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RESEARCH ARTICLE

Interaction of nitrogen availability in the soil with leaf physiological traits and nodule formation of *Robinia pseudoacacia*-rhizobia symbiosis depends on provenance

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Abstract

Aims The interaction between nitrogen (N) availability in the soil, rhizobia nodule formation and leaf physiological traits of *Robinia pseudoacacia* L. was explored at initial nodule development.

Methods We selected two *Robinia* provenances, one from Northwest (GS) and one from Northeast China (DB), and cultivated seedlings in the greenhouse with and without rhizobia inoculation at normal and high N supply in the soil. After ca. 2.5 months growth, nodule formation, plant biomass, CO_2 and H_2O gas exchange of the leaves, and foliar N contents and partitioning were analyzed.

Results Rhizobia inoculation strongly promoted the formation of root nodules independent of N availability in the soil, but this effect was more pronounced in the DB than for GS provenance. It reduced biomass accumulation of the GS provenance, but not for DB provenance at both, normal and high soil N availability. High N supply did not affect biomass accumulation independent of rhizobia inoculation. Leaf photosynthesis of both *Robinia* origins was enhanced by high N supply, but this effect was counteracted by rhizobia inoculation only in leaves of DB plants. In GS but not in DB plants, high N supply reduced not only nodule formation, but also stomatal conductance, but still enhanced transpiration without modifying the foliar water content. In addition, high N supply plus inoculation enhanced the organic N content in GS plants rather than DB plants.

Conclusion These results indicate that excess N availability in the soil interacts with the performance of *Robinia* provenances, as previously reported for drought and phosphorus (P) depletion.

Keywords Nitrogen availability · Nitrogen partitioning · Nodule formation · Photosynthesis · *Robinia pseudoacacia*-rhizobia symbiosis

Introduction

Trees developed in evolution on marginal soil and, therefore, had to explore strategies to cope with low nutrient availability in the environment (Raven and Andrews 2010). This is particularly important for nitrogen (N), as it is an essential macronutrient required for plant growth

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Hongguang Sun and Bin Hu contributed equally to this work.

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and development (Crawford 1995) and also plays an important role for numerous ecosystem functions (Millard and Grelet 2010; Rennenberg and Dannenmann 2015; Simon et al. 2017). As an essential macronutrient for plants, still today N availability constitutes a key limitation to plant growth in many forest ecosystems (Laliberté et al. 2012).

To counteract various environmental stresses, e.g., N limitation or high N deposition, plants have developed several regulatory pathways that reprogram metabolic and physiological processes, even at the cost of reduced growth and yield (Bailey-Serres et al. 2019). In the context, physiological responses of trees to N availability in the soil have been analyzed in numerous studies (Rennenberg and Dannenmann 2015; Bote et al. 2018; Kruse et al. 2020; LeBauer and Treseder 2008; Ni et al. 2020a; Song et al. 2019; Wang and Liu 2014; Wang et al. 2016). For instance, one strategy developed in trees to cope with low N availability in the soil includes N storage and mobilization to uncouple the actual demand for N from N availability in the soil (Zhang et al. 2019a). In addition, plants have developed mechanisms to use atmospheric N_2 as N source to counteract growth limitation by low N availability (Saikia and Jain 2007). Since fixation of atmospheric N_2 can only be performed by microorganisms, this mechanism requires the symbiosis of roots with N_2-fixing bacteria. Biological symbiotic N_2-fixation (BNF) has been developed by many plant species, including most legumes of the Fabaceae with rhizobia (Kalloniati et al. 2015; Liu et al. 2020; Matamaoros et al. 1999) and a large number of other woody plants with Frankia (Bissonnette et al. 2014; Jung-Tai and Sung-Ming 2018; Sharaya et al. 1987; Sprent et al. 2017). Thus, the legume-rhizobium symbiotic system provides an important N source for terrestrial ecosystems and agricultural production and is conducive to the sustainable development of both, agriculture and forestry (Sprent 2008). Still, the physiology and molecular biology of the legume-rhizobia interaction has mostly been studied with herbaceous plants, particularly with the model systems *Lotus* and *Medicago* (Li et al. 2020; Lorite et al. 2018), whereas such information on woody legumes is scarce. On the other hand, nitrogen (N) deposition in forests has become an important issue of global environmental change (Galloway et al. 2008). Nitrogen enrichment has already affected global ecosystems (Bai et al. 2010; Zhang et al. 2016) and has been projected to increase to 200 Tg year^{-1} by 2050 on a global scale (Vitousek et al. 1997). Excess N may lead to enhanced growth of invasive or alien species, thereby changing biodiversity and community structure of ecosystems (Pitcairn et al. 1995; Sala et al. 2000; Bai et al. 2010).

Compared with non-legumes, N metabolism in legumes is rather complex due to the use of atmospheric N_2 in addition to inorganic and organic N sources in the soil. N_2-fixation is often inhibited by excess NO_3^--N and NH_4^+-N in soil, since N_2-fixation is a costly process in terms of both, energy and carbon investments (Lambers et al. 2002). For instance, many studies in agriculture indicated that the application of N fertilizer or the presence of high levels of residual N in the soil severely limits both the formation of nodules and N_2-fixation (Streeter and Wong 1998; Miller et al. 2007). Excess N inhibits BNF not only by a decrease in nodule number, nodule mass, and N_2 fixation activity, but also by accelerated nodule senescence (Saito et al. 2014; Ferguson et al. 2018). Still little is known about the mechanisms that cause excess N application to influence nodule formation and development of legume species. In addition, it has not been elucidated, if these results achieved with herbaceous N_2-fixing species can be transferred to N_2-fixing woody plants. Therefore, further studies are needed to elucidate how excess N supply in the soil interacts with nodule development and the performance of BNF, particularly for woody legume species.

Generally, plant responses to the availability of macro-nutrients such as N in the soil are aimed to maintain regular growth and development. However, the mechanisms that determine these responses are not fully understood. For example, maximum root length and branching of the model plant *Arabidopsis* are reported at intermediate N limitation (Kiba and Krapp 2016), but branching also depended on the chemical form of N (NO_3^--N and NH_4^+-N) in the soil (Meller et al. 2020). In addition, the mechanisms determining growth and development vary not only with soil nutrient availability, but also between plant species and provenances. Often species or provenances originating from harsh environments with poor nutrient availability in the soil, show a superior performance under regular soil nutrient availability in terms of survival and growth than those from more favorable environments (Shi et al. 2015; Meller et al. 2020).

In this context, genetic variation and adaptive plasticity are the two main factors by which forest tree species adapt to environmental stress by nutrient limitation (Gandour et al. 2007; Ramírez-Valiente et al. 2014). There are considerable differences among populations in the adaptive characteristics due to divergent evolutionary processes under environmental variation in the original habitats. For example, Meller et al. (2020) reported that European beech (*Fagus sylvatica* L.) saplings originating from a site with low P-availability in the soil exhibited a provenance-specific high plasticity in adapting growth, architecture, and morphology of the root system to these conditions. Thus, differences in adaptive characteristics can develop in populations of woody species due to divergent evolutionary processes in response to the soil nutrient availability in their original habitats. Under current climate change, the survival of woody species may depend on this adaptation capability not only to limited N availability, but also to excess N availability by N deposition (Wang et al. 2018, 2021.). However, the responses of tree provenances to excess N availability are not fully understood.

Among woody legumes, symbiotic N_2-fixation has mostly been studied with *R. pseudoacacia* L. (Mantovani et al. 2015; Moshki and Lamersdorf 2011; Quinkenstein et al. 2012). *Robinia* originates from North America and was introduced to Europe and Africa in the seventeenth century as well as to China in the early nineteenth century (Fowells 1965; Ren et al. 2014a). Currently, it is widely cultivated in northeast, northwest and southwest China covering an area of ca. 70,000 ha (Du et al. 2019). *Robinia* is characterized by a high vitality, N_2-fixing capability, fast growth and high ability of vegetation restoration in degraded land systems. The positive performance of *Robinia* is partially attributed to its root symbiosis with atmospheric N_2-fixing rhizobia, one of the most effective symbiotic N_2-fixation systems of plants (Olesniewicz and Thomas 1999). In this context, N_2-fixing woody legume species, such as *Robinia* can provide more than half of the plant N demand thereby supporting biomass accumulation for forest restoration (Batterman et al. 2013). For instance, *Robinia* can greatly increase soil N availability in the planting area and its surroundings, thereby reducing nutrient limitation of plant species and of microorganisms, thereby increasing plant and microbial biodiversity as well as abundance (Luo et al. 2020). These features have made it a typical pioneer tree species for water and soil conservation and the regeneration of depleted and contaminated soil (Ren et al. 2014b; Hu et al. 2017). Recent field studies in the Loess Plateau area of northwest China with *Robinia* plantations on N and P depleted soil showed that its co-cultivation with the common walnut (*Juglans regia* L.), a non-N_2-fixing species, not only increased N and labile carbon levels in the soil, but also promoted N and P nutrient concentrations and root uptake of *Juglans* attributed to the formation of N_2-fixing nodules of *Robinia* in symbiosis with rhizobia (Hu et al. 2017; Du et al. 2019).

Using the ^{15}N isotope dilution method, Moshki and Lamersdorf (2011) showed that different provenances of *Robinia* strongly differ in N_2-fixation efficiency. From its widespread global distribution and the ca. 200-years of cultivation in China since its introduction in the early of nineteenth century, it can be assumed that *Robinia* has evolved a large variety of provenances adapted to specific local habitats. In previous studies, a *Robinia* provenance from harsh arid to semi-arid climate with poor nutrient availability in the soil showed more tolerance of physiological traits to drought and low phosphorus (P) availability than a provenance from temperate humid to semi-humid continental monsoon climate with fertile soil (Yuan et al. 2022; Zhou et al. 2022). However, little is known about provenance-specific responses of *Robinia* to excess N availability in the soil.

The present study was aimed to characterize the interaction of high soil N availability with initial nodule formation and leaf physiological traits of *Robinia* seedlings. Whereas previous studies showed the consequences of drought and P depletion for the performance of *Robinia* provenances and its interaction with nodulation (Yuan et al. 2022; Zhou et al. 2022), the present study intends to indicate that also N nutrition contributes to this interaction. For this purpose, foliar CO_2 and H_2O gas exchange as well as N contents and partitioning were investigated in addition to growth and nodule formation. We hypothesized that (i) high N availability in the soil inhibits nodule development, but enhances growth, photosynthesis as well as the N content of *Robinia* leaves without rhizobia inoculation; (ii) rhizobia inoculation counteracts these effects of high N availability on photosynthesis and N nutrition of *Robinia* leaves; and (iii) the effects of rhizobia inoculation and high N in the soil on photosynthesis and foliar N contents differ between *Robinia* provenances, with provenances from habitats with poor N-nutrition in the soil exhibiting a superior physiological and growth performance to excess N supply. To test these hypotheses, we selected seedlings of *Robinia* from two provenances with distinct nutrient availability in the soil of the original habitats, i.e., the Gansu Province (GS) of Northwest China with poor nutrient availability in the soil and the Dongbei region (DB) of Northeast China with fertile soil. Seedlings of these provenances were cultivated at normal and high N levels in the soil, with/without rhizobia inoculation in a greenhouse experiment.

Material and methods

Plant material

Robinia seeds used in the present experiment originated from commercial tree nurseries of two regions with contrasting climate and nutrient availability in the soil (i.e., Tianshui city (34°32′N, 105°44′E), Gansu Province (GS) in northwest China and Fushun city (41°51′N, 123°56′E), Liaoning province of the Dongbei region (DB) in northeast China). *Robinia* seeds of both origins were purchased at a seed store in Yangling, Shaanxi, China. Like for most *Robinia* plantations set up several decades ago, the exact genetic background of the provenances in unknown. The climate at the plantation in Tianshui city, Gansu Province, northwest China is mainly semi-arid to arid continental (Kottek et al. 2006). The average annual precipitation amounts to ca. 605 mm, the soil is infertile (field water capacity:15.8%; mean soil total N: 0.66 g kg^{-1}; mean soil total phosphorus (P): 0.75 g kg^{-1}; mean soil organic carbon (SOC): 10.2 g kg^{-1}; all at 0–10 cm soil depth) (Zhang et al. 2021a), and drought-tolerant plant species constitute the predominant vegetation (Kong et al. 2014; Zhang et al. 2021a, b). In contrast, the *Robinia* provenance originated from Fushun, city, northeast China (DB provenance) experiences temperate humid to semi-humid continental monsoon climate (Lin et al. 2017; Ge 2020). The annual precipitation is ca. 800 mm, and the soil is more fertile (field water capacity: 27.1%; mean soil total N: 1.8 g kg^{-1}; mean soil total phosphorus (P): 0.65 g kg^{-1}; mean soil organic carbon (SOC): 41.3 g kg^{-1}; all at 0–10 cm soil depth) (Duan et al. 2010; Ge 2020).

For germination of *Robinia* seeds, the seed coat must be broken. For this purpose, healthy seeds of similar size of both origins were selected at the beginning of June, 2020 in the laboratory and soaked in concentrated sulfuric acid for ca. 10 min. Subsequently, the concentrated sulfuric acid was replaced by 95% alcohol for 30 sec and seeds were rinsed with sterile water. For surface sterilization seeds were soaked in sodium hypochlorite for 10 minutes and rinsed in sterile water seven times. Finally, seeds were placed on petri dishes with wetted filter paper and inoculated in a climate chamber (RLD-1000E-4, Ledian Ltd., Ningbo, China) at 25 °C and 12 h illumination at 400 μE m^{-2} s^{-1} photosynthetic active radiation (PAR). After germination, 24 seedlings of each origin were selected and transplanted into pots with a diameter and height of 20 cm, each. All pots contained 2.5 kg of a mixed soil substrate consisting of 95% sand and 5% vermiculite (Jialing River, Chongqing, China). For sterilizing and controlling potential fungal and bacteria contamination, a carbendazim and thiodiazole-copper (95%) mixture was added at 0.016 kg L^{-1} (w/v) (Luyiyuan Technology Development Co., Beijing, China) (Heju 2005).

Experimental design

The present study was conducted in a greenhouse of the Research Center of Molecular Ecophysiology (CMEP) of the College of Resources and Environment, Southwest University (29°49′N, 106°25′E), Chongqing, China. All *Robinia* seedlings were pre-cultivated in the greenhouse from the beginning of July to the end of September, 2020. The average temperature in the greenhouse was 25 °C - 35 °C and artificial illumination was applied for 12 h at 500 μE m^{-2} s^{-1} photosynthetic active radiation (PAR) during the cultivation period. The water holding capacity of the mixed substrate was 15.33% referring to the ring knife measurement as described by Bi et al. (2014). From the 01st, July to the 01st, October, 2020 and according to the actual situation in the greenhouse, 250 ml full nutrient solution (0.05 mM K_2SO_4, 0.5 mM KH_2PO_4, 0.25 mM $MgSO_4·7H_2O$, 1 mM $CaCl_2$, 0.01 mM Ferric citrate, 0.3 mM KNO_3, 0.1 mM NH_4NO_3, 2 μM H_3BO_3, 1 μM $MnSO_4·H_2O$, 0.2 μM $CuSO_4·5H_2O$, 0.1 μM $CoSO_4·7H_2O$, 0.5 μM

$ZnSO_4·7H_2O$, 0.1 μM $Na_2MoO_4·2H_2O$) per pot every 14 days and 250 ml deionized H_2O per pot every 3–5 days were required for normal seedlings growth and applied without seepage to avoid leaching of nutrients from the bottom of the pots (slightly modified from Broughton and Dilworth 1971; Flemetakis et al. 2006; Mariangela et al. 2011). A watering scheme of 250 ml deionized H_2O per pot every 3–5 days and P concentration (0.5 mM) was used as sufficient water irrigation and P level for control (CK) plant growth as reported by Yuan et al. (2022) and Zhou et al. (2022), respectively. The N concentration of 0.5 mM was applied as sufficient N level for CK plant growth, which was adapted from a previous N supply experiment of *Robinia* seedlings grown in a greenhouse (Wang et al. 2018) and comparable with the nutrition-poor soil condition in the Loess Plateau area of northwest China (Dang et al. 2007; Liu et al. 2013; Cao and Chen 2017). Seedlings of similar size and height were selected for the subsequent high N supply experiment.

The high N experiment adopted a completely randomized block design with three factors, i.e., provenance, rhizobia inoculation and N supply in the soil. The roots of half of the *Robinia* seedlings were inoculated with rhizobia strain *Mesorhizobium huakuii* QD9 (Liu et al. 2019) which was isolated from a 50-year-old *Robinia* tree grown in the coastal area of Qingdao city, Shandong province, eastern China. *Robinia* was nodulated by *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* genera bacteria, but *Mesorhizobium* clearly predominated in root nodules (Mierzwa et al. 2010). The *M. huakuii* has been isolated in a variety of *Robinia* stands that represent different environmental conditions. Thus, this rhizobia species has a potential to form nodule with *Robinia* from different provenances (Liu et al. 2019; Wei et al. 2009). For inoculation, the isolated rhizobia strains of *M. huakuii* QD9 were firstly cultivated on YMA solid medium (mannitol 10 g L^{-1}, yeast extract 0.12 g L^{-1}, NaCl 1 g L^{-1}, $MgSO_4$ 0.2 g L^{-1}, K_2HPO_4 0.5 g L^{-1} and agar 20 g L^{-1}; pH = 6.8) (Vincent 1970) and then transferred into TY liquid medium (6 g of tryptone, 3 g of yeast extract, and 0.38 g of $CaCl_2$ in 1 L of dH_2O) (Franzini et al. 2013) with a sterilized bamboo stick, cultivated on a shaker at 28 °C for 48 hours, and centrifuged at 25 °C and 2000 g for 10 min. From the pellet, a bacterial suspension with $OD_{600} = 1.0$ was produced. The roots of the *Robinia* seedlings were inoculated with 10 mL of this bacterial suspension using a sterile syringe. Rhizobia-inoculation started on 1st September 2020 and was performed three times every 7–10 days. The N supply in the soil included two N levels, normal N (0.5 mM L^{-1}) and excess high N (7 mM L^{-1}) in the nutrient solution. The normal and high N treatments used potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3) as N sources, for normal N 0.3 mM KNO_3, 0.1 mM NH_4NO_3, for high N 5 mM KNO_3, 1 mM NH_4NO_3. Other nutrients were applied at the same level described above for the full nutrient solution. This protocol was adapted from a previous study on increased N supply of *Robinia* seedlings grown in a greenhouse (Wang et al. 2018). The high N treatment lasted from the 1st October until the 15th December 2020. The normal N and high N nutrient solutions were added every 14 days (five times in total) for 2.5 consecutive months at 240 ml per pot each time. Two N supply treatments of two provenances with/without rhizobia inoculation amounted to a total of 8 treatments with 6 replicate each, summing up to a total of 48 pots.

CO_2 and H_2O gas exchange

CO_2 and H_2O exchange parameters were determined on the 13rd December 2020 with the portable Li-6800 photosynthesis system (LI-COR, Lincoln, Nebraska, USA) by enclosing a leaf completely in a closed, transparent leaf chamber according to the manufacturer's instructions. The PAR in the leaf chamber was set at 1500 μmol m^{-2}·s, the CO_2 concentration at 400 μmol mol^{-1}, the temperature was 25 °C, and the RH was 60%. The parameters measured included the net rate of photosynthesis (P_n), stomatal conductance (G_s), intracellular CO_2 concentration (C_i) and transpiration (T_r). For this purpose, three well grown *Robinia* plants of each treatment were selected and three healthy, fully expanded leaves in the middle and upper part of each plant were analyzed ($n = 3$). All measurements were repeated three times technical replicates for each leaf on each plant and for each treatment.

Plant tissue sampling

Plant tissue sampling was carried out on 15th December 2020 after the CO_2 and H_2O gas exchange measurement. Firstly, the whole seedlings were taken out carefully from the pots to prevent any damage of the roots and washed several times with distilled water. After counting the number of nodules and weighing, the leaves were dissected, transferred to 10 ml centrifuge tubes, immediately frozen in liquid N_2, transferred to the laboratory and stored at −80 °C. After ca. 7 days, leaves samples were ground to a fine powder with mortar and pestle under liquid N_2 or with a refrigerated grinder at −80 °C (JX-CL, Jing Xin Ltd., Shanghai, China). All samples were stored in a freezer at −80 °C for ca. 10 days until subsequent biochemical analyses.

Biochemical analyses

Water content

Water contents of leaf samples were calculated based on the following equation: (Jin et al. 2017).

$$Water\ content\left(mgH_2O/mgFW\right) = \frac{FW - DW}{FW}$$

FW fresh weight

DW dry weight

δ^{13}C and δ^{15}N signatures, total N, and total C contents

Aliquots of 100~300 µg dried and powdered leaf material (moisture content <2%) were weighed into tin capsules and δ^{13}C and δ^{15}N signatures as well as total N and C contents were quantified with an elemental analyses (Flash EA, Thermo Fisher Scientific Ltd., Massachusetts, USA) coupled with Conflo IV multi-purpose interface to an isotope ratio mass spectrometer (253 plus, Thermo Fisher Scientific Ltd., Massachusetts, USA) as previously described (Vallano and Sparks 2013). Measurements were performed by Nanjing Convinced-test Technology Co., Ltd.

Quantification of total soluble protein

Total soluble protein in the leaves was extracted as previously reported by Hu et al. (2013). For this purpose, ~0.05 g frozen homogenized leaf material was mixed with 0.15 g polyvinylpolypyrrolidone (PVPP 6755; Jin Kelong, Beijing, China) to remove phenolic substances. For total soluble protein extraction, 1.5 ml buffer (50 mm Tris-HCl, pH 8.0, 1 mm EDTA, 15% glyc-erol (v/v), phenylmethysulphonyl fluoride (PMSF, (0.1 M), dithiothreitol (DTT, 1 M) and 1 ‰Triton X-100) were added. The samples were incubated for 30 min at 4 °C, then centrifuged at 14,000 g for 10 min at 4 °C. Aliquots of 500 µl supernatant were mixed with 500 µl 10% trichloroacetic acid, followed by 10 min incubation at room temperature for protein precipitation. After 10 min centrifugation at 14,000 g and 4 °C, the leaf protein in the precipitate was dissolved with 1 ml 1 M KOH. Quantification of total soluble protein was conducted by adding 4 ml Bradford reagent (Biofroxx, Saiguo biotech Co., Ltd., Guangzhou, China) to 200 µl extract. Quantification was achieved with a UV-1800 spectrophotometer (AOE Instruments, Shanghai, China) at 595 nm. Bovine serum albumin (BSA; Solarbio Technology Co., Ltd., Beijing, China) was used as standard.

Quantification of total amino acids

Amino acids were extracted using the method described by Hu et al. (2013) . For this purpose, 1 ml of a methanol: chloroform mixture (3.5:1.5, v: v) and 0.2 ml Hepes buffer (containing 20 mM Hepes, 5 mM EGTA, 10 mM NaF, pH 7.0) were added to ca. 0.05 g homogenized frozen leaf material and incubated on ice for 30 min. Subsequently, 600 µl distilled water were added and the mixture was centrifuged for 5 min at 14,000 g and 4 °C. The supernatant was transferred to a new tube and stored on ice. The extraction was repeated once and the supernatants were combined. The combined solutions were used to quantify the total amino acid content with ninhydrin reagent (Yuanyebio, Yuanye Bio-Tecnology Co., Ltd., Shanghai, China). For this purpose, aliquots of 240 µl extract and 240 µl ninhydrin reagent (1:1 mixture of solution A containing 4.2 g monohydrate citric acid and 0.134 g anhydrous $SnCl_2$ in 40 ml 1 M NaOH, made up to 100 ml with distilled water (pH 5.0) and solution B containing 4 g ninhydrin in 100 ml ethylene glycol-monomethyl ether) were boiled at 100 °C for 30 min. For stabilization of the ninhydrin derivative, isopropanol (3 ml, 50%) was added to the samples, followed by 15 min incubation at room temperature in the dark. Finally, a UV-1800 spectrophotometer (AOE Instruments, Shanghai, China) was used to determine the optical density at 570 nm. L-glutamine was used as standard.

Foliar nitrate and ammonium contents and nitrate reductase activity

Nitrate (NO_3^-) and ammonium (NH_4^+) were extracted and measured using a plant Nitrate Nitrogen Assay Kit (Comin, Biotechnology Co. Ltd., Suzhou, China) and a plant Ammonium Nitrogen Assay Kit (Comin, Biotechnology Co. Ltd., Suzhou, China), respectively. Nitrate and ammonium contents were determined according to the methods described by Rizwan et al. (2019) and the absorbance was read at 410 and 580 nm for NO_3^- and NH_4^+, respectively, and expressed in mg g^{-1} DW for both parameters. Nitrate reductase (NR) was extracted and the in vitro activity of the enzyme was measured using a Nitrate Reductase (NR) Assay Kit NR-2-Y (Solarbio, Biotechnology Co. Ltd., Beijing, China) applying the manufacturer's instructions. For NR activity determination, fresh plant leaf samples (1.0 g) were ground in a chilled mortar containing 6 ml of extraction buffer. The crude supernatant was analyzed for NR activity according to the modification of the method reported by Savidov and Lips (1997).

Calculation of structural N

The structural N content of leaves was estimated by subtracting soluble protein N, amino acid N, nitrate N and ammonium N from total N (Hu et al. 2013). Calculations were based on the dry weight of the samples.

Statistical analyses

Differences between N supply, rhizobia inoculation and provenances were analyzed by three-way analysis of variance (ANOVA). Raw data were first tested by either Kolmogorov-Smirnov or Shapiro-Wilk tests for normal distribution. Where necessary, data were transformed using either log- or square-roots transformation to satisfy the assumptions of normality and homogeneity of variance. Statistical significance of differences was determined by least square significant difference (LSD) analyses at a significance level of $p < 0.05$. Data still failing to match normal distribution after transformation were subjected to Kruskal-Wallis ANOVA followed by Dunn's *post*-hoc test (i.e., water contents, $\delta^{13}C$ and $\delta^{15}N$ abundance). All statistical tests were based on six individual seedlings ($n = 6$, exception for photosynthesis and leaf gas exchange: $n = 3$ for individual plants). The SPSS19.0 software was used (SPSS Inc., Chicago, IL, USA) to analyze all data. Figures were generated using SigmaPlot 12.0 (Systat Software, Erkrath, Germany). Biochemical and photosynthetic data were subjected to partial least squares discriminant analysis (PLS-DA) ($n = 6$; exception for photosynthesis and leaf gas exchange data: $n = 3$) using the MetaboAnalyst 5.0 software (http://www.metaboanalyst.ca).

Results

Nodule development on Robinia roots and biomass accumulation depend on rhizobia inoculation, N supply, and provenance

In both provenances, the highest number of root nodules were observed for inoculated plants supplied with normal N in the nutrient solution. In inoculated plants of the GS provenance, high N supply strongly reduced the number of nodules on the roots, but this effect was not observed for the DB provenance. Irrespective of N supply, initial nodule development was minute on plants of both provenances that were not inoculated with rhizobia (Supplementary Table S1, Table 1). In both provenances, high N supply did not affect *Robinia* biomass, irrespective of inoculation. Irrespective of N supply, inoculation with rhizobia strongly reduced *Robinia* biomass in GS, but not in DB plants. In inoculated plants, *Robinia* biomass was higher for DB than for GS plants irrespective of N supply, this effect was not observed in non-inoculated plants (Supplementary Table S1, Table 1).

Nitrogen supply and rhizobia inoculation modifies foliar traits of Robinia provenances

General analyses of the foliar traits studied by PLS-DA showed a clear separation between normal N and high N supply for CO_2 and H_2O exchange parameters

Table 1 Three-way ANOVA analyses of physiological characteristics in different N treatments, rhizobia inoculation treatments and different provenances

	N	Inoculation	Provenance	Provenance× N×Inoculation	Inoculation×N	Provenance×N	Provenance×Inoculation
Number of nodules	0.140	**<0.001**	0.546	0.386	0.337	0.605	0.061
Plant biomass (g FW)	**<0.001**	**<0.001**	**<0.001**	**0.012**	0.293	**<0.001**	**<0.001**
P_n (μmol m^{-2} s^{-1})	**<0.001**	**0.023**	**<0.001**	**0.039**	**0.003**	**<0.001**	**0.001**
G_s (mmol m^{-2} s^{-1})	0.781	0.115	0.408	0.097	0.780	**0.014**	**0.030**
T_r (mol m^{-2} s^{-1})	0.960	**0.009**	0.367	0.131	0.240	**0.005**	**0.013**
C_i (μmol/mol)	0.593	**0.012**	0.661	0.283	0.616	0.336	**0.003**
δ^{13}C (‰)	0.538	**0.002**	0.891	0.447	0.642	0.758	0.334
δ^{15}N (‰)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.739	0.077	**<0.001**
Water content (%)	0.489	0.276	**0.014**	0.140	0.565	0.512	0.121
Total C (mg g^{-1} DW)	**0.026**	0.333	0.428	**0.005**	**<0.001**	0.821	0.862
Total C/N ratio	0.636	0.118	0.248	0.342	0.585	0.970	0.066
Total N (mg g^{-1} DW)	0.256	0.123	**0.039**	0.733	0.096	0.973	0.096
Total soluble protein N (mg·g^{-1}DW)	**0.002**	0.344	**0.001**	**<0.001**	**0.004**	**<0.001**	**0.009**
Total amino acid N (mg·g^{-1}DW)	0.513	**0.007**	**<0.001**	**0.002**	0.720	0.070	**<0.001**
Nitrate N (mg·g^{-1}DW)	0.265	**0.001**	**<0.001**	0.783	**0.040**	0.560	**<0.001**
Ammonium N (mg·g^{-1}DW)	**0.009**	**<0.001**	**<0.001**	0.213	**0.001**	**0.001**	**<0.001**
Structural N (mg·g^{-1}DW)	**0.041**	0.120	0.104	**0.035**	0.790	0.079	0.669
NR	**0.006**	0.145	0.815	**0.031**	0.591	0.768	**0.001**

*Bold values indicate the significance level at $p < 0.05$; $n = 6$, exception for gas exchange parameters: $n = 3$

in GS plants irrespective of inoculation; this separation was less pronounced in DB plants irrespective of inoculation (Fig. 1a). Between rhizobia inoculated and non-inoculated plants, foliar CO_2 and H_2O exchange traits were not clearly separated at high N supply irrespective of provenance. At normal N supply, such clear separation was observed for DB plants, but not for GS plants. Foliar CO_2 and H_2O exchange traits were clearly separated between inoculated GS and DB plants irrespective of N supply (Fig. 1a). Without inoculation, CO_2 and H_2O exchange traits of GS and DB plants were clearly separated at normal N supply, but not at high N supply (Fig. 1a).

For the biochemical traits studied in the leaves, there was a clear separation between normal and high N supply in inoculated GS plants and non-inoculated DB plants. Between rhizobia inoculated and non-inoculated plants, the biochemical traits studied were not clearly separated at normal N supply, irrespective of plant origin, and at high N supply for DB plants. However, at high N supply such a separation was observed for GS plants. There was a clear separation of biochemical traits between the GS and the DB provenance at normal N supply for rhizobia inoculated plants and for non-inoculated plants at high N supply (Fig. 1b). To identify the particularly parameter(s) that determine the separation of foliar traits between N supply, inoculation and plant origin, the significance of differences between individual parameters was analyzed.

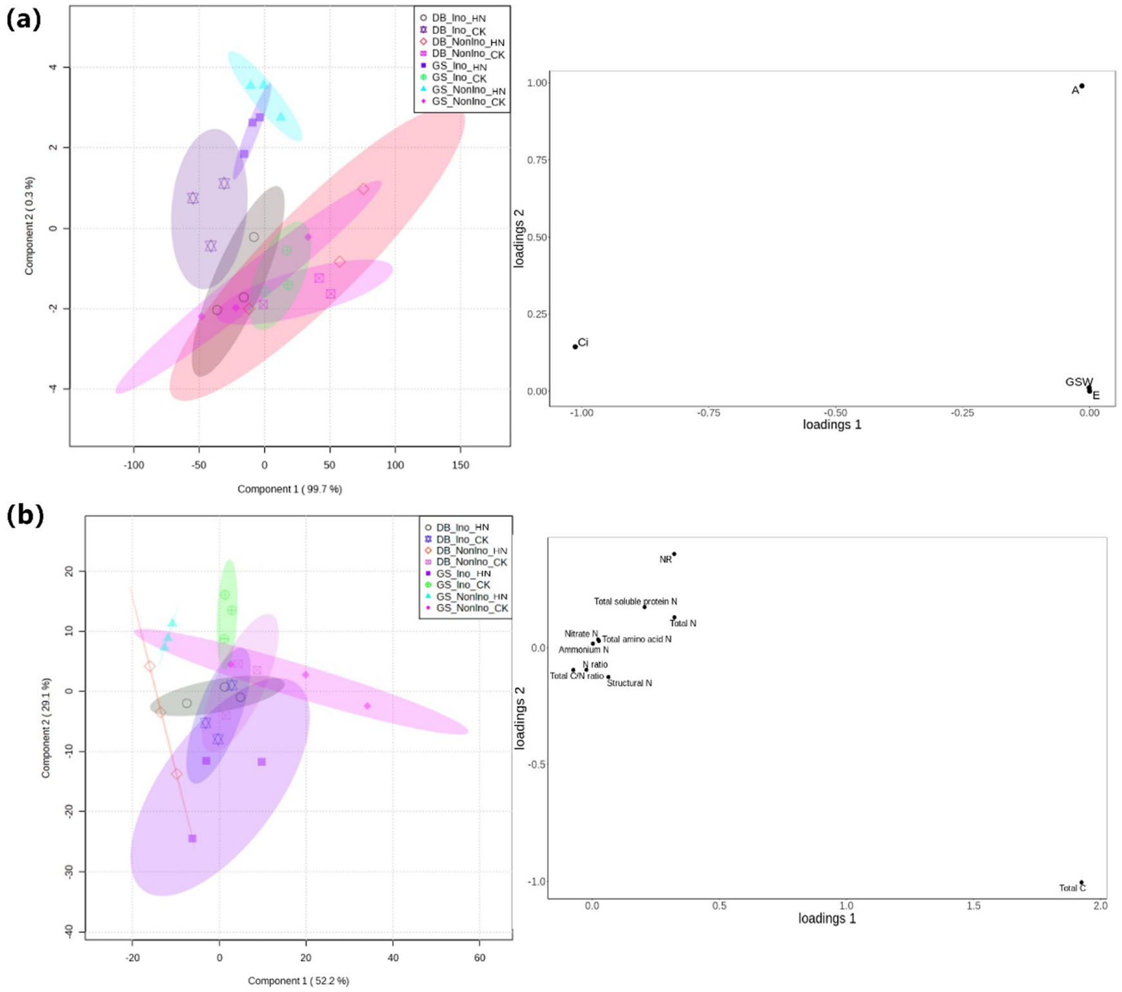


Fig. 1 Partial least squares-discriminant analysis (PLS-DA) and loading plots of foliar traits of *Robinia* provenances subjected to different N supply and rhizobia inoculation according to 18 photosynthetic and physiological parameters. **a** CO_2 and H_2O exchange traits (n = 3); **b** physiological biochemical traits ($n = 6$). CO_2 and H_2O exchange traits in (**a**) include the net rates of photosynthesis (P_n), the stomatal conductance (G_s), the intracellular CO_2 concentration (C_i), and the transpiration rates (T_r). Physiological and biochemical traits in (**b**) include whole plant biomass, foliar water contents, numbers of nodules, total C and N contents, C:N ratio, $\delta^{13}C$ and $\delta^{15}N$ signatures, NR activity, total protein N, total amino acids N, ammonium and nitrate N, structural N contents. GS, Gansu provenance; DB, Dongbei provenance. Ino, rhizobia inoculation; NonIno, no inoculation. HN, high N supply; CK, normal N supply. All ellipses indicate the 95% confidence range

Effects of N supply and rhizobia inoculation on foliar CO_2 and H_2O gas exchange differ between Robinia provenances

At high N supply, GS plants showed higher foliar P_n compared to normal N supply irrespective of inoculation. Inoculated DB plants showed greatly higher foliar P_n at high N supply than at normal N. A significant negative effect of inoculation on foliar P_n was only found for DB plants at high N supply. Among the two tested *Robinia* provenances, GS plants showed higher foliar P_n compared to DB plants irrespective of inoculation and N supply (Tables 1 and 2). In the GS provenance, high N supply reduced foliar G_s, but this effect was only significant for inoculated plants. A similar effect was observed for inoculated DB plants, but not for DB plants without inoculation due to an already high G_s at high N supply. Inoculation reduced G_s of DB plants, but did not affect G_s of GS plants. Foliar G_s was significantly lower in inoculated GS

Table 2 Effects of treatments on photosynthesis of *Robinia* leaves

Treatment	P_n (μmol/m^2·s)	G_s (mmol/m^2·s)	C_i (μmol/mol)	T_r (mol/m^2·s)
GS-Ino-HN	7.02 ± 0.23 a	$1.76 \times 10^{-3} \pm 8.1 \times 10^{-5}$ ab	302.24 ± 6.02 abc	0.13 ± 0.002 a
GS-Ino-CK	3.12 ± 0.28 bc	$7.20 \times 10^{-4} \pm 5.5 \times 10^{-5}$ cd	280.50 ± 9.80 bc	0.05 ± 0.004 b
GS-NonIno-HN	7.74 ± 0.36 a	$1.60 \times 10^{-3} \pm 4.4 \times 10^{-4}$ abc	292.39 ± 11.72 abc	0.11 ± 0.035 a
GS-NonIno-CK	3.21 ± 0.27 b	$7.70 \times 10^{-3} \pm 2.0 \times 10^{-5}$ bcd	304.75 ± 41.78 ab	0.09 ± 0.034 ab
DB-Ino-HN	5.60 ± 0.46 a	$1.43 \times 10^{-3} \pm 5.0 \times 10^{-4}$ bc	312.60 ± 14.51ab	0.07 ± 0.027 ab
DB-Ino-CK	3.47 ± 0.46 b	$3.12 \times 10^{-3} \pm 8.6 \times 10^{-4}$ a	335.05 ± 12.04 a	0.20 ± 0.045 a
DB-NonIno-HN	3.24 ± 0.52 bc	$6.07 \times 10^{-4} \pm 1.1 \times 10^{-4}$ d	251.81 ± 46.34 c	0.04 ± 0.007 b
DB-NonIno-CK	2.43 ± 0.18 c	$7.30 \times 10^{-4} \pm 1.9 \times 10^{-4}$ cd	261.65 ± 27.80 bc	0.04 ± 0.012 b

GS refers to the *Robinia* provenance from Gansu, Northwest China; DB refers to the *Robinia* provenance from the Dongbei region, Northeast China. Ino, rhizobia inoculation; NonIno, no inoculation. HN, high N supply; CK, normal N supply. The results shown are means ± SE. Different lowercase letters indicate significant differences between different treatments ($p < 0.05$; $n = 3$)

compared to DB plants at normal N supply, but significantly higher in non-inoculated GS plants at high N supply (Tables 1 and 2).

In general, the foliar C_i was not significantly affected by the N supply. Inoculation significantly enhanced C_i of DB, but not of GS plants irrespective of the N supply. Significant differences in C_i between provenances were only found at normal N supply for inoculated plants, with higher values for the DB compared to the GS provenance (Tables 1 and 2). In the GS provenance, high N supply increased T_r despite reduced G_s, but this effect was only significant for inoculated plants. A similar effect was not observed for DB plants. A significant effect of inoculation was only found in DB plants normal N supply, with higher values for inoculated plants. A consistent effect of provenance on T_r was not observed. T_r of the GS provenance was significantly lower than for the DB provenance in inoculated plants at normal N supply, but significantly higher in non-inoculated plants at high N supply (Tables 1 and 2).

Effects of N supply and rhizobia inoculation on foliar water content, natural $\delta^{13}C$ and $\delta^{15}N$ abundance of different Robinia provenances

Despite significant differences in G_s and T_r between the treatments, the foliar water contents were mostly maintained and only slightly increased at high N supply in non-inoculated GS plants (Fig. 2a, Table 1). Similar foliar water relations irrespective of treatments and plant provenances were also indicated by the leaf $\delta^{13}C$ signatures that showed similar values below −30 ‰ (Fig. 2b, Table 1).

High N supply showed lower $\delta^{15}N$ abundance compared to normal N supply in inoculated GS plants and non-inoculated DB plants. Inoculation with rhizobia decreased $\delta^{15}N$ in GS plants irrespective N supply and at normal N supply in DB plants. DB plants showed a higher $\delta^{15}N$ abundance compared to GS plants in inoculated plants irrespective N supply and at normal N supply in non-inoculated DB plants (Fig. 2c, Table 1).

Effects of N supply and rhizobia inoculation on foliar N partitioning of Robinia provenances

The total N content of the leaves was not significantly affected by the different N supplies and inoculation treatments in both *Robinia* provenances (Fig. 3a, Table 1). High N supply significantly reduced the foliar soluble protein N content compared to normal N supply in inoculated GS, but not in DB plants. Inoculation reduced the soluble protein N content significantly in GS plants at high N supply, but enhanced soluble protein content at normal N supply. A similar effect was not observed for DB plants, where inoculation even reduced the foliar soluble protein content at normal N supply. Between the provenances, soluble protein N content of the leaves was significantly higher in inoculated GS compared to DB plants at normal N supply (Fig. 3b, Table 1).

Total amino acid N contents were reduced by high N supply in inoculated GS, but enhanced in inoculated DB plants. Inoculation with rhizobia enhanced total amino acid N content in the leaves of GS plants irrespective of N supply, but reduced total amino acid N content in the leaves of DB plants at normal

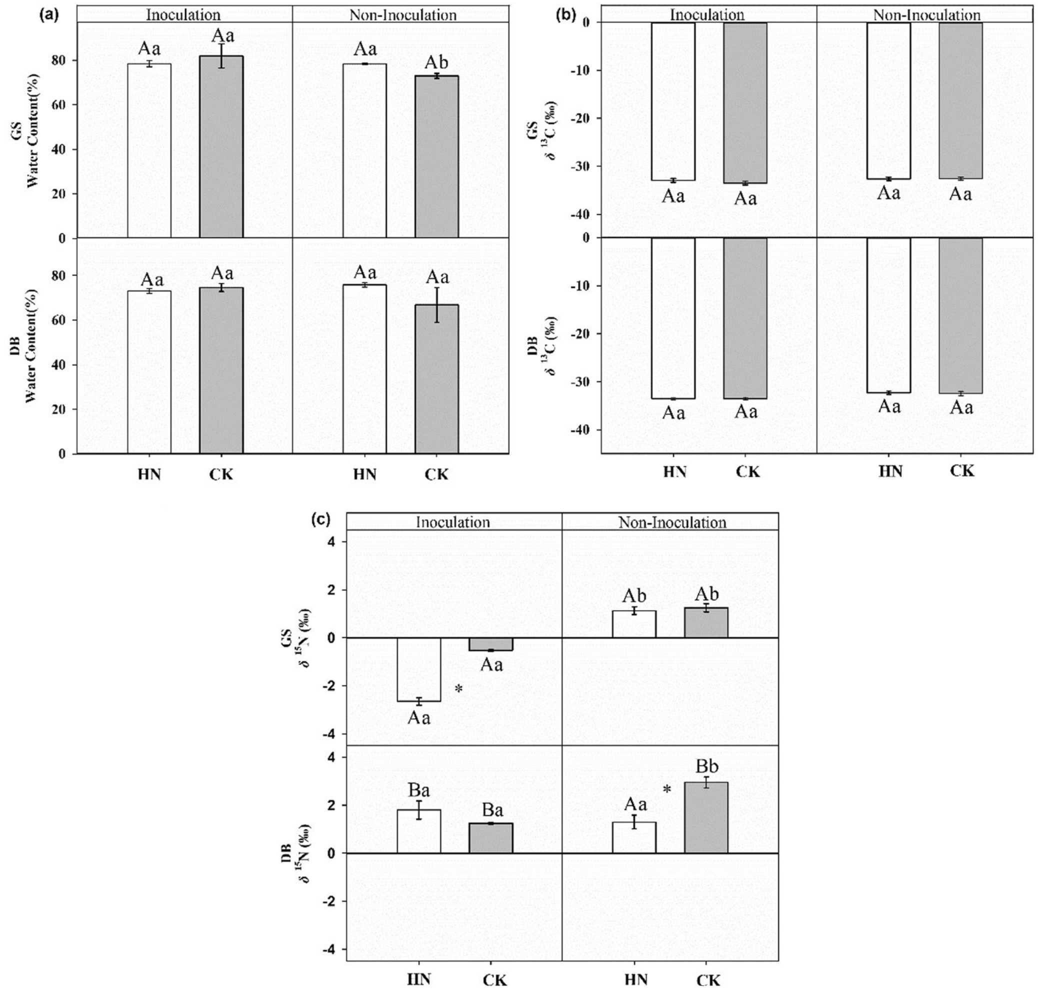


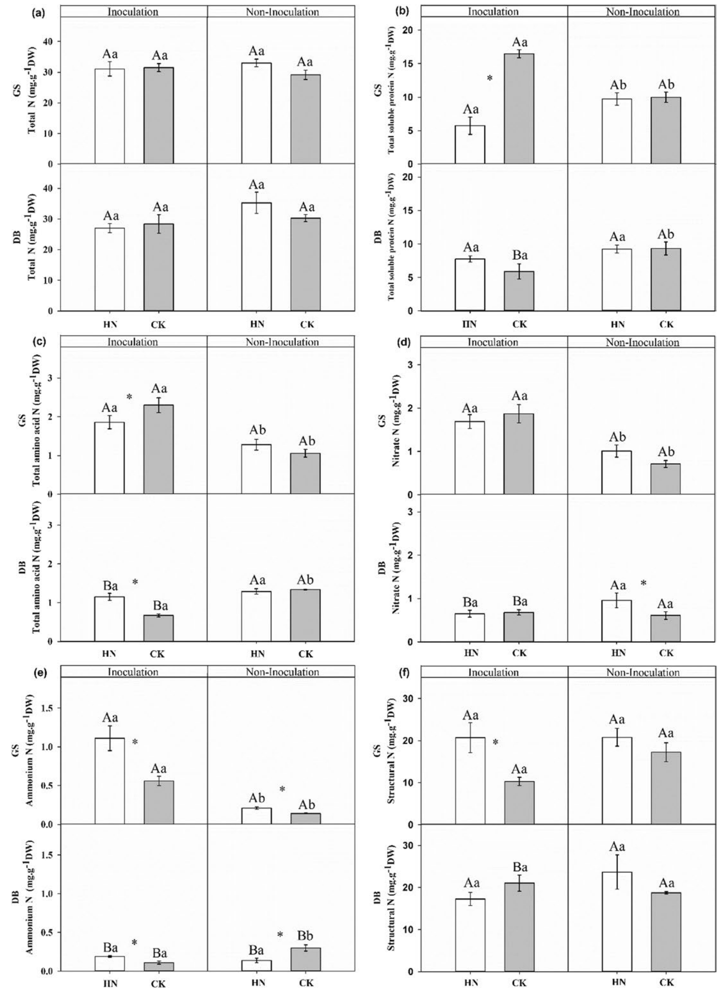
Fig. 2 Water content (%), δ^{13}C (‰), and δ^{15}N (‰) in leaves of *Robinia* provenances subjected to different N supplies and rhizobia inoculation treatments. Bars represent means ± SE. Asterisk indicates significant differences between different N supplies of the same provenance with the same rhizobia inoculation treatment. Different lowercase letters indicate significant differences between rhizobia inoculated and non-inoculated plants of the same provenance subjected to the same N supply. Different capital letters indicate significant differences between different provenances at the same N supply with the same rhizobia inoculation treatment ($p < 0.05$; $n = 6$)

N supply. Between provenances, total amino acid N contents were higher in leaves of inoculated GS compared to DB plants, but similar in non-inoculated plants of both provenances (Fig. 3c, Table 1).

High N supply enhanced the foliar nitrate N content only in non-inoculated DB, but not in GS plants. Rhizobia inoculation enhanced the foliar nitrate N content in GS, but not in DB plants. In inoculated plants of the GS provenance, foliar nitrate N contents were significantly higher than in the DB provenance, irrespective of N supply (Fig. 3d, Table 1).

High N supply enhanced the foliar ammonium N content in GS plants irrespective of inoculation and in inoculated DB plants, but enhanced the ammonium N content of the leaves in non-inoculated DB plants. Rhizobia inoculation enhanced the foliar ammonium N content of GS plants irrespective of N supply, but reduced the ammonium N content in leaves of DB plants at normal N supply. In general, foliar ammonium N contents were higher in GS compared to DB plants, irrespective of N supply and rhizobia inoculation (Fig. 3e, Table 1).

Fig. 3 Total N content and partitioning (mg·g^{-1}DW) in leaves of *Robinia* provenances subjected to different N supplies and rhizobia inoculation treatments. Bars represent means ± SE. Asterisk indicates significant differences between different N supplies of the same provenance with the same rhizobia inoculation treatment. Different lowercase letters indicate significant differences between rhizobia inoculated and non-inoculated plants of the same provenance subjected to the same N supply. Different capital letters indicate significant differences between different provenances at the same N supply with the same rhizobia inoculation treatment ($p < 0.05$; n = 6)



High N supply promoted the structural N content in inoculated GS plants, but this parameter was not significantly affected by the N supply in other treatments. Rhizobia inoculation did not significantly affect structural N contents of the leaves of both provenances. At high N supply, structural N content of inoculated DB plants was lower compared to inoculated GS plants (Fig. 3f, Table 1).

Effects of N supply and rhizobia inoculation on foliar nitrate reductase (NR) activity in two Robinia provenances

High N supply plants enhanced foliar NR activity compared to normal N supply in inoculated GS plants and non-inoculated DB plants. Inoculation with rhizobia enhanced NR activity in GS plants irrespective of N supply, but not in DB plants that even showed reduced activity of the enzyme in inoculated plants at high N supply. At high N supply, inoculated GS plants showed higher foliar NR activity compared than inoculated DB plants, whereas non-inoculated GS plants had a lower foliar NR activity than non-inoculated DB plants at high N supply. A similar effect was not observed at normal N supply (Fig. 4, Table 1).

Effects of N supply and rhizobia inoculation on foliar total C contents and the C/N ratio of different Robinia provenances

In leaves of the GS provenance, high N supply significantly reduced total C content in inoculated plants, but increased total C content in non-inoculated plants. Inoculation with rhizobia increased the foliar total

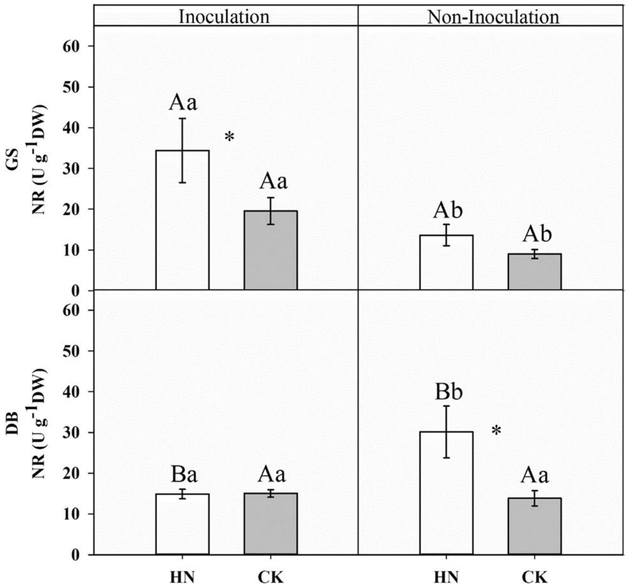


Fig. 4 Nitrate reductase activity (U·g^{-1}DW) in leaves of two *Robinia* provenances at different N supplies and rhizobia inoculation treatments and. Bars represent mean ± SE. Bars represent means ± SE. Asterisk indicates significant differences between different N supplies of the same provenance with the same rhizobia inoculation treatment. Different lowercase letters indicate significant differences between rhizobia inoculated and non-inoculated plants of the same provenance subjected to the same N supply. Different capital letters indicate significant differences between different provenances at the same N supply with the same rhizobia inoculation treatment ($p < 0.05$; n = 6)

C content at normal N supply, but decreased total C content at high N supply in GS plants. The provenances did not differ significantly in total C contents irrespective of the treatment (Fig. 5a, Table 1). In leaves of the DB provenance, inoculation significantly increased the C/N ratio at high N supply. A similar effect was not observed for GS plants (Fig. 5b, Table 1).

Discussion

Response to high N supply largely differ between Robinia provenances

In the present study, seedlings of two *Robinia* provenances were exposed to high and normal N availability in the soil at full water supply to exclude responses to water limitation. Different to previous studies, the present investigation combined different levels of N supply and rhizobia inoculation for two *Robinia* provenances. Because interpretation of plant responses to soil nutrient availability under natural field conditions is often hampered by interactions with other soil properties (Weemstra et al. 2017a), environmental factors (Hertel et al. 2013; Minotta and Pinzauti 1996), or differences in stand age (Finér et al. 2007), the present study was conducted under greenhouse conditions. Consistent with our hypothesis (i), our results show that high N supply significantly decreases root nodulation of inoculated GS plants as previously observed in other studies (Talaat and Abdallah 2008). However, different to this hypothesis high N supply did not affect initial root nodule development of DB plants. Reduced root nodulation of GS rather than DB provenance seedlings was also observed under drought (Yuan et al. 2022). Apparently, the responses of nodule development to changes in soil N availability and water limitation differs between the *Robinia* provenances studied reflecting an adaptive strategy to the soil nutrient and water availability at the original habitats (hypothesis iii). This conclusion is in line with a report from different provenances of European beech saplings growing under contrasting soil nutrition and precipitation conditions (Meller et al. 2020).

Independent of provenance and inoculation, high N supply neither changed whole plant biomass nor foliar total N content. This result is in line with the previous finding that leaf N concentrations and

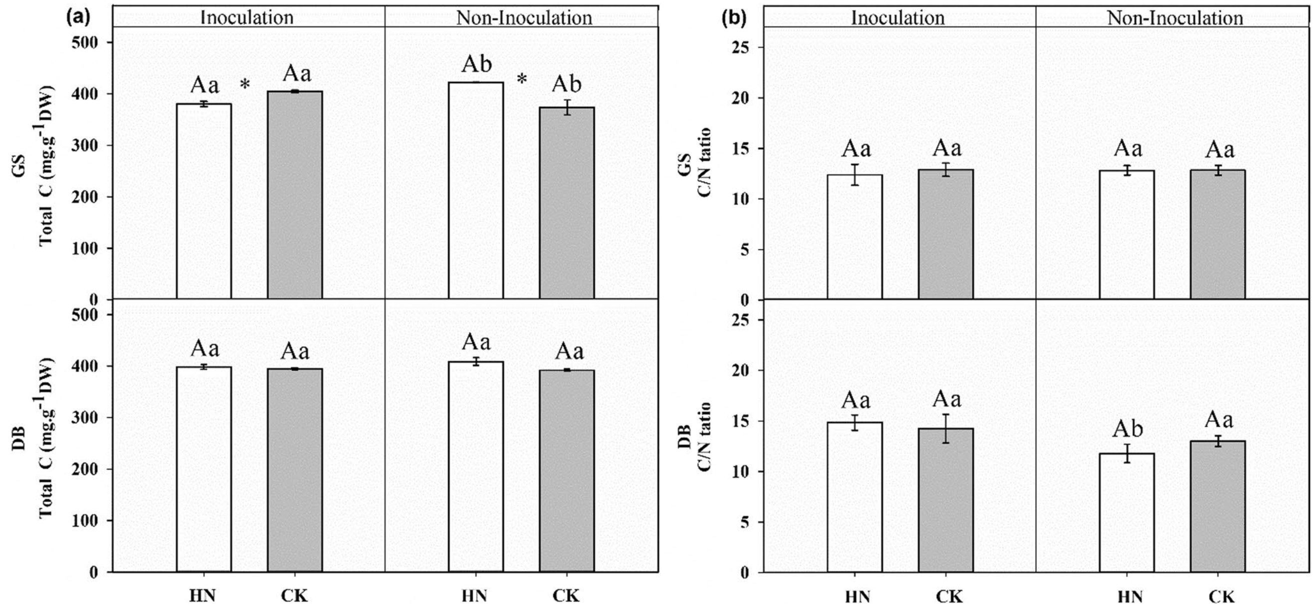


Fig. 5 Total C (mg·g^{-1}DW) and C/N ratio of *Robinia* provenances subjected to different N supplies and rhizobia inoculation treatments. Bars represent means ± SE. Asterisk indicates significant differences between different N supplies of the same provenance with the same rhizobia inoculation treatment. Different lowercase letters indicate significant differences between rhizobia inoculated and non-inoculated plants of the same provenance subjected to the same N supply. Different capital letters indicate significant differences between different provenances at the same N supply with the same rhizobia inoculation treatment ($p < 0.05$; n = 6)

plant biomass of *Robinia* seedlings remained unaffected by different N supply (Wang et al. 2021). We assumed that in the present study this observation is due to N-accumulation and saturation by the combined action of N uptake from the soil and of N_2-fixation already before shifting the plants to high N supply as well as the relatively short time (ca. 10 weeks) of the subsequent high N treatment. Thus, further studies are needed to address growth and physiological responses of *Robinia* seedlings under long-term N supply. Still, in non-inoculated GS plants, high N supply significantly increased P_n. Increased P_n can be attributed to the higher CO_2 influx, consistent with previous findings on poplar (Luo et al. 2015b). In the present study photosynthetic efficiency of the *Robinia* GS provenance was higher than that of the DB provenance at high N supply. This observation may be attributed to reduced P_n as a consequence of stomatal closure at high N supply in DB plants to protect foliar tissues from damage, similar to the effect of drought stress (Fang and Xiong 2014; Tardieu et al. 2018). Hence, the sensitive and fast stomatal response of the DB provenance could help to provide effective protection of photosynthesis under high N supply (Meller et al. 2020). Apparently, such a response has not been developed by the GS provenance originated from a habitat with poor N-nutrition in the soil. Such a provenance specific response of photosynthesis was also observed in leaves of *Cunninghamia lanceolata* originating from habitats with different precipitation under drought stress (Gao et al. 2021).

Foliar δ^{13}C abundance indicates the long-term water status of plants at undisturbed CO_2 fixation and is related to stomatal conductance (Hu et al. 2013). In the present study, high N supply did not significantly affect foliar δ^{13}C, but reduced foliar G_s. This result indicates that the water balance of *Robinia* trees was not affected by high N availability at normal water supply in the soil. This result is consistent with the unaffected water use efficiency in leaves of sunflower plants at different N supply (Cechin and Fumis 2004). In this context, reduced foliar G_s may be induced by soil acidification and depletion of other nutrients at high N supply (Azuchi et al. 2014). In addition, it may also be related to increased photosynthetic capacity of plants at high N supply, which reduces the intercellular CO_2 concentration, as in line with hypothesis (i) (Azuchi et al. 2014; Wang et al. 2021).

Consistent effects of high N supply on N partitioning were not observed in the two provenances. Although physiological constrains were reported under N excess in previous studies (Ni et al. 2020b), plants may be able to adapt to environments that reflect the soil-nutrient status of its original habitat. Changes of organic-N partitioning induced by excess N-supply were also reported to be less pronounced in the leaves of poplar (*Populus simonii* L.) (Luo et al. 2015a). However, in the present study, high N supply increased the accumulation of ammonium-N in leaves, as previously reported for mulberry and poplar (Luo et al. 2015a; Ni et al. 2020b). Ammonium is incorporated into amino acids via the GS/GOGAT system and this process requires energy and reductant from photosynthesis (Ni et al. 2020b). Therefore, the joint increases of photosynthesis and N assimilation observed in the present study can be considered a useful response to high N supply. This view is supported by the observation that high N can lead to increased NR enzyme activity, which will also increase the foliar N content and can support photosynthesis (Guiboileau et al. 2013; Zhang et al. 2014). As indicated by decreased foliar δ^{15}N abundance, high N also leads to increased N_2-fixation capability of inoculated GS provenance seedlings. Although the inhibition of N_2-fixation by the availability of soil high N was reported previously (Voisin et al. 2002), our results indicated that more N was acquired mainly from the atmospheric N_2-fixation, as also reported in other species (e.g., *Trifolium pratense* and *Trifolium repens*) (Oberson et al. 2013). In this context, high N supply seemed not to fulfil the nutrient demand for enhanced growth of *Robinia*. Increased N_2-fixation capability of *Robinia* plants under high N treatment was also found in a previous study (Wang et al. 2021).

Rhizobia inoculation induces nodule development, but has different effects on foliar traits in the two provenances

Inoculation with rhizobia resulted in strongly enhanced abundance of root nodules in both provenances without affecting foliar N contents. However, a change in the foliar N source by rhizobia inoculation was indicated by the significantly decreased δ^{15}N abundance in GS plants (ca. –3-fold irrespective of N supply, Fig. 2c). Apparently, previously accumulated N from soil sources still played a dominant role for initial foliar N nutrition in inoculated DB, but not in inoculated GS plants, where products of N_2 fixation contributed to foliar N contents. The latter has also been observed in other studies of legume trees inoculated with rhizobia (Pereyra et al. 2015). However, also previous studies with mature trees in the Loess Plateau of China showed that *Robinia* mainly uses N in the soil as a N source (Du et al. 2019). Despite unaffected total N contents, foliar N partitioning in the leaves of the GS provenance was altered in favor of amino acids and inorganic N by the rhizobia inoculation in the present study. Also, previous studies showed that *Robinia* inoculation with rhizobia contributed to the accumulation of inorganic N (Zhang et al. 2019b). The latter can be interpreted as a reduced requirement for N assimilation in the leaves because of enhanced allocation of amino acids from the nodules to the leaves (Zhang et al. 2019b). The enhanced nitrate content of the leaves was accompanied by enhanced NR activity. This is in line with results on N nutrition of poplar (Rennenberg et al. 2010).

Consistent with our hypothesis (ii), the initial development of nodules reduced total plant biomass accumulation in GS plants, as also observed upon inoculation of soybean varieties with rhizobia (Saito et al. 2014). This is probable due to the belowground organic carbon drain for nodule production and for the generation of energy required for N_2 fixation in the nodules at initial inoculation. Apparently, the new belowground carbon sink operates at the expense of growth, particularly at unaffected P_n as initial, but probably transient, effect of inoculation. However, positive effects of nodulation on biomass accumulation in the long term, as frequently observed for host legume crop species (Guo et al. 2010; Gicharu et al. 2013; Mothapo et al. 2013; Allito et al. 2021), can also be expected for *Robinia* trees. Previous studies showed that inoculation with effective and appropriate rhizobia strains is essential for efficient symbiotic nitrogen fixation and optimum legume crop productivity (McKenzie et al. 2001; Allito et al. 2021). Our results show that the GS provenance tended to enhance its N uptake and N_2-fixation (increased foliar NR activity and more negative δ^{15}N abundance), but reduced nodule development under high N supply compared with the DB provenance. Thus, the effectiveness of Robinia-rhizobia symbiosis seems to depend on both, the macro and micro-symbiont, i.e., on differences in the compatibility between *Robinia* provenances and rhizobium strains. As most legumes benefit from a nitrogen-fixing symbiosis, access to compatible strains of nodule bacteria in the soil of new environments constitute a critical factor for their ecological success (Parker et al. 2007). Invasive legumes were found to either interact efficiently with locally adapted indigenous symbiotic bacteria, or carry along appropriate symbionts into the new environment (Batzli et al. 1992; Ulrich and Zaspel 2000; Weir et al. 2004; Wei et al. 2009). Obviously, the rhizobia strain of *Mesorhizobium huakuii* from the coastal area of eastern China used in the present study showed different symbiotic compatibility with the *Robinia* provenance analyzed. In a previous study, the lack of shoot growth promotion by rhizobia inoculation of soybean was attributed to the inefficiency of the introduced rhizobia strain in nitrogen fixation (Ulzen 2018). More attentions should be given on nodulation with adapted indigenous rhizobia strains from local habitat soils of *Robinia* provenances in future studies.

In the present study, the carbon contents of the leaves of GS plants were not affected by rhizobia inoculation. This effect can be attributed to the substantial proportion of total C in belowground tissues of woody legume seedlings that is ca. 3–5-folder higher than reported for pasture legumes (Ohyama and Kumazawa 1980; Kohl et al. 1989). Unaffected leaf carbon contents were also observed by rhizobia inoculation of *Leucaena leucocephala* (Pereyra et al. 2015).

Provenance-specific response to rhizobia inoculation

Inoculation with rhizobia at high N still resulted in the development of a significant number of nodules in the two provenances of *Robinia*, indicating that *Robinia* can use atmospheric N_2 even at high N supply in the soil (Zhang et al. 2019b). Previous studies showed that high N in the soil can significantly decrease nodule number, nodule mass and N_2 fixation activity, even accelerating nodule senescence (Ferguson et al. 2018). In the present study, we selected *Robinia* seedlings from two provenances with distinct climate and soil nutrient availability, i.e., GS seedlings originating from Northwest China with low precipitation and poor nutrient availability, including low total N and SOC contents in the top 0–10 cm soil layer compared to DB seedlings originating from Northeast China with higher precipitation and fertile soil. However, in the present study, the responses of the two *Robinia* provenances to inoculation with rhizobia at high N in the soil differed as hypothesized (iii), indicating the confounding effects of the habitat of origin and current plant performance (Meller et al. 2020). At high soil N, the DB provenance tended to slightly increase photosynthesis, but did not significantly alter nodule development and foliar N partitioning. Under this condition, the GS provenance showed significantly increased P_n and enhanced N-uptake and N_2-fixation capability (indicated by increased foliar NR activity and more negative $\delta^{15}N$ abundance). These differences in performance of GS compared to DB seedlings indicated of a more flexible resource use strategy of the *Robinia* seedlings from the harsh and nutrient-poor habitat of the GS plants (Craine 2009; Weemstra et al. 2017b). This result is surprising since decreased photosynthesis and leaf growth of European beech saplings originated from a nutrient-poor habitat was observed upon growth under high nutrient availability in the soil (Yang et al. 2016; Zavišić et al. 2018). The more flexible resource use strategy on *Robinia* seedlings may be of particular significance for its role as pioneer plant.

Although rhizobia inoculation did not cause changes in total N content of GS plants, it promoted the synthesis of organic N and enhanced inorganic N accumulation at reduced nodule development. In this context, genotypic differences between provenance related to adaptation to specific site conditions have often been found to include adaptive traits to environmental stress (e.g., Cuervo-Alarcon et al. 2018). Thus, genetic diversity of provenances could significantly affect above- and below-ground traits of plants at contrasting nutrient availability in the soil, e.g., photosynthesis activity, leaf morphology, leaf nutrient concentrations and ratios, plant growth and root morphology. As a consequence, responses of plant populations adapted to sites differing in resource availability, will differ when growing in soils with nutrient supply that does reflect the habitat of origin (Craine 2009; Kremer et al. 2012). This conclusion from the present study is consistent with reports with European beech saplings from habitats with contrasting nutrient availability in the soil (Mellert and Göttlein 2012; Göttlein 2015; Zavišić et al. 2018; Meller et al. 2020). In *Robinia*, nodule formation and the foliar traits studied were much more responsive to rhizobia inoculation in the GS compared to the DB provenance (hypothesis iii).

Conclusion

The present results show that rhizobia inoculation initially modified foliar N partitioning of the GS *Robinia* provenance, probably to offset the negative effects of high N availability. A similar effect was not found in the DB *Robinia* provenance. Obviously, not only the responses to rhizobia inoculation, but also to high N supply differ between the GS and the DB provenance of *Robinia*. Whereas the DB provenance developed a similar number of nodules upon rhizobia inoculation at high N, the number of nodules was strongly reduced in the GS provenance. These results show that both, nodulation and foliar traits of the GS provenance were more responsive to high N availability in the soil than in the DB provenance. However, given the lack of reports on acclimation to changes in nutrient supply, the ability of *Robinia* provenances from habitats with different climate and soil fertility to acclimate to high N exposure and other environmental stress conditions in the long term still require more attention in future studies.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hongguang Sun, Rui Liu, Zhenshan Liu, Mi Zhou and Hui Yuan. The first draft of the manuscript was written by Hongguang Sun and Bin Hu. Heinz Rennenberg and Bin Hu contributed to the finalization and revision of the manuscript. All authors read and approved the final manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interests.

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