

Morphological and physiological responses of *Spatholobus suberectus* Dunn to nitrogen and water availability

Z.-X. ZHANG^{*,**}, J. ZHANG^{*,**,**}, Y. GONG[#], G.-Z. LIU^{*}, L.-L. GAO^{*}, P. ZHANG[#], and C.-T. CAI^{*,##,+}

CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Mengla, 666303 Yunnan, China^{*}

University of Chinese Academy of Sciences, 100049 Beijing, China^{**}

Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences, Kunming, 650200 Yunnan, China^{***}

Zhuzhou Qianjin Pharmaceutical Co., Ltd., Zhuzhou, 412007 Hunan, China[#]

Center of Economic Botany, Core Botanical Gardens, Chinese Academy of Sciences, Menglun, Mengla, 666303 Yunnan, China^{##}

Abstract

A pot study was conducted to elucidate morphological and physiological responses of *Spatholobus suberectus* Dunn accessions to four nitrogen application rates (0, 10, 20, and 30 mmol L⁻¹) and two watering regimes (daily irrigation and irrigation every 10 d for a month). The results showed that water deficit led to marked decreases in leaf water relations and photosynthetic parameters regardless of the N treatments. Under drought conditions, the reductions in net photosynthesis occurred mainly due to stomatal factors. Although the leaf N content and stem mass ratio were positively correlated with N application, other morphological and physiological characteristics were negatively correlated or nonsignificantly correlated with N application under limited water supply. As the root nodules of *S. suberectus* can provide sufficient N supply, and high N applications made *S. suberectus* plants more vulnerable to drought stress, we suggest that no N supplement is required for *S. suberectus* seedlings.

Additional key words: fertilizer; gas exchange; nitrogen-use efficiency; water-use efficiency; water stress.

Introduction

Spatholobus suberectus Dunn is a perennial woody liana belonging to the Leguminosae family. It is indigenous to tropical and subtropical forests in China and other Southeast Asian countries, in a nonarid climate (Li 2017). The vine stem of *S. suberectus* has been widely used in traditional and folk medicine in China, Korea, and Japan (Lee *et al.* 2011). It exhibits various pharmacological activities, including improvement of hematopoiesis (Liu *et al.* 2004), antithrombosis (Lee *et al.* 2011), antibacterial (Fu *et al.* 2017), and antitumor activities (Wang *et al.* 2011, Li *et al.* 2015). Due to its remarkable pharmacological activities and low toxicity, *S. suberectus* is considered as a medicine with a great potential (Lee *et al.* 2011, Wang *et al.* 2011, Li *et al.* 2015). However, over the past 30 years, *S. suberectus* is identified as a vulnerable species and is listed in the Red List of Biodiversity in China (Ministry of Ecology and

Environment of the People's Republic of China and Chinese Academy of Sciences 2013), as a result of its low regeneration rate and human activities (overexploitation and deforestation). Moreover, market demand for *S. suberectus* is large, but the wild resources can no longer meet this growing need. Therefore, artificial planting of this species has been started in mountainous areas in Xishuangbanna in southwest Yunnan, China, which is characterized by a climate of distinct, alternating rainy and dry seasons. One major limitation for the cultivation of *S. suberectus* in these areas is the erratic seasonally low soil water status and high temperature during the dry season, particularly at its early growth stage.

Drought, in conjunction with high temperature and radiation, poses the most important environmental constraints to plant survival and to crop productivity (Boyer 1982). Water deficit can trigger a series of physiological and biochemical responses in plants to reduce leaf photo-

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⁺Corresponding author; phone/fax: +86 (0) 871 65161066, e-mail: caict@xrbg.ac.cn

Abbreviations: C_i – CO₂ concentration at the substomatal cavity; DM – dry mass; E – transpiration rate; FM – fresh mass; g_s – stomatal conductance; HW – high water; LMR – leaf mass ratio; LW – low water; N – nitrogen; P_N – net photosynthetic rate; PNUE – photosynthetic nitrogen-use efficiency; P/S – palisade tissue thickness to spongy tissue thickness ratio; RMR – root mass ratio; R/S – root to shoot ratio; RWC – relative water content; SLA – specific leaf area; SMR – stem mass ratio; SWC – soil water content; TM – turgid mass; W – water; WUE – instantaneous water-use efficiency ($= P_N/E$); WUE_i – intrinsic water-use efficiency ($= P_N/g_s$); $\delta^{13}\text{C}$ – carbon isotopic composition ratio; ψ_{md} – leaf midday water potential; ψ_{pd} – leaf predawn water potential; ψ_w – leaf water potential.

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synthesis and, in turn, yield (Dias *et al.* 2007, Yousefzadeh Najafabadi and Ehsanzadeh 2017, Nadal and Flexas 2019). Stomatal closure is the first reaction of plants upon a decline in soil moisture (Chaves *et al.* 2003, Flexas *et al.* 2004) to prevent excessive water loss. As water stress progresses, CO_2 diffusion is also restricted by the reduction in both stomatal and mesophyll conductance and accounts for most of the photosynthesis decrease (Cano *et al.* 2013). Under severe water stress conditions, a number of photosynthetic components and/or functions is damaged, including leaf pigment, enzymes, and CO_2 fixation capacity, biochemical constraints begin to affect photosynthesis (Waraich *et al.* 2011, Yousefzadeh Najafabadi and Ehsanzadeh 2017, Nadal and Flexas 2019). In a water-limited scenario, enhancing water-use efficiency is not only a major prerequisite for plant survival and growth (Costa *et al.* 2007, Morison *et al.* 2007), but also a good mean for saving water in agriculture (Gago *et al.* 2014).

Extensive approaches are being used to enhance water-use efficiency and to minimize the detrimental effect of water stress in crop plants (Deng *et al.* 2006, Gago *et al.* 2014). Fertilizer application to a reasonable extent has been reported to be a good management for drought mitigation in dry land agriculture (Deng *et al.* 2006, Waraich *et al.* 2011). Adequate nutrition is essential for plant growth and key physiological processes. Especially nitrogen (N), a major macronutrient, being an integral part of chlorophyll, enzymes, and proteins, plays an important role in photosynthesis, antioxidant and metabolic activities, leading to alleviation of adverse effects imposed by environmental stress (Zhao *et al.* 2008, Waraich *et al.* 2011, Hassan *et al.* 2015). Under water-stress conditions, supplemental N can increase osmotic adjustment (DaMatta *et al.* 2002a), maintain higher leaf turgor and water status (Tesha and Kumar 1978, DaMatta *et al.* 2002a), expand root growth (Liu *et al.* 1998), allocate accumulation of dry matter more into roots (He *et al.* 2010, Molla *et al.* 2019), increase chlorophyll contents (Tesha and Kumar 1978, Hassan *et al.* 2015), and significantly ease water stress-induced depression in the net photosynthetic rate (He *et al.* 2010). These physiological and morphological changes in the N-fertilized plants facilitate greater tolerance to drought.

In spite of the positive effects of fertilizer in regulating water utilization, which are well documented, little is known about the physiological and morphological characteristics of N application on *S. suberectus* plants under drought stress. In this work, pot experiments were conducted to investigate the effects of N nutrition on growth, photosynthesis, and water-use efficiency in *S. suberectus* under well-watered and drought conditions. The results can give insight into a better understanding of reasonable water and fertilizer management practices for cultivation of *S. suberectus*.

Materials and methods

Study site, plant material, and treatments: This study was conducted in the Xishuangbanna Tropical Botanical Garden (XTBG; 21°56'N, 101°15'E, 560 m a.s.l.) in Yunnan,

southwest China. The annual mean temperature in this area is 21.5°C, with a total mean annual precipitation of 1,500 mm. The climate is dominated by tropical southern monsoons from the Indian Ocean between May and October and by subtropical jet streams between November and April. Three seasons are apparent in this area: a rainy season from May to October, a foggy cool season from November to January, and a hot dry season from February to April. Rainfall during the rainy season (mean temperature of 25°C) accounts for approximately 84% of the total annual precipitation. The foggy cool season is the coldest period, with dense fog in the morning and night. The hot dry season is a transitional period, with less rainfall and higher air temperature (exceeding 38°C). The foggy cool and hot dry seasons are collectively referred to as the dry season owing to the lack of rainfall during these periods.

Seeds of *S. suberectus* were sown in a nursery in December 2016. In July 2017, when the seedlings were approximately 20 cm tall, uniformly sized seedlings were selected and cultivated in a shade house (with 35% PPFD of full sunlight, created using neutral-density screen on a steel frame) in 11-L pots (24 cm in diameter and 24 cm in height) filled with forest soil. The soil was a yellow-brown type and the characteristics of the soil (0–20 cm) were: pH 4.68; 7.55 g(organic matter) kg^{-1} ; 5.09 g(total carbon) kg^{-1} ; 0.87 g(total nitrogen) kg^{-1} ; 0.32 g(total phosphorus) kg^{-1} ; 13.27 g(total potassium) kg^{-1} ; 68 mg(available nitrogen) kg^{-1} ; 0.79 mg(available phosphorus) kg^{-1} ; 87 mg(available potassium) kg^{-1} . Organic fertilizer (3 g) was applied per pot as a base fertilizer.

All pots were rotated at a 20-d interval to avoid rooting into ground and local variation in light availability. Weeds were removed regularly and insecticides were used when necessary. Since *S. suberectus* is a liana species, the plants were supported by bamboo poles.

Initially, plants were irrigated with tap water on days without rain to the level close to field capacity. Two months after planting, N treatments were imposed, with plants receiving monthly 1 L of either Hoagland's solution without N (N0) or complete Hoagland's solution plus extra N: 10 mmol L^{-1} (N10), 20 mmol L^{-1} (N20), and 30 mmol L^{-1} (N30) supplied as ammonium nitrate. Nutrient solutions were supplied until water treatment. Seven months after planting, when the dry season begins in 2018, a half of the pots of each N treatment were continuously irrigated (high-water treatment, HW) while the other half was moved to a spot with waterproof plastic sheet in the shade house, and was submitted to cyclic water stress (low-water treatment, LW). Drought was allowed to progress until predawn wilting became visible, whereupon the plants were reirrigated. Three such drought cycles, each lasting 10 d, were completed. In LW plants, measurements were made after the end of the third cycle of dehydration. During the cycle of water stress, pot soil surfaces were covered with a plastic sheet to minimize evaporation, thus allowing a slower establishment of the water stress, to simulate more realistic responses to drought. At the end of water treatment, soil water content (SWC) was measured at 5 cm below the ground using conductivity probe (*Theta probe MPM-160B, ICT International Pty Ltd.*, Armidale,

New South Wales, Australia) between 08:00 and 09:00 h. The SWC remained at 37.5% in HW conditions, while it ranged from 4.6 to 7.2% under LW supply.

The plants were distributed over a completely randomized design, with eight treatments (four N application rates and two watering regimes). Each treatment contained 15 *S. suberectus* seedlings.

Leaf relative water content (RWC): At the end of water treatment, five plants per treatment were randomly chosen to determine RWC. Ten top-most fully expanded leaves of each plant were picked and quickly weighed for fresh mass (FM). Then the samples were immediately hydrated in deionized water for 24 h until fully turgid under normal room light and temperature. Afterwards, the hydrated samples were surface-dried with filter tissue paper and weighed for turgid mass (TM). Samples were oven-dried at 70°C for 24 h to constant dry mass (DM). The following formula was used to calculate RWC: $RWC = [(FM - DM) / (TM - DM)] \times 100$.

Leaf water potentials (ψ_w) were measured at predawn (ψ_{pd} , 04:00–6:30 h) and midday (ψ_{md} , 12:30–14:30 h) using a *Pump-Up* pressure chamber (*PMS Instruments*, Albany, USA). Five plants per treatment were randomly chosen to measure the ψ_w , two ternately compound leaves of each plant were picked and measured *in situ*. Only leaves with a healthy appearance and recently fully expanded were collected.

Carbon isotopic composition ratio ($\delta^{13}\text{C}$), leaf carbon and nitrogen content: Three plant leaf samples were collected for each treatment, dried to a constant mass at 60°C, and homogenized to a fine powder with an 80-mesh sieve to determine their $\delta^{13}\text{C}$ compositions, leaf C and N concentrations. The $\delta^{13}\text{C}$, C, and N values of the leaf samples were measured using a stable isotope ratio mass spectrometer (*IsoPrime100*, *Isoprime*, Stockport, UK) at the Central Laboratory, XTBG. Each isotope ratio is expressed in parts per thousand relative to V-PDB for ^{13}C . C/N stoichiometry was calculated based on the leaf C and N concentrations.

Photosynthetic parameters: The net photosynthetic rate (P_N), stomatal conductance (g_s), CO_2 concentration at the substomatal cavity (C_i), and transpiration rate (E) of six plants per treatment were measured on top-most fully expanded leaves from 09:00 to 11:30 h, using a portable open-flow photosynthetic system (*LI-6400*, *LI-COR*, Lincoln, USA). These parameters were measured under the following conditions: 6400-02B LED light source with PPFD of 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$; flow rate was 500 $\mu\text{mol s}^{-1}$; leaf temperature was $28.1 \pm 1.2^\circ\text{C}$; leaf-to-air vapour pressure deficit was $1.84 \pm 0.31 \text{ kPa}$. One leaf was measured per plant. Instantaneous water-use efficiency (WUE) was calculated as the ratio of P_N to E . Intrinsic water-use efficiency (WUE_i) was calculated as the ratio of P_N to g_s . Photosynthetic nitrogen-use efficiency (PNUE) was computed as the ratio of P_N to unit of leaf N.

Leaf microstructure: Five recently fully expanded leaves were picked randomly from each treatment to measure anatomical traits and stomatal density. Transverse sections were hand-cut using a blade, made into water tablets. Sections were observed with a *Leica* biological microscope (*DM1000*, *Leica Microsystems*, Wetzlar, Germany), and six images of each section were captured at magnifications of 40×10 using a *Leica* microscope camera (*DFC295*, *Leica Microsystems*, Wetzlar, Germany). The image analysis software *ImageJ* (*ImageJ version 1.8.0, National Institutes of Health*, USA) was used to measure the following anatomical parameters: leaf thickness, thickness of palisade tissue, thickness of spongy tissue, thickness of upper and lower epidermis. The ratio of palisade tissue thickness to spongy tissue thickness (P/S) was calculated. The same group of leaves was used to investigate the stomatal density. Imprints of the upper and lower epidermis at maximum leaf width near the central vein were taken using clear nail varnish and adhesive cellophane tape. All imprints were fixed onto glass slides and photographed under *Leica DM1000* at magnifications of 20×10 , and counted the number of stomata in the grid scale. The stomatal density per leaf was estimated as the mean of the number of stomata in six randomly selected areas of $500 \times 500 \mu\text{m}^2$.

Growth traits: At the end of the experiment, five plants per treatment were harvested and separated into leaves, stems, roots, and root nodules. Ten recently fully expanded leaves without obvious damage were picked randomly from each plant. Each leaf was scanned, and the area measured using *ImageJ* software and then dried to a constant mass at 70°C. Specific leaf area (SLA) was calculated as the leaf blade area divided by the leaf dry mass. Roots and root nodules were washed thoroughly with tap water above a 0.5-mm screen sieve. Plant tissues were then oven-dried to a constant mass at 70°C, after which dry matter of leaves, stems, and roots was determined. Based on these data, the following plant growth traits were calculated: total biomass, aboveground biomass (leaf mass + stem mass), leaf mass ratio (LMR, leaf mass divided by total biomass), stem mass ratio (SMR, stem mass divided by total biomass), root mass ratio (RMR, root mass divided by total biomass), root to shoot ratio (R/S, root mass divided by aboveground biomass).

Statistical analyses were performed in *SPSS* (*version 16.0, IBM SPSS*, USA). Results from analysis of variance (*ANOVA*) and post-*ANOVA* *Student's t*-test were used for examining significance ($p < 0.05$). To study the relationships between the parameters with N application rate, linear regression analysis was employed.

Results

Water relations: Withholding irrigation significantly reduced the soil water content, reaching values lower than 7.2% after three water stress cycles. Water deficit brought about significant decrease in RWC, and RWC was negatively correlated with N application rate in either

watering treatment (Fig. 1A). Under HW conditions, the highest RWC value (74.8%) was found in no additional N-applied plants (N0), and significantly higher than the three additional N treatments (N10–N30). Under LW supply, similar trends were observed, the highest RWC value (62.2%) was in N0, the lowest RWC value (54.1%) was in N30, but no significant differences were found (Fig. 1A).

Nitrogen treatments had no obvious effects on leaf ψ_w in each watering regime, whereas water stress significantly

decreased the ψ_w (Fig. 1B,C). The ψ_{pd} was always greater than -0.40 MPa under HW conditions. Under LW supply, the lowest ψ_{pd} value (-0.72 MPa) was found in N30 treatment; in contrast, the highest ψ_{pd} values (-0.64 MPa) were found in N0 and N20 treatments (Fig. 1B). Similar trends were observed for the ψ_{md} , although the values were more negative (Fig. 1C).

Leaf carbon and nitrogen status: Leaf C, N content, and C/N ratio did not change significantly with the watering treatments, but was notably affected by the N application rate (Fig. 2). Under HW conditions, leaf C content ranged from 481.00 mg g $^{-1}$ (N30) to 490.33 mg g $^{-1}$ (N10), no significant differences were found. Under LW conditions, leaf C was negatively correlated with N

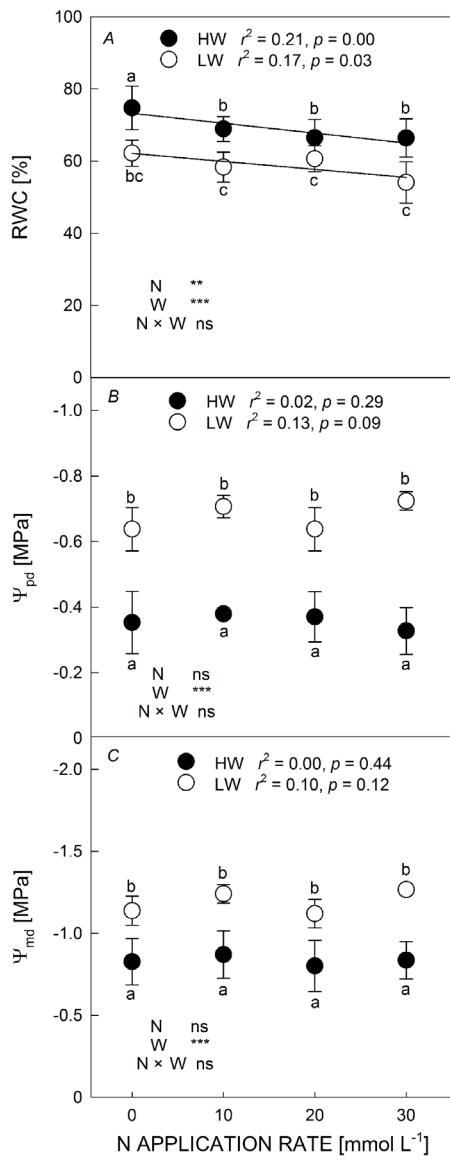


Fig. 1. Leaf water relation variables of *Spatholobus suberectus* plants grown under four nitrogen (N) application rates and two watering regimes. RWC – relative water content; ψ_{pd} – leaf predawn water potential; ψ_{md} – leaf midday water potential; HW – high water; LW – low water; W – water; N – nitrogen; r^2 – regression coefficient. Values are means \pm SD, $n=5$. Different letters in each point represent significant difference ($p<0.05$). Analysis of two-way ANOVA significance level was shown (* – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$; ns – not significant).

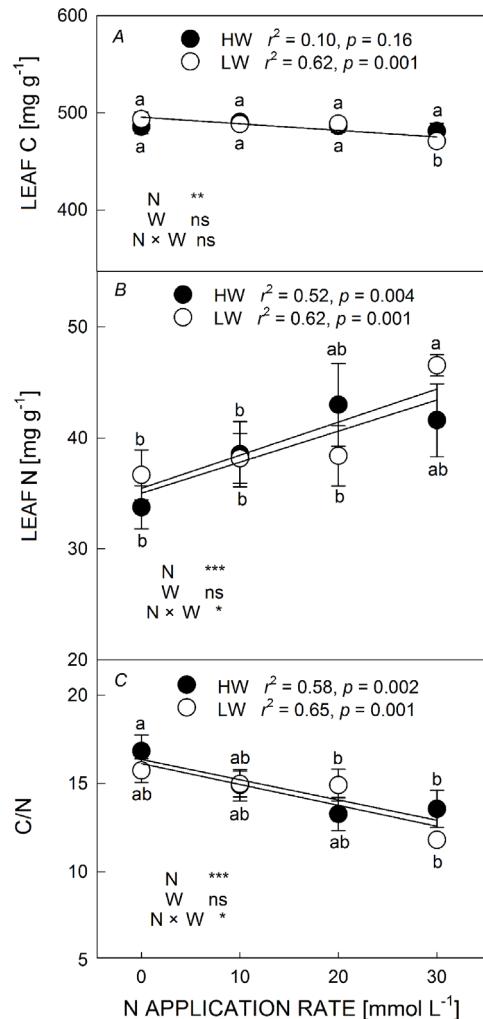


Fig. 2. Leaf carbon (C) and nitrogen (N) content and leaf C/N ratio of *Spatholobus suberectus* plants grown under four nitrogen (N) application rates and two watering regimes. HW – high water; LW – low water; W – water; N – nitrogen; r^2 – regression coefficient. Values are means \pm SD, $n=5$. Different letters in each point represent significant difference ($p<0.05$). Analysis of two-way ANOVA significance level was shown (* – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$; ns – not significant).

application rate; the lowest leaf C content (470.67 mg g^{-1}) was in N30 treatment, significantly lower than in other treatments (Fig. 2A). However, leaf N concentration was positively correlated with N application rate in both watering regimes, as expected. The highest leaf N content (46.52 mg g^{-1}) was in N30 plants under LW condition; in contrast, the lowest leaf N content (33.74 mg g^{-1}) was in N0 plants under HW condition (Fig. 2B). Notably, the C/N ratio was correlated negatively with N application rate in either watering regime. Moreover, the highest C/N ratio (16.83) was found in N0 plants under HW condition, while the lowest C/N ratio (11.81) was in N30 plants under LW condition (Fig. 2C).

Gas exchange and isotopic signatures: Regardless of the soil water availability, P_N , g_s , C_i , and E did not differ significantly between the N treatments, and no significant correlation was found between these parameters with N application rate (Fig. 3A–D). However, N0 plants had higher P_N than that of N10 and N30 plants under HW conditions; meanwhile, the highest P_N value was found in N0 plants under LW supply (Fig. 3A). In contrast, water stress provoked dramatic reductions in P_N , g_s , and E , but it caused an increasing trend in C_i , especially in N20 treatment (Fig. 3A–D). Neither N nor watering treatments had significant effects on WUE and WUE_i (Fig. 3E,F). However, the WUE and WUE_i were slightly higher in the three additional N treatments than that in N0 treatment under HW conditions. Moreover, the four N application treatments showed different changes under LW supply. Water deficit increased the WUE and WUE_i of N0 and N10 treatments, and this increment for N30 treatment was found only in WUE_i. In N20 treatment, water deficit brought about a slightly greater decrement in P_N than in g_s and E , altogether, these changes provoked a reduction in WUE and WUE_i (Fig. 3A,B,D–F).

PNUE was significantly altered by N applications in either watering treatment (Fig. 3G). Under HW conditions, N0 plants had the largest PNUE ($80.62 \mu\text{mol mol}^{-1} \text{ s}^{-1}$), higher than the three additional N treatments, while no significant correlation was found between PNUE with N application rate. Water deficit caused pronounced reduction in PNUE. Under LW supply, PNUE was negatively correlated with N application rate; also, the largest PNUE value ($37.30 \mu\text{mol mol}^{-1} \text{ s}^{-1}$) was in N0 plants and significantly higher than the three additional N treatments (Fig. 3G).

The $\delta^{13}\text{C}$ values ranged between -28.34 and -27.07‰ . Regardless of the soil water availability, the $\delta^{13}\text{C}$ varied only slightly among the N treatments, and showed no response to the water limitation (Fig. 3H).

Leaf microstructure: Except for the spongy tissue thickness, all other leaf anatomical structures including the leaf thickness were affected by water and/or N and the interaction of water and N (Table 1). Under HW conditions, the leaf thickness, palisade tissue thickness, epidermal thickness (upper and lower), and P/S ratio were positively correlated with the N application rate. Under LW supply, the palisade tissue thickness and P/S ratio were negatively

correlated with N application rate. Also, the thickness of leaf, palisade tissue, upper epidermis, and the P/S ratio in N30 treatment were all significantly lower than that in other treatments. In addition, except for N30 treatment, all the treatments had higher epidermal thickness in LW plants than that in HW plants (Table 1).

Leaf stomata of *S. suberectus* are only found in the lower epidermis. Nitrogen did not alter stomatal density, whereas water and the interaction of N and water had significant effects. Under HW conditions, stomatal density was 17.5% greater in the three additional N treatments than that in N0 treatment. Under LW supply, stomatal density of N20 plants was significantly lower than that of other plants, and showed a 24.1% decrease in relation to HW conditions; also, 21.0% reduction for N10 plants. However, N0 and N30 plants exhibited some rises under LW supply compared with their control counterparts (Table 1).

Growth traits: There were no significant effects of N and water on the biomass accumulation and the pattern of biomass allocation (Table 2). Nevertheless, under HW conditions, the total biomass, aboveground biomass, and LMR were positively correlated with N application rate. N30 and N20 plants grew faster than N10 and N0 plants, showing larger total biomass accumulation (about 44.1% for N30 and 34.0% for N20) and aboveground biomass (46 and 35.6%) relative to N10 and N0 plants. The LMR in the three additional N treatments was 58.5 (N30), 31.6 (N20), and 37.4% (N10) greater than that in N0, respectively. In contrast, SMR was negatively correlated with N application rate, the SMR in the three additional N treatments was 21.6 (N30), 13.1 (N20), and 14.4% (N10) lower than that in N0, respectively. The RMR and R/S decreased with increasing N supply (Table 2).

Under LW supply, only SMR showed positive correlation with the N application rate, no significant correlation was found between N application rate with total biomass, aboveground biomass, LMR, RMR, and R/S (Table 2). However, the plants with additional N application treatments exhibited 13.1 and 18.6% increase in RMR and R/S in relation to N0 plants in water stress. Total biomass, aboveground biomass and LMR in N20 and N30 plants decreased due to water deficit. In addition, the interaction between water and nitrogen did not alter the biomass accumulation and partitioning pattern above and below the soil surface, although it significantly affected the SMR and LMR (Table 2).

The number of root nodules was not significantly altered by N and watering treatments. However, the number of root nodules under LW supply was somewhat higher than that under HW conditions; also, the number of root nodules in the N0 treatment was higher than that in the three additional N treatments (Table 2).

Nitrogen elicited differential responses on SLA with water availability (Table 2). Under HW conditions, the SLA ranged from $309.60 \text{ cm}^2 \text{ g}^{-1}$ (N20) to $332.43 \text{ cm}^2 \text{ g}^{-1}$ (N30), varied only slightly among N treatments. Under LW supply, SLA was negatively correlated with N application rate, N20 and N30 plants exhibited 26.0 and 28.4% decline

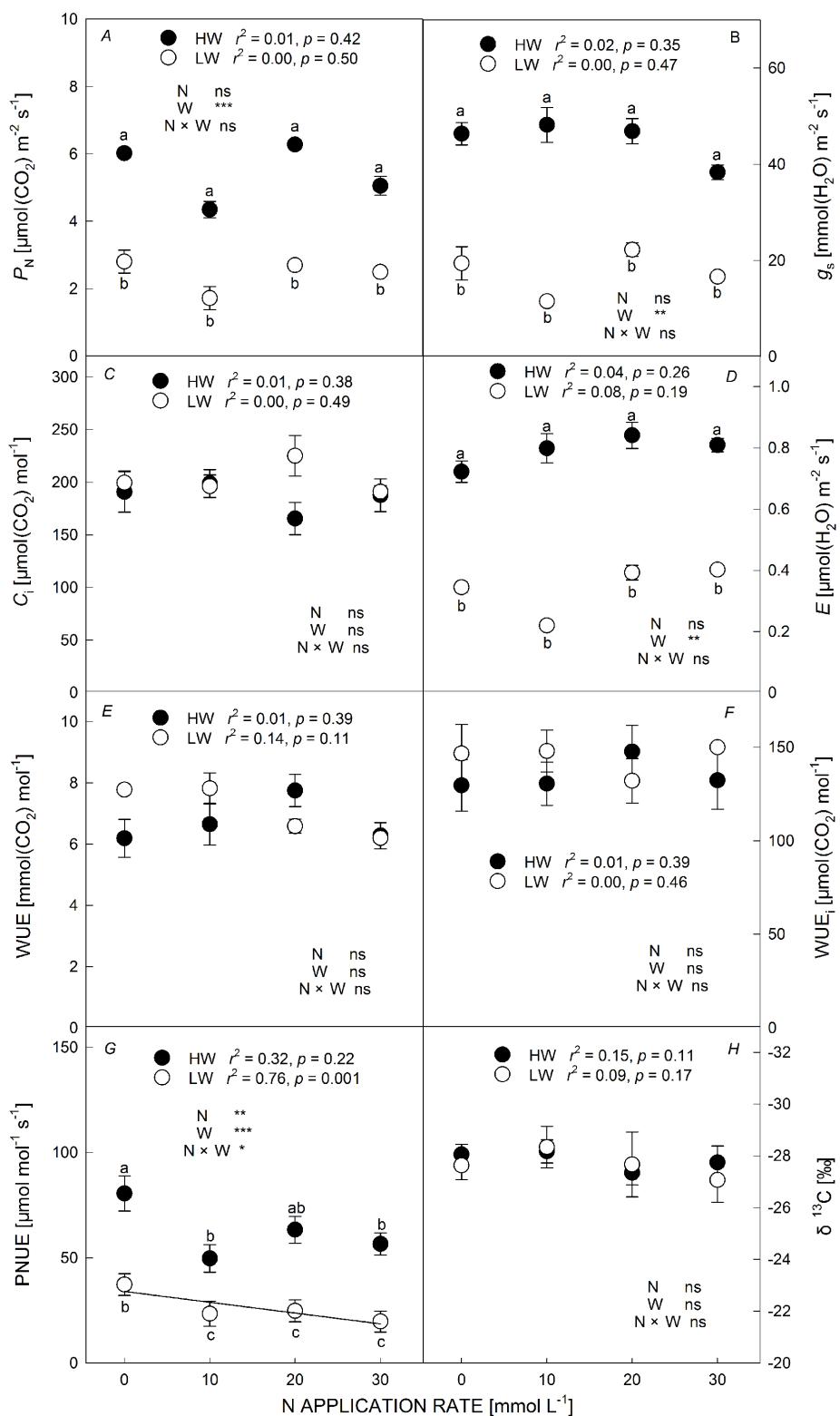


Fig. 3. Leaf photosynthetic parameters and carbon isotopic composition ratio ($\delta^{13}\text{C}$) of *Spatholobus suberectus* plants grown under four nitrogen (N) application rates and two watering regimes. P_N – net photosynthetic rate; g_s – stomatal conductance; C_i – CO_2 concentration at the substomatal cavity; E – transpiration rate; WUE – instantaneous water-use efficiency; WUE_i – intrinsic water-use efficiency; PNUE – photosynthetic nitrogen-use efficiency; HW – high water; LW – low water; W – water; N – nitrogen; r^2 – regression coefficient. Values are means \pm SD, $n = 6, 3$ (PNUE, $\delta^{13}\text{C}$). Different letters in each point represent significant difference ($p < 0.05$). Analysis of two-way ANOVA significance level was shown (* – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; ns – not significant).

Table 1. Leaf anatomical traits of *Spatholobus suberectus* plants grown under four nitrogen (N) application rates and two watering regimes. P/S – palisade tissue thickness to spongy tissue thickness ratio. Values are means \pm SD, $n = 5$. Different letters in each column represent significant difference ($p < 0.05$). Analysis of two-way ANOVA and Pearson's correlation significance level were shown (* – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; ns – not significant).

Watering regime	N application rate [mmol L ⁻¹]	Leaf thickness [μm]	Palisade tissue [μm]	Spongy tissue [μm]	Upper epidermis [μm]	Lower epidermis [μm]	P/S ratio	Stomatal density [mm^{-2}]
High water	0	110.18 \pm 9.88 ^c	43.56 \pm 1.77 ^c	40.78 \pm 1.80 ^a	13.84 \pm 0.65 ^c	12.00 \pm 0.21 ^c	1.10 \pm 0.28 ^c	175.40 \pm 50.50 ^c
	10	122.81 \pm 12.78 ^a	55.37 \pm 2.86 ^a	37.33 \pm 1.79 ^a	16.12 \pm 0.99 ^b	13.99 \pm 0.49 ^b	1.51 \pm 0.28 ^a	224.37 \pm 40.48 ^a
	20	119.93 \pm 19.82 ^{ab}	49.61 \pm 1.41 ^b	38.17 \pm 2.64 ^a	16.74 \pm 0.80 ^b	15.40 \pm 0.95 ^{ab}	1.32 \pm 0.25 ^b	203.48 \pm 45.66 ^b
	30	126.68 \pm 22.52 ^a	55.44 \pm 2.12 ^a	40.81 \pm 1.90 ^a	15.45 \pm 0.51 ^{bc}	14.99 \pm 0.97 ^{ab}	1.45 \pm 0.46 ^a	190.26 \pm 43.27 ^{bc}
	Low water	0	116.53 \pm 8.77 ^b	47.45 \pm 2.38 ^b	38.13 \pm 2.29 ^a	16.46 \pm 0.65 ^b	14.49 \pm 0.64 ^b	187.21 \pm 39.64 ^{bc}
	10	115.81 \pm 14.60 ^b	46.69 \pm 1.59 ^b	36.92 \pm 1.03 ^a	16.64 \pm 0.10 ^b	15.57 \pm 0.59 ^{ab}	1.30 \pm 0.25 ^b	177.33 \pm 32.48 ^{bc}
20	20	116.67 \pm 10.20 ^b	47.43 \pm 2.17 ^b	34.55 \pm 2.93 ^a	18.68 \pm 0.17 ^a	16.01 \pm 0.56 ^a	1.43 \pm 0.38 ^a	147.96 \pm 25.84 ^d
	30	110.67 \pm 12.60 ^c	40.84 \pm 1.24 ^c	40.60 \pm 2.71 ^a	14.50 \pm 0.92 ^c	14.73 \pm 0.94 ^{ab}	1.03 \pm 0.21 ^c	201.30 \pm 49.73 ^b
	Two-way ANOVA (significance level)							
Nitrogen (N)		ns	**	ns	***	***	***	ns
Water (W)		**	***	ns	*	**	*	**
N \times W		***	***	ns	*	ns	***	***
<i>Pearson's correlation coefficient (r)</i>								
High water		0.31***	0.33***	0.03 ^{ns}	0.14*	0.33***	0.26***	0.05 ^{ns}
Low water		-0.13 ^{ns}	-0.26**	0.06 ^{ns}	-0.07 ^{ns}	0.04 ^{ns}	-0.16*	0.02 ^{ns}

Table 2. Growth traits of *Spatholobus suberectus* plants grown under four nitrogen (N) application rates and two watering regimes. SMR – stem mass ratio; LMR – leaf mass ratio; RMR – root mass ratio; R/S – root to shoot ratio; SLA – specific leaf area. Values are means \pm SD, $n = 5$. *Different letters* in each column represent significant difference ($p < 0.05$); ** – $p < 0.01$; *** – $p < 0.001$; ns – not significant).

Watering regime	N application rate [mmol L ⁻¹]	Total biomass [g]	Aboveground biomass [g]	SMR	LMR	RMR	R/S	Nodule number	SLA [cm ² g ⁻¹]
High water	0	31.02 \pm 1.36 ^a	24.24 \pm 1.25 ^a	0.54 \pm 0.07 ^a	0.23 \pm 0.01 ^b	0.31 \pm 0.03 ^a	12.20 \pm 0.16 ^a	322.56 \pm 67.49 ^a	
	10	29.00 \pm 1.67 ^a	23.48 \pm 1.97 ^a	0.47 \pm 0.06 ^{ab}	0.31 \pm 0.01 ^{ab}	0.21 \pm 0.02 ^a	0.28 \pm 0.02 ^a	8.20 \pm 0.04 ^a	
	20	40.18 \pm 1.84 ^a	31.38 \pm 1.05 ^a	0.48 \pm 0.04 ^{ab}	0.30 \pm 0.04 ^{ab}	0.22 \pm 0.03 ^a	0.29 \pm 0.01 ^a	8.80 \pm 0.25 ^a	
	30	43.18 \pm 2.95 ^a	34.82 \pm 1.44 ^a	0.45 \pm 0.04 ^b	0.36 \pm 0.05 ^a	0.20 \pm 0.03 ^a	0.24 \pm 0.01 ^a	11.67 \pm 0.17 ^a	
	0	30.60 \pm 1.41 ^a	23.72 \pm 1.82 ^a	0.50 \pm 0.03 ^a	0.28 \pm 0.03 ^b	0.23 \pm 0.03 ^a	0.29 \pm 0.02 ^a	23.20 \pm 0.09 ^a	
Low water	0	33.32 \pm 2.83 ^a	25.70 \pm 1.89 ^a	0.46 \pm 0.05 ^{ab}	0.32 \pm 0.02 ^{ab}	0.23 \pm 0.04 ^a	0.29 \pm 0.01 ^a	9.40 \pm 0.15 ^a	
	10	31.42 \pm 1.71 ^a	23.54 \pm 1.67 ^a	0.53 \pm 0.06 ^a	0.21 \pm 0.02 ^b	0.26 \pm 0.02 ^a	0.36 \pm 0.01 ^a	8.80 \pm 0.29 ^a	
	20	33.38 \pm 1.41 ^a	25.32 \pm 1.84 ^a	0.54 \pm 0.05 ^a	0.23 \pm 0.02 ^b	0.23 \pm 0.02 ^a	0.31 \pm 0.02 ^a	14.80 \pm 0.13 ^a	
	30								263.10 \pm 56.04 ^b
Two-way ANOVA (significance level)									
Nitrogen (N)	ns	ns	ns	ns	ns	ns	ns	ns	**
Water (W)	ns	ns	ns	ns	ns	ns	ns	ns	ns
N \times W	ns	ns	*	*	ns	ns	ns	ns	**
Pearson's correlation coefficient (<i>r</i>)									
High water	0.44*	0.43*	-0.52**	0.47*	-0.23 ^{ns}	-0.26 ^{ns}	-0.00 ^{ns}	0.05 ^{ns}	
Low water	0.09 ^{ns}	0.05 ^{ns}	0.40*	-0.37 ^{ns}	0.10 ^{ns}	0.14 ^{ns}	-0.26 ^{ns}	-0.43**	

in SLA than that in N0 and N10 plants, and dramatically lowered under LW supply compared with their control counterparts (Table 2).

Discussion

After three drought cycles, the SWC decreased below 7.2%, the RWC, ψ_{pd} , and ψ_{md} significantly decreased (Fig. 1). Under LW supply, neither ψ_{pd} nor ψ_{md} was significantly altered by N, and N0 plants had higher RWC than those after the three additional N treatments (N10–N30) (Fig. 1). Thus, N supply did not improve leaf water relation in *S. suberectus*, as reported in other species (Tesha and Kumar 1978, DaMatta *et al.* 2002a), which probably depends on species. Meanwhile, leaf ψ_{pd} ranged from -0.72 MPa (N30) to -0.64 MPa (N0) under LW conditions, without any significant difference (Fig. 1B). These results indicate that the moderate water deficit conditions were established by the cycle of water stress (Eamus and Prior 2001, Isarangkool Na Ayutthaya *et al.* 2011), and a similar degree of internal plant water status was experienced by the plants under LW supply.

Leaf N concentrations of N0 plants were 33.74 mg g⁻¹ (HW) and 36.66 mg g⁻¹ (LW) (Fig. 2B), higher than that of nonleguminous plants grown under conditions with high N supply (DaMatta *et al.* 2002a,b; Bruno *et al.* 2011). This could be related to the root nodules in the *S. suberectus* root region near the soil surface, and indicate that the root nodules of *S. suberectus* can provide well-balanced N nutrition for plant growth without additional N supply. The nodule formation and nitrogen fixation capacity are sensitive to water supply (Doku 1970, Sprent 1971, Kirda *et al.* 1989). Additionally, high N concentration also inhibits the root nodule formation and nitrogenase activity (Daimon *et al.* 1999, Saito *et al.* 2014). In the present study, we found nodulation under HW supply lesser than that under LW supply, maybe due to oxygen deficiency. Additional N supply also reduced the number of root nodules in *S. suberectus* plants (Table 2).

Stomata control CO₂ and H₂O exchange between land plants and the atmosphere. The leaf of *S. suberectus* is hypostomatus, the stomata are only found on the lower epidermis. Plants with high stomatal density have a greater capacity for nitrogen uptake when water is plentiful, while low stomatal density could reduce transpiration, conserve soil moisture, and plants are highly drought-tolerant but show little or no reduction in nitrogen concentrations, especially when water is limited (Hepworth *et al.* 2015). The results of our study showed that stomatal density was greater in the three additional N treatments than that in N0 treatment under HW conditions (Table 1), and correspondingly with higher concentrations of leaf N (Fig. 2B). The differences in the leaf N content should occur due to the effect of fertilization treatment, but the effect of stomatal density should not be ignored. Under LW supply, the change of stomatal density caused by water treatment corresponded to the same trend in leaf N concentration (Table 1, Fig. 2B). Stomatal density in N20 treatment was significantly lower than that of other treatments in LW plants (Table 1). Similar results have

been reported for *Coffea arabica* by Tesha and Kumar (1978). They pointed out that applying appropriate extra nitrogen could maintain the lower stomatal frequency and loose water relatively slowly under conditions of limited water supply.

Leaves are known to be highly plastic in response to variations in nutrient and/or moisture availability, varying greatly in anatomy, morphology, and physiology (Lu *et al.* 2019). In our study, the thicknesses of leaf, palisade tissue, epidermis, and the P/S ratio were all positively correlated with N application rate under HW conditions (Table 1). Our results demonstrated the positive role of nitrogen in anatomical changes of leaf under well-watered conditions (Sorin *et al.* 2016). Generally, more protective tissues and palisade parenchyma are common characteristics found in stress-tolerant plants (Nobel and Walker 1985). Under LW supply, the epidermal thickness of N30 plants was significantly lower than that in other treatments, and decreased compared to HW conditions; in addition, the palisade tissue thickness and P/S ratio were negatively correlated with N application rate (Table 1). This anatomical trade-off (investment in photosynthetic machinery *vs.* protective tissues) in our study indicates that N30 plants are more susceptible to drought stress. Apparently, N application *per se* did not demonstrate the positive role in anatomical changes of *S. suberectus* leaf upon drought stress, as reported in *Populus* (Lu *et al.* 2019).

Specific leaf area (SLA, the ratio of leaf area to leaf mass) is a key leaf trait reflecting the trade-off between resource capture and conservation (Long *et al.* 2011), consists of two components: leaf thickness and density (Meziane and Shipley 1999). Usually, plants in resource-rich environments tend to have larger SLA than those in environments with resource stress (Cornelissen *et al.* 2003), particularly for soil moisture (Long *et al.* 2011). In this study, SLA did not differ significantly among the N treatments under HW conditions (Table 2), indicating that *S. suberectus* plants were not N deficient. The reductions in SLA of N20 and N30 treatments have to be attributed to the notable decrement of leaf area (data not shown) by water deficit.

The relationship between photosynthetic capacity and N nutrition is positively correlated (Ripullone *et al.* 2003, Belane and Dakora 2011), but once above critical maxima, N can potentially deactivate the photosynthetic machinery (Cheng and Fuchigami 2000), and even increase stress sensitivity (Zhu *et al.* 2005, He *et al.* 2010). In this study, no significant correlation was found between P_N with N application rate in either watering treatment, and N0 treatment had slightly, but not significantly, higher P_N than additional N treatments, particularly under LW supply (Fig. 3A). This indicates that root nodules of the *S. suberectus* can provide sufficient N nutrient for its photosynthetic N requirements, and additional N supply did not improve the P_N of *S. suberectus* under drought. Meanwhile, N also did not affect g_s in any watering treatment (Fig. 3B). This is in accordance with data from coffee (DaMatta *et al.* 2002a,b). The values of P_N , g_s , and E were significantly diminished by water-stress treatments

in all N application treatments, and the reduction in g , and E were relatively greater than that in P_N , except the N20 treatment (Fig. 3A,B,D). Thus, the reductions in P_N due to drought must be mainly attributed to stomatal constraints; maybe nonstomatal limitations also occurred in N20 treatments. Usually, high N nutrient increases instantaneous water-use efficiency and/or long-term water-use efficiency in wheat, coffee, and *Rauvolfia vomitoria* (Shangguan *et al.* 2000, DaMatta *et al.* 2002a,b; He *et al.* 2010). In the present experiment, WUE, WUE_i, and $\delta^{13}\text{C}$ all did not differ significantly between N treatments under each watering regime (Fig. 3E,F,H). Nitrogen supply did not improve the water-use efficiency of *S. suberectus* seedlings.

Photosynthesis can be simply described as the process of CO_2 diffusion, CO_2 fixation, and conversion into energy (Flexas *et al.* 2004). The leaf C/N ratio means the ability of plants to absorb nutrients and assimilate C, which reflects the nutrient-utilization efficiency of plants to a certain extent (Güsewell *et al.* 2003, McGroddy *et al.* 2004). In this study, the leaf C ranged from 470.67 to 493.33 mg g⁻¹ (Fig. 2A), higher than 492 terrestrial plants in the world (Elser *et al.* 2000), which shows that *S. suberectus* has a good capacity for carbon sequestration. However, leaf C content and C/N ratio were correlated negatively with N application rate, especially under LW conditions (Fig. 2A,C). It means that additional N application did not enhance the carbon assimilation capacity of *S. suberectus* leaves. Excessive N transfer in plants may lead to premature leaf senescence and decrease in photosynthetic capacity (Osaki *et al.* 1995). At the same time, leaf nutrient uptake is affected by soil nutrient supply (Aerts and Chapin 1999). When plants grow under conditions of elevated soil N, they exhibit luxury consumption of N, leading to elevated tissue N concentration (Tripler *et al.* 2002). Leaf N content was positively correlated with soil N application rate in this study (Fig. 2B). However, leaf organic N is not all bound in the photosynthetic apparatus (Chapin *et al.* 1987). It may expend much N in nonphotosynthetic functions, such as defense and tenacity, and leading to a low photosynthetic nitrogen-use efficiency (PNUE) (Field and Mooney 1986). Our study showed that PNUE decreased with increasing N supply (Fig. 3G). Additionally, PNUE was found to be positively correlated with drought tolerance ability in tropical crops (Hoang *et al.* 2019), indicating that N0 treatment had better drought resistance.

Nitrogen fertilizer can counterbalance the stress-induced decreases in the photosynthetic attributes (Ögren 1988, Shangguan *et al.* 2000), enhance development of the root system (He *et al.* 2010), and hence has long been the preferred fertilizer for semiarid and mountainous plants (Deng *et al.* 2006, Waraich *et al.* 2011, Hoang *et al.* 2019). However, in the present study, the effect of N application on overcoming drought on *S. suberectus* was not as significant as that of previous studies on nonleguminous plants (Tesha and Kumar 1978, Ögren 1988, Cechin 1998, He *et al.* 2010). Nitrogen had no significant effects on the biomass accumulation and the pattern of biomass partition (Table 2). Under HW conditions, N30 and N20 plants grew faster than N10 and N0 plants, but no

significant differences were found. Water deficit brought about decreases in total biomass, aboveground biomass, and LMR in N20 and N30 plants. Moreover, we found relative increases in the biomass of the stem and root at the expense of leaf biomass under LW supply (Table 2).

Conclusions: *S. suberectus* is a nodulated legume and has good capacity for carbon sequestration. Leaf N concentrations in *S. suberectus* plants were high enough for its photosynthetic N requirements when no additional N was supplied. Nitrogen application did not improve the leaf water status, photosynthesis, and water-use efficiency of *S. suberectus* plants. High N treatment increased drought sensitivity, aggravating photosynthetic and growth depressions of *S. suberectus*. Therefore, we suggest the cultivation of *S. suberectus* does not require additional N application for the growth, at least during the seedling stage.

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