



Article Differences in Characteristics of Photosynthesis and Nitrogen Utilization in Leaves of the Black Locust (*Robinia pseudoacacia* L.) According to Leaf Position

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Abstract: Robinia pseudoacacia L. has been widely planted worldwide for a variety of purposes, but it is a nonindigenous species currently invading the central part of Japanese river terraces. To understand and control this invasion, we investigated how this species invests nitrogen resources in different functions depending on the leaf location, and how these resources are used in physiological reactions such as photosynthesis. The Tama river terrace was examined in Tokyo, Japan. The leaf nitrogen (N) concentration, chlorophyll (Chl) concentration, Chl a/b ratio, leaf mass per unit area (LMA) and ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) concentration were all significantly lower in shade leaves than in leaves exposed to the sun. Conversely, the net photosynthetic rate in saturated light conditions (Pmax), the net photosynthetic rate under enhanced CO2 concentration and light saturation (A_{max}), the maximum carboxylation rate of RuBisCo (V_{cmax}) and the maximum rate of electron transport driving RUBP regeneration (J_{max}) were all significantly lower in shade leaves than in leaves exposed to the sun. We also found that RuBisCo/N and Chl/N were significantly less in shade leaves, and values of J_{max}/N , V_{cmax}/N less in shade leaves than in sun leaves, but not significantly. Allocation of nitrogen in leaves to photosynthetic proteins, RuBisCo (NR) was broadly less in shade leaves, and NL (light-harvesting complex: LHC, photosystem I and II: PSI and PSII) and $N_{\rm E}$ (electron transport) were also lower. The N remaining was much greater in shade leaves than in sun leaves. We suggest that N remobilization from RuBisCo is more efficient than remobilization from proteins of N_E , and from N_L . This study shows that *R. pseudoacacia* has an enhanced ability to adapt to environmental changes via characteristic changes in N allocation trade-offs and physiological traits in its sun and shade leaves.

Keywords: invasive plant; RuBisCo; N allocation pattern; legume; chlorophyll fluorescence; Fv/Fm

1. Introduction

Robinia pseudoacacia L. (the black locust) is a deciduous legume and is a native tree species in North America. This is one of the most widely planted woody species in the world used as a pioneer during the early stage of succession, because of its adaptability to environmental stress, its high photosynthetic rate and its rapid growth [1,2]. Furthermore, *R. pseudoacacia* is a nitrogen (N) fixing tree, which is able to fix N₂ from the atmosphere with the help of N-fixing bacteria. In forest ecosystems it can therefore increase the soil N concentration and N cycling by producing N-rich litter [3–5]. For these reasons, this species has been frequently planted in Japan in degraded areas, roadsides, seacoasts and riverbanks for greening since 1873, when Japanese encountered it at the 1873 Vienna International Exposition [6–8]. It is also used today as the main source of nectar, providing half of the domestic honey production in Japan.



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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). *R. pseudoacacia* has high growth and distribution potential, and its habitat area has been expanding through natural regeneration in recent years in Japan and elsewhere [6,9,10]. Since *R. pseudoacacia* not only fixes N₂ with large carbon (C) and N soil deposition, but also produces allelochemicals, where *R. pseudoacacia* forests have been introduced there is usually low biodiversity. *R. pseudoacacia* propagates by seeds and root suckers, and it has become a large-sized naturalized invasive plant having large interconnected colonies in untended areas and river terraces, leading to a negative impact on biodiversity in the Japanese ecosystem [11–13]. A reduction in plant species richness in coastal pine forests and river terraces has been observed after invasion by *R. pseudoacacia* [6]. Similar phenomena have been described worldwide [5,14,15]. This species reduces biodiversity in river terrace forest ecosystems, narrows river width and changes the original landscape, leading to difficulties in river management [6,13]. *R. pseudoacacia* is listed as one of the 100 most invasive alien species in Japan [16].

A decrease in biodiversity has been reported in the Tama river basin in the central part of Japan [17,18]. The Tama river is a class A river and forms a 1240 km² watershed along its 138 km length, flowing through suburban and urban areas of Tokyo Metropolis and Kanagawa Prefecture having a population of about 5 million. *R. pseudoacacia* was planted in the headwaters of the upper Tama river before World War II as part of a soil conservation project for land damaged by the Great Kanto Earthquake of 1923 [17,19]. In this area, wood land expansion and afforestation have progressed rapidly since the 1980s due to *R. pseudoacacia* [20,21]. Since the construction of the Ogochi Dam on the Tama River in 1957, the invasion of exotic plants has accelerated in the floodplains downstream, and many pioneering native species have disappeared [22]. This early successional tree spreads rapidly, creates dark shade stands and has interesting biological properties that surpass nearby plants. Expansion of *R. pseudoacacia* 's area of distribution is expected to be even faster under environmental changes such as increasing atmospheric CO₂ concentration [23]. So, the invasive spread of *R. pseudoacacia* is not just a problem in Japan, but more widely.

There is growing awareness of the seriousness of the biodiversity decline and of the dominance of *R. pseudoacacia* in the Tama river basin, but only limited understanding of its ecophysiological traits in natural environments. Solar radiation under natural conditions is a limiting resource for many trees, as photosynthesis and growth depend on light. The spectral quality of photosynthetically active radiation (PAR) and irradiance inevitably change in the natural environment. The energy capture of a whole single tree depends on the photosynthetic reactions in its various leaves and on their inclusion in an effective canopy and the cost of generating and maintaining their photosynthetic capacity under limited nitrogen resources. Multiple studies have reported that differences between sun and shade leaves in leaf nitrogen (N) concentration, chlorophyll (Chl) concentration and a/b ratios, leaf architecture and anatomy such as leaf mass per area (LMA) and photosynthetic rates are all strongly correlated with the vertical light gradient within tree canopies [24–27]. Sun leaves often have higher LMA and N concentration, thicker palisade parenchyma tissues, and more mesophyll cells, which together support higher photosynthetic rates on an area basis than shade leaves. The results of these studies therefore support the optimal resource partitioning hypothesis of maximized canopy photosynthetic production. Additionally, this may be associated with the changes in the internal structure of the leaf (to permit a rapid rate of gas exchange) and less investment of carbon and nitrogen into RuBisCo. Consequently, it is also important that trees partition resources between sun and shade leaves optimally and arrange for these leaves to have differing traits. The effect upon leaf N allocation of differing irradiance regimes within similar canopies has scarcely been studied [28].

The purpose of this study is to investigate the physiological responses of sun and shade leaves in *R. pseudoacacia* in the Tama river basin, depending on irradiance. This study focuses on the physiological traits of sun leaves and shade leaves in *R. pseudoacacia*, looking at LMA, leaf N concentration, photosynthetic parameters relating to RuBisCo, chlorophyll and N concentration, and N allocation patterns in the whole single tree. We hypothesized that sun leaves would have the greater photosynthetic capacity due to higher Chl, RuBisco

and N concentrations, whereas, shade leaves would have the lower net photosynthetic capacity, because of very low Chl, RuBisCo and N concentrations. In addition, sun and shade leaves are expected to change leaf morphological traits and to have different N utilization properties to effectively use limited resources such as light and N effectively.

2. Materials and Methods

2.1. Study Site and Plant Material

The study was conducted on the river terrace forest of Tama river in Fuchu city, Tokyo, Japan ($35^{\circ}39'10''$ N, $139^{\circ}28'20''$ E). The Tama river is one of the Japanese national class A river running through Kanagawa prefecture and Tokyo metropolitan, and the headwaters are on Mt. Kasatori in Yamanashi prefecture. The mean annual temperature and mean annual precipitation at the study site for the last 5 years were 15.7 °C and 1608.5 mm. The mean atmospheric CO₂ concentration in Japan for the previous 5 years was 403.6 ppm [29]. In the upper reaches of the Tama river was afforested with *Pinus thunbergii* and *R. pseudoacacia* ca. 50 years ago, although the ratio of planting density of *P. thunbergii* to *R. pseudoacacia* was not recorded. In recent years, there has been a remarkable expansion of the distribution *R. pseudoacacia* in this study site.

In early September of 2018, six healthy *R. pseudoacacia* trees with no visible damage (e.g., disease, yellowing or defoliation) were randomly selected at the river terrace. The selected six *R. pseudoacacia* trees were similar in tree height (about 4.5 m) and diameter at breast height (5.2 ± 0.5 cm), and estimated to have spread naturally about 10 years ago. We collected sun-exposed leaves and shade leaves from randomly selected three branches of six individual *R. pseudoacacia* trees. For each measurement, three sun leaves and three shaded leaves from the upper and lower leaves of the canopy in each tree, and six trees were selected. Sun leaves and shade leaves in the canopy received over 1500 µmol m⁻² s⁻¹ and up to 200 µmol m⁻² s⁻¹ of the photosynthetic photon flux density (PPFD). Sampling and measurements on the sun and shade leaves of six individual *R. pseudoacacia* trees were made on clear rainless days after measuring the net photosynthetic rate. Leaves grown at each sunlight condition were collected for chemical analysis. We determined the N, Chl and RuBisCo concentration, and made morphological measurements, namely the LMA and observation of transverse sections from the six individual trees in early September.

2.2. Photosynthetic Rate

The photosynthetic light response curves ($P_N/PPFD$ curves) were studied for sunexposed leaves and shade leaves from three branches of six individual *R. pseudoacacia* trees using an open gas exchange system (Li-6400, Li-Cor, Lincoln, NE, USA) combined with an integrated fluorescence chamber head (Li-6400-40, Li-Cor) between 09:00 and 14:00 local time. The $P_N/PPFD$ curves for sun exposed leaves and shade leaves were measured under chamber conditions of 25 °C, a CO₂ concentration of 400 µmol·mol⁻¹, and relative humidity 60–70%. We changed the photosynthetic photon flux density (PPFD) from high to low (1800, 1500, 1000, 700, 350, 200, 100, 50 and 0 µmol m⁻²s⁻¹). $P_N/PPFD$ curves and the net photosynthetic rate at light saturate (P_{max}) were constructed from the resulting data, using the formula [30].

$$P_{\rm N} = \frac{\alpha I + P_{\rm max} - \sqrt{(\alpha I + P_{\rm max})^2 - 4\theta \alpha I P_{\rm max}}}{2\theta} - R_{\rm d}$$
(1)

where P_N is the net photosynthetic rate, P_{max} is the maximum photosynthetic rate at light saturation, α is the initial gradient of the curve, I is the PPFD, θ is the convexity of the light response curve and R_d is the respiration rate at 0 µmol m⁻² s⁻¹ PPFD [31].

The same samples were used to measure net photosynthetic rate/intercellular CO_2 concentration (P_N/C_i) curves, after confirming that no photoinhibition had occurred during the measurements of the $P_N/PPFD$ curve by remeasuring the photosynthetic rate at 1500 µmol m⁻²s⁻¹, which is considered to be optimal for RuBisCo activity [23,31–34].

The PN/Ci curve was measured with a CO₂ mixer for the sequence of concentrations 400, 300, 200, 100, 50, 100, 200, 400, 600, 800, 1200 and 1500 μ mol·mol⁻¹. Measurements were performed under chamber conditions at a relative humidity of 60–70% and leaf temperature 25 °C. The stomatal conductance to water vapor (g_s, mmol m⁻²s⁻¹) was measured after ca. 20 min of exposure to saturating irradiance (1500 μ mol m⁻²s⁻¹) when the stomata had fully opened. From the P_N/C_i curves, we calculated the maximum carboxylation rate of RuBisCo (V_{cmax}), the maximum rate of electron transport (J_{max}), the net photosynthetic rate under enhanced CO₂ concentration and light saturation (A_{max}) and other relevant parameters were using the Farquhar–von Caemmerer–Berry (FvCB) model [35–39]. Additionally, V_{cmax} was calculated to the initial slope of the P_N/C_i curves (C_i < 300 µmol m⁻²s⁻¹) with the least square method using Kaleida Graph software (Ver 4.11, Synergy Software, PA, USA). After measurement of the photosynthetic rate, three disk samples (1 cm²) were punched out of each leaf, avoiding the midrib, for determination of the LMA, nitrogen content and total Chl. The rest of the leaves were immediately stored in a portable liquid nitrogen tank and placed in a deep freeze ($-85 \, ^{\circ}$ C) prior to measurement of the RuBisCo concentration.

2.3. Chlorophyll Fluorescence

After the photosynthetic rate measurements on individual sun-exposed and shade leaves from three branches of six *R. pseudoacacia* trees, chlorophyll fluorescence was measured. The minimum fluorescence (F_o) and maximum fluorescence (F_m) were determined following 30 min of acclimation to darkness. The maximal quantum yield of photosystem II (F_v/F_m) was calculated (where $F_v = F_m - F_o$). Thirty measurements were made for each of sun and shade leaves. Chlorophyll fluorescence was measured with a white leafclip to prevent overheating of leaf tissue upon dark acclimation, together with a chlorophyll fluorometer (OS-30P, OptiSciences, Hudson, NH, USA) [40].

2.4. LMA and Leaf Nitrogen Concentration

After measurement of the photosynthetic rate, the 1 cm² disks were used to determine the leaf mass per unit area (LMA, g(leaf)·m⁻²(leaf)). Leaf N concentration per unit leaf area (N, g(N) m⁻²(leaf)) was determined using a N-C analyzer (NC-900, Shimadzu, Kyoto, Japan) after the samples had been dried at 60 °C for one week. The N concentration was calibrated and checked again a known standard (acetanilide: N = 10.36%, C = 71.09%; Wako Chemical, Osaka, Japan). In most data sets, leaf N and LMA were determined using the same leaf as was used in the photosynthesis measurements.

2.5. Chlorophyll and RuBisCo Concentration in Leaves

Chlorophyll in the sun and shade leaves from three branches of six *R. pseudoacacia* trees was extracted with dimethyl sulfoxide (DMSO) and measured using a spectrophotometer (1800U, Hitachi, Tokyo, Japan). The following formula was used to calculate the concentration of chlorophyll a and b [41,42]:

Chl a
$$(\mu g \cdot m g^{-1}) = (14.85 \text{ A665} - 5.14 \text{ A648}) \times (a/b)$$

Chl b $(\mu g \cdot m g^{-1}) = (25.48 \text{ A665} - 7.36 \text{ A648}) \times (a/b)$ (2)

The RuBisCo content was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from eighteen leaf disks [23,43–45]. The leaves were stored at -85 °C prior to this observation. Leaf disks (4 cm²) were punched out of sun and shade leaves, and were homogenized in 10 mM Na-phosphate buffer (pH 7.5) containing 0.4 M sorbitol, 2 mM magnesium chloride (MgCl₂), 10 mM sodium chloride (NaCl), 5 mM iodoacetic acid (C₂H₃IO₂), 1% (w/v) β -mercaptoethanol (C₂H₆OS) and 2% (w/v) polyvinylpyrrolidone ((C₆H₉NO)_n), together with acid-washed quartz sand. The homogenate was centrifuged at 10,000 × g for 1 min at 4 °C. The resulting supernatant fluid was treated with SDS solution for 1 min at 100 °C. The gel was stained with Coomassie Brilliant Blue R-250 (CBB), and the RuBisCo large subunit was extracted with formamide, facilitating spectrophoto-

metric determination of RuBisCo. A calibration curve was made with bovine albumin (Sigma-Aldrich, St. Louis, MO, USA) [23,46].

2.6. N Allocation Pattern

Nitrogen allocation to N_L (light-harvesting complex (LHC), PSI and PSII), N_E (electron transport proteins except N_L and the carbon cycle proteins except N_R) and N_R (RuBisCo) as components of the total N concentration were calculated using the formula of Kitaoka and Koike [44]:

$$N_{\rm L} = [\rm Chl] \times 37.1 \times 14 \tag{3}$$

where [Chl] is the calculated Chl a + b concentration (mmol m^{-2}) [47],

$$N_{\rm E} = J_{\rm max} / 156 \times 10 - 3 \times 9530 \times 14 \tag{4}$$

Here, N_E is calculated according to the method of Hikosaka and Terashima [48] and Niinemets and Tenhunen [49].

The value of J_{max} is calculated from Farquhar et al. [35] as follows:

$$P_{\rm N} = J \frac{C_{\rm i} - \Gamma^*}{4(C_{\rm i} + 2\Gamma^*)} - R_{\rm d} \tag{5}$$

where P_N is the net photosynthetic rate, J is the potential electron transport rate, C_i is the intercellular concentration of CO₂ (µmol mol⁻¹), Γ^* is the CO₂ compensation point (µmol mol⁻¹), R_d denotes day respiration (µmol mol⁻¹) and $\Gamma^* = 3.69$ (kPa) [50]. Additionally,

$$J = \frac{\alpha Q}{\sqrt{1 + \frac{\alpha^2 Q^2}{J_{max^2}}}}$$
(6)

In this formula α is the quantum utilization light conversion efficiency, Q is the photosynthetic photon flux density of light saturation (µmol mol⁻¹) and $\alpha = 0.18$ [51]. Finally,

$$N_{\rm R} = {\rm RuBisCo} \left(g \, m^{-2} \right) \times 0.204 \tag{7}$$

where N is calculated by the method of Evans and Seemann [47].

The method of calculation of N allocation was described in detail in our previous paper [23].

2.7. Statistical Analysis

Statistical analyses were performed using the R software (version 4.0.3; RStudio Desktop 1.4.1103; http://cran.r-project.org/). The effects of leaf position (sun and shade leaves) on N, Chl and RuBisCo concentration, LMA and other leaf traits were analyzed through the analysis of variance (ANOVA). The ANOVA procedure (proc ANOVA) and lm command were used for R, and test results are displayed as mean \pm SE.

3. Results

3.1. Nitrogen, RuBisCo and Chl Concentration, and Structural Characteristic in Sun and Shade Leaves

Significantly higher N and RuBisCo concentrations were observed in sun leaves than in shade leaves (Figure 1). Sun leaves had 23% greater N concentration than shade leaves (p < 0.001). Sun leaves contained 2.8 times greater RuBisCo concentration than shade leaves (p < 0.001). Sun leaves tended to have higher RuBisCo concentration at the same level of leaf N concentration. Sun leaves tended to have 2856 mg m⁻² additional RuBisCo content in given N content than shade leaves, but the slopes of the regression lines between sun and shade leaves were not different (p = 0.190; Figure 2a). Significant differences were found between sun and shade leaves in Chl a, b, their sum, and the a/b ratio (Table 1; Figure 2b). Sun leaves had significantly higher Chl a, b and higher Chl a/b ratio than shade





Figure 1. (a) N and (b) RuBisCo contents in sun and shade leaves of *Robinia pseudoacacia*. Values represent mean \pm SE. ANOVA was used to test the difference between the leaf position at the 0.05 alpha level.



Figure 2. Relationship between N vs. (**a**) RuBisCo and N vs. (**b**) Chl concentrations in sun and shade leaves of *Robinia pseudoacacia*. Dashed and solid lines stand for (**a**) estimated regression lines and (**b**) means of sun and shade leaves, respectively.

Table 1. Chlorophyll (Chl) content (a, b, a + b), Chl a/b ratio, maximal quantum yield of photosystem II (F_v/F_m), and leaf mass per area (LMA) for sun and shade leaves of *Robinia pseudoacacia*. Values represent mean (SE in parentheses). ANOVA was used to test the difference between the leaf position at the 0.05 alpha level.

Parameter	Leaf Position		Shade/Sun	p value
	Sun Leaf	Shade Leaf	(Ratio)	
Chl a (mg m $^{-2}$)	248.24 (29.34)	163.60 (24.26)	0.66	< 0.001
Chl b (mg m ^{-2})	52.18 (8.45)	44.92 (10.99)	0.86	0.034
Chl a + b (mg m ^{-2})	300.42 (32.62)	208.53 (26.06)	0.69	< 0.001
Chl a/b ratio	4.85 (0.83)	3.88 (1.23)	0.80	0.010
F_v/F_m	0.81 (0.01)	0.78 (0.05)	0.96	0.006
$LMA (g m^{-2})$	146.53 (23.5)	115.31 (29.55)	0.78	< 0.001

3.2. Photosynthetic Responses and Physiological Traits

The photosynthetic light response curve ($P_N/PPFD$ curve) for sun and shade leaves of *R. pseudoacacia* are shown in Figure 3, and parameter estimates are set out in Table 2. Based

on these parameter estimates, the P_{max} value for sun and for shade leaves were respectively 25.84 and 13.49 µmol m⁻²s⁻¹ (48% less, p < 0.001); see Table 2. At saturated PPFD, P_{max} was linearly correlated ($P_{max} = 0.0437g_s$; $R^2 = 0.98$) with maximum stomatal conductance (g_s), as shown in Figure 4. The g_s values of sun leaves were always significantly higher than for shade leaves. The value of R_d for sun leaves was 1.81 µmol m⁻² s⁻¹, and for shade leaves was 0.74 µmol m⁻² s⁻¹ (59% less than sun leaves, p < 0.001). The net photosynthetic rate under enhanced CO₂ concentration and light saturation (A_{max}) for sun and shade leaves was respectively 35.43 and 25.02 µmol m⁻²s⁻¹ (p < 0.001) (Table 2; Figure 5). The V_{cmax} values estimated from the P_N/C_i curve for sun and shade leaves were 15.92 and 10.37 µmol m⁻² s⁻¹, respectively. The J_{max} values were estimated as 148.26 and 103.67 µmol m⁻² s⁻¹ for sun and shade leaves in the estimates of V_{cmax} and J_{max} were statistically significant (p < 0.001 in each case).



Figure 3. Response of the net photosynthetic rate to photosynthetic photo flux density (PPFD) for sun and shade leaves of *Robinia pseudoacacia*. Values represent mean \pm SE.

Table 2. Estimated parameters of the photosynthetic rate curves for sun and shade leaves of *Robinia pseudoacacia*. Values represent mean (SE in parentheses). ANOVA was used to test the difference between the leaf position at the 0.05 alpha level.

Parameter	Leaf Position		Shade/Sun	<i>p</i> value
	Sun Leaves	Shade Leaves	(ratio)	
P_{max} (µmol m ⁻² s ⁻¹)	25.84 (0.85)	13.49 (0.47)	0.52	< 0.001
A_{max} (µmol m ⁻² s ⁻¹)	35.43 (1.80)	25.02 (1.91)	0.71	< 0.001
$R_d \ (\mu mol \ m^{-2} s^{-1})$	1.81 (0.33)	0.74 (0.31)	0.41	< 0.001
J_{max} (µmol m ⁻² s ⁻¹)	148.26 (22.28)	103.67 (16.88)	0.70	< 0.001
V_{cmax} (µmol m ⁻² s ⁻¹)	15.92 (0.99)	10.37 (0.48)	0.65	< 0.001



Figure 4. Relationship between maximum CO₂ assimilation rate (P_{max}) and maximum stomatal conductance (g_s) at saturated photosynthetic photon flux density (PPFD = 1500 µmol m⁻²s⁻¹).



Figure 5. Response of the net photosynthetic rates to intercellular CO_2 concentration for sun and shade leaves of *Robinia pseudoacacia*. Values represent mean \pm SE.

Table 3 shows the RuBisCo/N, Chl/N, J_{max}/N, V_{cmax}/N and J_{max}/V_{cmax} of sun and shade leaves of *R. pseudoacacia*. The RuBisCo/N of *R. pseudoacacia* for sun leaves was 2.45 g m⁻²/g m⁻², and for shade leaves was 1.05 g m⁻²/g m⁻². The RuBisCo/N in shade leaves was significantly less (by 57%) than in sun leaves (p < 0.001). Values of J_{max}/N and V_{cmax}/N were sharply higher in sun leaves than in shade leaves, but the difference was not significant. Conversely, Chl/N was significantly lower (by 22%) in shade leaves than in sun leaves (p = 0.012). The ratio of the capacities of RuBP regeneration to RuBP carboxylation (J_{max}/V_{cmax}) was similar value in sun and shade leaves. A higher maximal quantum yield of photosystem II (F_v/F_m) was also observed in sun leaves than shade leaves (p = 0.006).

Table 3. RuBisCo/N, Chl/N, V_{cmax}/N , J_{max}/N and J_{max}/V_{cmax} of *Robinia pseudoacacia* in sun and shade leaves, and relative changes between these leaf positions. Values represent mean (SE in parentheses). The significance of the leaf position is indicated by the *p* value (*ns* = nonsignificant). ANOVA was used to test the difference between the leaf position at the 0.05 alpha level.

Parameter	Leaf Position		Shade/Sun	<i>p</i> value
	Sun Leaf	Shade Leaf	(Ratio)	
$\frac{1}{\text{RuBisCo/N} (\text{g m}^{-2}/\text{g m}^{-2})}$	2.45 (0.42)	1.05 (0.17)	0.43	< 0.001
$Chl/N (g m^{-2}/g m^{-2})$	0.14 (0.03)	0.11 (0.01)	0.78	0.012
$J_{max}/N (\mu mol m^{-2} s^{-1}/mg m^{-2})$	66.30 (19.84)	55.26 (10.61)	0.83	ns
$V_{cmax}/N (\mu mol m^{-2} s^{-1}/mg m^{-2})$	7.14 (1.79)	5.60 (1.13)	0.71	ns
J_{max}/V_{cmax} (µmol m ⁻² s ⁻¹ /µmol m ⁻² s ⁻¹)	9.39 (1.84)	10.02 (1.74)	1.06	ns

3.3. Nitrogen Allocation Patterns

The total N concentration in sun and shade leaves was 2.3 and 1.9 g m⁻² respectively. The most notable difference in N allocation patterns was observed in the proportion of RuBisCo and remaining N (Figure 6). N allocated to RuBisCo was less in shade leaves than in sun leaves, from 49.9% to 21.5%. Remaining N was greater in shade leaves (66.4%) than in sun leaves (35.8%). Slightly lower proportions of N were allocated for chlorophyll and electron transport (5.4% and 7.0%, respectively) in shade leaves than sun leaves (6.0% and 8.3% for N_L and N_E, respectively).



Figure 6. N allocation patterns in sun and shade leaves (%) of *Robinia pseudoacacia*. The black part of the column denotes N allocated to RuBisCo (N_R); the white portion denotes N allocated to the light-harvesting complex (LHC), PSI and PSII (N_L); the dark-gray part denotes N allocated to electron transport proteins (N_E), and the diagonally hatched box denotes the rest. Values represent mean \pm SE.

4. Discussion

Leaf traits change due to the light gradient from the top to the lower parts of the crown and a variety of traits have evolved for efficient light harvesting and utilization. The leaf structure changes represented by LMA is very responsive to the light gradient set up within the canopy, and is positively correlated with the leaf N concentration of the sun and shade leaves calculated on a leaf area basis. Our results show that the sun leaves of *R. pseudoacacia* are characterized by higher levels of LMA and N concentration (Figure 1, Table 1) than shade leaves. Similar observations have been made in N-fixing trees and other broadleaf tree species, in which the LMA decreases in specimens in shaded light conditions [28,43,52,53]. High LMA is a leaf property that regulates light suppression under full irradiance in the sunlight canopy layer (sun leaves), whereas low LMA assists in maintaining and optimizing the light absorbing surface of leaves grown in the shaded environment of the low canopy layer (shade leaves) [54,55]. As well as LMA, the Chl concentrations (a, b and a+b) and Chl a/b ratio on a leaf area basis were significantly greater in sun leaves than in shade leaves of *R. pseudoacacia* (p < 0.05) (Figure 2, Table 1). Significantly higher values of the Chl a/b ratio were also found in sunlit leaves at the upper canopy level of four broadleaf tree species, relative to leaves in the lower canopy level [56,57].

Additionally, sun leaves had higher rates of physiological activity than shade leaves (Figures 3–5; Table 2). Differences in the photosynthetic rate and the light-saturated photosynthetic rate (P_{max}) between sun and shade leaves per unit leaf area matched those in several other broadleaf tree species [56–60] and coniferous tree species [61,62]. In general, the photosynthetic rate in sun leaves is consistent with a greater amount of N per unit leaf area in sun leaves than in shade leaves, and these contain a high level of Chl per leaf area (Figures 2 and 3). Leaf Chl concentration is also a key factor in determining the photosynthetic rate [31,63]. On a leaf area basis, sun leaves have significantly higher Chl concentration than shade leaves. It is suggested that because the leaf structure of the sun and shade leaves is different. The reductions we observed in the Chl a/b ratio in shade leaves relative to sun leaves are due mainly to significant decreases in Chl a (34%) rather than Chl b (14%), most likely caused by changes in the organization of light harvesting and electron transport components; see Table 2 [64]. Additionally, the Chl concentration was less in shade leaves, suggesting that light intensities greater than that of shaded conditions may seriously impair or totally inactivate the photosynthetic system [65]. A further point is that R_d is significantly less in shade leaves than in sun leaves. Modification of R_d has been widely proposed as part of acclimation to light availability [66]. Leaves grown under shaded conditions are known to optimize their effectiveness of light absorption by increasing their pigment density per unit leaf area, and suffer lower carbon losses via R_d than sun leaves [66,67]. It has also been suggested that the associated advantages of high photosynthetic capacity (high metabolic potential) cannot be realized in shaded conditions [68,69]. It is further possible that the higher N level per unit leaf area in sun leaves results in a higher

RuBisCo and Chl concentration than in shade leaves, and subsequently in the stimulation of the CO₂ assimilation rate with higher stomatal conductance at high irradiance (Figures 1–3), consistent with previous findings [27,57,70,71]. Moreover, sun leaves have a much higher maximum stomatal conductance (g_s) than shade leaves (Figure 4). This may indicate that they can open their stomata much more than shade leaves. Leaf temperature is determined by ambient temperature and light intensity, but also under control of leaf physical traits and transpiration (e.g., stomatal conductance). When leaves are exposed to sunlight, they can avoid high light damage by reducing the amount of radiation they receive via heat convection and transpiration. As a result, sun leaves had higher g_s and P_{max} than shade leaves. This is of course an essential prerequisite for higher photosynthetic rates.

Analysis of leaf physiological parameters such as Jmax, Vcmax, etc., for sun and shade leaves is crucial to understanding the physiological properties of R. pseudoacacia. Values of V_{cmax}, J_{max} and photosynthetic activity are strongly correlated with RuBisCo concentration, leaf structure (LMA), cytochrome and particularly leaf N concentration [23,30,72–74]. RuBisCo is the rate limiting enzyme in photosynthetic carbon assimilation and in photorespiratory oxygen reduction [23]. In this study, J_{max} was 30% less in shade leaves than in sun leaves, and V_{cmax} was 35% less. Moreover, A_{max} was 29% less in shade leaves than in sun leaves. It is showed that the decrease in the photosynthetic rate, inclusive of V_{cmax} and J_{max}, Chl and RuBisCo concentration in shade leaves, is due to a decrease in leaf N concentration with stomatal conductance (g_s) (Figures 4 and 5; Table 2). However, shade leaves have a low photosynthetic rate, but given the low g_s, shade leaves are expected to be more undisturbed to water stress than sun leaves. These characteristics of sun and shade leaves of R. pseudoacacia are considered to be an important factor in adapting to the changing environment. Additionally, it is indicated that the increase in J_{max}/V_{cmax} reduces the stimulation of photosynthesis by CO_2 , and that the photosynthetic rate is limited by RuBP carboxylation rather than RuBP regeneration in R. pseudoacacia in its shade leaves. Furthermore, RuBisCo/N in shade leaves was 57% less than in sun leaves (Table 3). Additionally, V_{cmax}/N and J_{max}/N were respectively reduced in shade leaves by 29% and 17% relative to their values, with corresponding RuBisCo content. This observation illustrates that the reason for the reduction in RuBisCo content is that leaf N is not allocated in sufficient quantities to RuBisCo in shade leaves (Table 3; Figure 2).

As a result of the acclimation response to low irradiance, shade leaves of R. pseudoacacia with changed N allocation pattern considerably reduce P_N on a leaf area basis compared with sun leaves. Changes in the N allocation patterns within the photosynthetic apparatus are an important mode of acclimation to changes in light, temperature and atmospheric CO_2 ([23,75,76]. The N allocation to RuBisCo (N_R) in shade leaves decreased dramatically from 49.9% to 21.5% compared to sun leaves; this is not true for N allocation to other photosynthetic proteins such as N_E and N_L (Figure 5). Shade leaves often have a lower LMA and N on an area basis, but a greater amount of N is allocated to Chl to enhance light capture and compensate for the lower irradiance in maintaining photosynthetic capability; N allocated to Chl (N_L) decreased slightly, however [43,77,78]. The explanation is that R. pseudoacacia leaves grown in low irradiance conditions allocate N less rapidly to RuBisCo $(N_R; 49.9-21.5\%)$ than to other photosynthetic proteins, such as N_E (8.3–7.0%) and N_L (6.0–5.1%). The N remaining in shade leaves was 66.4%, far greater than the value of 35.8% in sun leaves. The N allocation pattern within leaves suggests that RuBisCo is negatively correlated with low irradiance under natural environmental conditions, due to a trade-off between CO_2 fixation and remaining proteins [47,48].

These results indicate that less N is allocated to RuBisCo in shade leaves. The shade leaves did not produce RuBisCo in low irradiance conditions and are left as remaining N. As a result, the RuBisCo concentration in shade leaves was significantly lower than sun leaves. RuBisCo is a soluble protein that is easily degraded, but the reaction center and LHC protein are thylakoid membrane proteins [45,75]. Remobilizing the N from RuBisCo may therefore be more efficient than remobilizing it from light harvesting proteins. These responses indicate that shade leaves of *R. pseudoacacia* have decreased RuBisCo and leaf N

content, resulting in a low carbon gain under low irradiance conditions, since there is not enough light energy to maintain the same photosynthetic carbon assimilation rate as in sun leaves. These observations imply that shade leaves of *R. pseudoacacia* are well prepared for sudden changes in environmental conditions, such as changes in light, temperature or atmospheric CO₂, with N stored in soluble form in soluble proteins. The consequent rapid response of *R. pseudoacacia* to environmental stressors increases its adaptability, so that this is a superior species at regenerating itself after disturbance.

5. Conclusions

We found clear differences in the physiological characteristics and morphological leaf traits of sun and shade leaves of *R. pseudoacacia*. This species has very different patterns of N utilization in sun and shade leaves. Since shade leaves do not receive enough light energy to assimilate photosynthetic carbon, the proportion of N invested in RuBisCo is less. We also believe that *R. pseudoacacia* has increased the proportion of water-soluble proteins in shade leaves, increasing its adaptability to various environmental changes. As well as the N₂-fixing ability of *R. pseudoacacia*, the strategic uses of N in sun and shade leaves play a major role in expanding the area of distribution in changing environments.

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