drought treatment resulted in decreased GS activity in all plants, while a higher level was maintained in AN plants than in LN and NN plants. GS activity was further decreased in LN and AN plants, with a lower level in LN plants after rewatering (Figure 4A). AN plants had higher GOGAT activity than others, while NN plants had a higher level than LN plants under control conditions (Figure 4B). GOGAT activity was decreased in AN plants after drought treatment, but not in other plants. The activity was further decreased with a higher level in AN and NN plants compared with LN plants (Figure 4B). GDH activity was higher in AN plants compared with LN and NN plants under control conditions, while LN had higher activity than NN plants (Figure 4C). It was decreased in AN and LN plants after drought treatment and rewatering, while a higher level was maintained in AN plants than in LN plants. GDH activity was unaltered in NN plants in response to drought (Figure 4C). The results indicated that GS, GOGAT, and GDH activities were more responsive to drought in AN and LN plants than in NN plants, and that higher levels were maintained in AN plants than in LN pants.

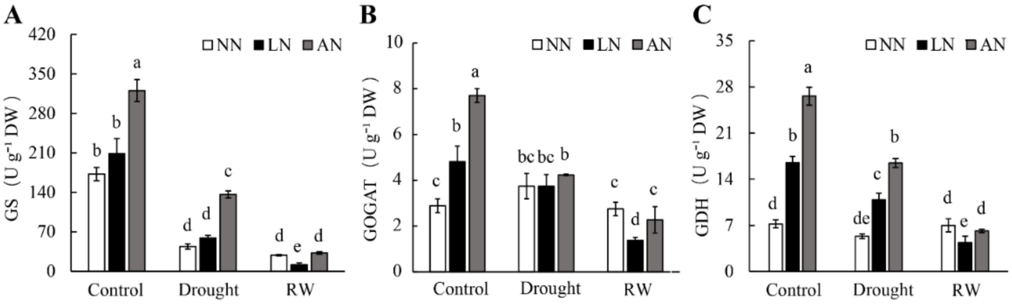

Figure 4. The effects of rhizobia inoculation on GS, GOGAT, and GDH activities in roots of Chinese milk vetch in response to drought stress. Ninety-day-old plants were withheld irrigation for 11 days (drought), followed by 2 days of rewatering (RW). Activities of GS (**A**), GOGAT (**B**), and GDH (**C**) in leaves were determined, while non-stressed plants were used as a control. AN, LN, and NN indicate the plants with active nodules, low-activity nodules, and no nodules, respectively. The data presented are means and standard errors of three replicates, and the same letter above the column indicates no significant difference at $p < 0.05$.

3.4. Nodulation Led to Altered Free Amino Acids in Milk Vetch

The ammonia produced by N_2 fixation is assimilated by the GS/GOGAT system to glutamic acid, which is in turn transaminated to amino acids [38]. Free amino acids in 90-day-old milk vetch plants were analyzed. Compared to NN plants, higher levels of proline (Pro), arginine (Arg), and alanine (Ala) were observed in AN and LN plants, while AN had higher levels than LN plants. The glutamate (Glu) level was higher in AN plants than in LN and NN plants, while the glycine (Gly) level was higher in LN than in NN and AN plants. Lysine (Lys), leucine (Leu), valine (Val), isoleucine (Ile), tyrosine (Tyr), and methionine (Met) concentrations showed no difference between AN and LN plants, with lower levels than in NN plants. Phenylalanine (Phe), cysteine (Cys), and histidine (His) concentrations showed no significant differences among plants (Figure 5).

3.5. Polyamines in Milk Vetch as Affected by Nodulation and Drought Stress

Putrescine level was higher in LN plants than in NN plants under control conditions (Figure 6A). Compared to NN plants, Spd level was higher in AN plants, but lower in LN plants under control conditions (Figure 6B). Drought treatment resulted in increased Put and Spd concentrations in all plants, while higher levels were observed in AN plants than in LN and NN plants (Figure 6A,B). Spm level was increased in all plants after drought treatment, but showed no significant difference among the tested plants (Figure 6C).

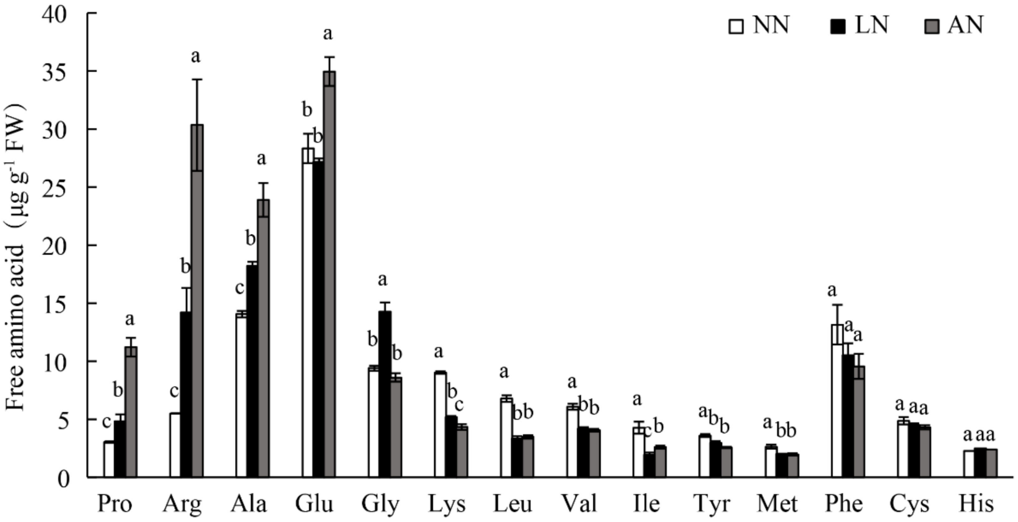

Figure 5. Free amino acids as affected by rhizobia inoculation in Chinese milk vetch. Free amino acids from leaves in plants with active nodules (AN), low-activity nodules (LN), and no nodules (NN) under unstressed control conditions were measured. The presented data are means and standard errors ($n = 3$). The same letter above the column indicates no significant difference at $p < 0.05$.

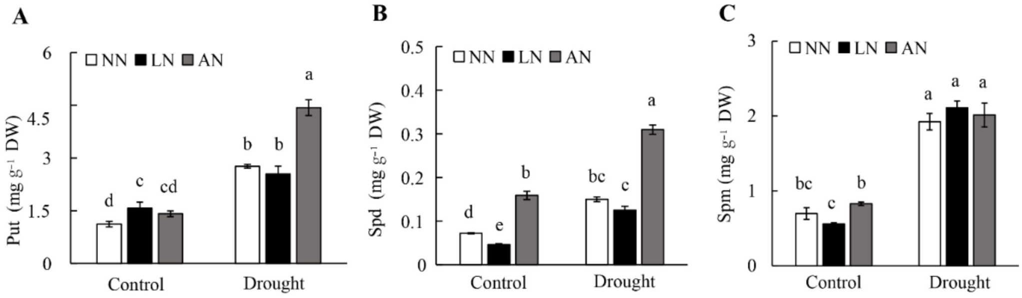

Figure 6. The effects of rhizobia inoculation on free polyamines in response to drought stress. Three-month-old plants were withheld irrigation for 11 days, while unstressed plants were used as a control. Putrescine (Put) (**A**), spermidine (Spd) (**B**), and spermine (Spm) (**C**) were measured in leaves. AN, LN, and NN indicate the plants with active nodules, low-activity nodules, and no nodules, respectively. The presented data are means and standard errors ($n = 3$). The same letter above the column indicates no significant difference at $p < 0.05$.

4. Discussion

The essential role of rhizobium symbiosis in improved drought tolerance in milk vetch was documented in the present study. Low nitrogen is required for nodulation in leguminous plants [39]. Pink active nodules with higher nitrogenase activity were obtained in milk vetch under low-nitrogen conditions after inoculating with *Mesorhizobium huakuii* strain 7653R, while smaller and white nodules with low nitrogenase activity were observed under high-nitrogen conditions. The AN plants had an improved survival rate and higher RWC after drought stress compared with LN and NN plants, suggesting that nodulation leads to increased drought tolerance in milk vetch. The result was consistent with the observations that rhizobium symbiosis leads to increased drought tolerance in *Lotus corniculatus*, common bean, pea, alfalfa, and *M. truncatula* [16,19–22]. Our study also showed that LN plants exhibited the lowest drought tolerance. One previous study in alfalfa reported that plants with nonfunctional nodules had the lowest fitness compared with AN and NN plants after salt tolerance [17]. We supposed that the nitrogen offered by nodules could not compensate for the energy cost of forming nodules in LN plants. As a result, the inactive nodules competed for the energy used for response to stress.

Nitrogen is fixed to produce ammonium in leguminous plants through rhizobium symbiosis, while ammonium is assimilated to produce glutamine and glutamate for use by higher plants, catalyzed by GS and GOGAT [2,3,40,41]. GDH catalyzes the production of glutamate via the reductive amination in the presence of the cofactor NAD(P)H [4,5]. Nitrogen assimilation declined in nodulated plants and nitrate-fed pea plants after drought stress [20]. GS, GOGAT, and GDH activities in milk vetch decreased after drought stress, which is similar to the results in pea [20], with higher activities of GS, GOGAT, and GDH in AN plants than in LN and NN plants under control and drought stress conditions. Glutamine and glutamate may be converted to other amino acids. AN and LN plants had higher levels of proline, arginine, and alanine than NN plants, and the highest levels were maintained in AN plants, indicating that the increased amino-acid level was associated with nodulation. In addition, AN plants had a higher level of glutamate than LN and NN plants. Proline is an important compatible solute in the regulation of multiple abiotic stress tolerance and an antioxidant involved in scavenging reactive oxygen species [13,42,43]. A higher level of proline is accumulated in nitrogen-fixing *Lotus corniculatus* after drought stress [21]. In addition to proline, some free amino acids are involved in plant adaptation to abiotic stresses via osmotic adjustment, modulation of membrane permeability and ion uptake, and regulation of gene expression and redox homeostasis [44]. Some amino acids are increased in plants in response to drought, for instance, alanine in *Phaseolus mungo* [45] and *Cryptomeria* [46], as well as glutamate in rice [47] and cabbage [48]. Nevertheless, the higher levels of proline, arginine, and glutamate, as well as nitrogen assimilation, were associated with the increased drought tolerance in nodulated milk vetch plants.

Arginine is increased in plants in response to drought stress [49,50]. Arginine is the precursor of Put biosynthesis [51], while Put is further sequentially converted to Spd and Spm [52]. Put, Spd, and Spm are major polyamines in plants that are involved in the regulation of multiple abiotic stress tolerance [53–56]. Consistent with arginine, higher levels of Put and Spd was observed in AN plants than in LN and NN plants, indicating that the increased Put and Spd levels were associated with nodulation causing arginine accumulation. Put and Spd are involved in the regulation of drought tolerance. Put and Spd are accumulated in plants in response to drought with higher levels in drought-tolerant cultivars than drought-sensitive ones [32,57], while exogenous application of Put and Spd leads to improved drought tolerance in multiple plant species [32,53,58,59]. Transgenic plants with improved Put and Spd accumulation have increased drought tolerance [60,61]. It is suggested that the enhanced drought tolerance in milk vetch plants with active nodules is associated with Put and Spd.

5. Conclusions

In summary, rhizobia inoculation led to active nodulation in roots of milk vetch under low-nitrogen conditions and improved drought tolerance. The increased drought tolerance in AN plants was associated with its increased nitrogen fixation and ammonium assimilation, which in turn led to increased Pro, Arg, Ala, and Glu levels, as well as Put and Spd concentrations.

Author Contributions: Z.G. and H.S. contributed to the study conceptualization and design; Y.L. performed material preparation and data collection and analysis; Y.L., H.S. and Z.G. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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