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How Does Deforestation Affect the Growth of *Cypripedium* (Orchidaceae) Species? A Simulation Experiment in Northeast China

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Abstract: Due to wild habitat destruction, *Cypripedium* is among the most endangered groups in China. Determining how *Cypripedium* respond to environmental changes is curial to their conservation. However, less is known about the effect of deforestation on the growth of *Cypripedium*. In this study, we selected four *Cypripedium* species in Northeast China, and conducted conservation-based transplantation simulating deforestation to explore the impact of increased light intensity on the growth of *Cypripedium*. After three years, the maximum net photosynthetic rate was decreased by 15.9%, 11.5%, 13.6% and 5.3% for *C. calceolus* L., *C. guttatum* Sw., *C. macranthos* Sw. and *C.*×*ventricosum* Sw., respectively, resulting in poor viability, manifesting as shorter and thinner shoots, and smaller leaves. Unexpectedly, no significant traits shifts were found in the roots across four species, which may be related to the long root lifespan and conservation. Our research confirmed that increased light intensity caused by deforestation would lead to an increase in respirate cost and a decrease in photosynthate accumulation, and consequently the recession of plant growth. Except for habitat loss, individual plant reduction caused by deforestation could be responsible for the population decline of *Cypripedium*.

Keywords: *Cypripedium*; transplantation; morphology; anatomy; physiology; trait shift; conservation

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1. Introduction

Deforestation is one of the most relevant causes of biodiversity loss and has direct and dramatic effects on individual plant growth and population development [1,2]. Within angiosperms, orchids are particularly vulnerable to environmental changes worldwide, because of their complicated interactions with other organisms [3–5]. Specifically, *Cypripedium* is an orchid genus that is widely distributed worldwide, and nearly two-thirds of its known species can be found in China [6]. Globally, over-collection and deforestation are the main drivers of the significant decrease in many *Cypripedium* [7]; nearly all these species have been listed on the IUCN Red List [6,8].

Previous studies showed that in the absence of anthropogenic disturbance, wild populations of *C. lentiginosum* P.J.Cribb and S.C.Chen [9], *C. guttatum* Sw. and *C. macranthos* Sw. [10] were stable and expansive, and could achieve the maximum carrying capacity over a long period (12, 200 and 150 ramets/m^2 after 50–60 years, respectively). This suggests that the ex situ conservation of those orchids is strongly dependent on the conservation of their habitat. Many researchers have reported that both the vegetative and reproductive traits of *Cypripedium* respond significantly to habitat changes. For example, the light-saturated photosynthetic rate (A_{max}) of *C. guttatum* was highest in thickets, while the A_{max} was decreased by 9.9% and 14.9% in the open and forest habitats, respectively [11]. Similarly, the A_{max} of *C. tibeticum* King ex Rolfe was highest at the forest edge, and the A_{max} was decreased by 10.2% and 46.7% in the forest gap and understory, respectively [12]. Moreover,

with decreased light intensity, significant increases were found in shoot height, leaf area and floral size in *C. tibeticum* [12]. Additionally, in the ex situ cultivation after transplanting (40–50% of full sunlight), the photo-saturated net photosynthetic rates were increased by 3.0%, 7.7%, and 15.7% in *C. flavum* P.F.Hunt and Summerh, *C. guttatum* and *C. tibeticum*, but decreased by 33.2% and 17.8% for *C. lichiangense* S.C.Chen and P.J.Cribb and *C. yunanense* Franch, respectively [13]. In South Korea, when *C. japonicum* Thunb was transplanted into a restored site with low light intensity, Cho et al. [14] found that the ratio of chlorophyll a:b and the net photosynthesis rate were significantly lower than those of natural ones, leading to poor long-term viability. The authors identified that the difference in dominant tree species, lower light availability and drier soil were the main drivers of the population recession [14].

Additionally, despite their key roles in nutrient absorption and carbohydrate storage in *Cypripedium* [9], the literature on belowground organs is limited in comparison with that on aboveground. This lack of knowledge can be explained by the tight association with fungal communities [15]. Generally, fine roots (less than 1 or 0.5 mm in diameter) in woody species are always more amenable to environmental changes than coarse roots (thicker in diameter) [16]. Meanwhile, *Cypripedium* roots are relatively thick (more than 1 mm in diameter), so it would be interesting to assess root plasticity in response to a changed habitat.

Although great efforts have been made towards the in vitro seed germination of *Cypripedium*, as well as in in and ex situ conservation worldwide, such as for *C. calceolus* L. [17], to date, if and how above- and belowground organs respond to deforestation in a local site is still relatively unclear, and whether the direction and magnitude of these trait shifts are generalized across different species remains to be evaluated. The potential responding patterns would ground more practical guidelines for future conservation actions against the threats to these *Cypripedium* species.

Herein, we compared how four different *Cypripedium* species (*C. calceolus, C. guttatum, C. macranthos*, and *C.* ×*ventricosum* Sw.) react to increased light intensity caused by deforestation. The objectives of this study were: (1) investigating the responses of individuals exposed to full sun by collecting data on leaf and root morphology, anatomy and physiology, and (2) determining the variations in growth and survival strategies in response to altered environmental conditions in *Cypripedium*.

2. Materials and Methods

2.1. Study Site

The study was conducted in a secondary forest in Jixi (44°59′~45°11′ N, 130°49′~131°02′ E) in Heilongjiang, China. The site has a continental temperate monsoon climate with a mean January, July and annual temperature of −19.2, 21.8 and 3.2 °C, respectively. The annual precipitation is 545 mm and the mean growing season is 120 d. The soils are Hap-Boric Luvisols with high organic matter content and well-developed horizons [18]. Secondary forest is the main type in this area, and the dominant woody species include *Quercus mongolica* Fisch. ex Turcz., *Betula dahurica* Pall., *Corylus heterophylla* Fisch. and *Corylus heterophylla* Fisch., while dominant herb species include *Convallaria majalis* L., *Fragaria orientalis* Lozinsk., *Viola acuminata* Ledeb., *Artemisia stolonifera* (Maxim.) Komar., *Paris verticillata* M.-Bieb., *Clematis terniflora* var. *mandshuria* (Rupr.) Ohwi., and *Adenophora tetraphylla* (Thunb.) Fisch.

2.2. Studies Species

The four selected *Cypripedium* species are typical terrestrial orchids in Northeast China, and are widespread in this area. Specifically, *C.* ×*ventricosum* is the natural hybrid of *C. calceolus* and *C. macranthos* [6]. Four species overwinter with rhizomes and dormant buds. Generally, two leaves are attached to the relatively short erect stem in *C. guttatum*, and four to five leaves attach to the high erect stems for the other three species. One flower is common in *C. guttatum* and *C. macranthos*, and one or two in *C. calceolus* and

C. ×*ventricosum*. For each species, the number of ramets within a population varies widely, from one to hundreds. All these *Cypripedium* species are insect-pollinating, and their full bloom is June [6]. Due to the low rates of fruit setting and seed germination, vegetative reproduction occurs by rhizome ramification in most *Cypripedium* populations [6].

According to China's List of Wild Plants under State Priority Conservation, published by the National Forestry and Grassland Administration and the Ministry of Agriculture and Rural Affairs on 7 September 2021, four selected *Cypripedium* species are national grade-2 protected plants. Additionally, four species are also listed in Appendix II in CITES (Convention on International Trade in Endangered Species). On the basis of Version 3.1 of the Red List Categories and Criteria of IUCN, the threatened status of *C. guttatum* and *C. macranthos* is EN, *C. calceolus* is NT, and *C.* ×*ventricosum* is VU [19].

In May 2016, due to the planned design of Shengli Forest Station, land-use changes occurred in some local sites, including road construction and crop cultivation. In order to avoid the immediate extinction of the *Cypripedium* populations, Forestry Station workers transplanted four species (Figure 1) from the understory to a nursery at the forest edge, which was free of trees and shrubs and exposed to full light, 150–200 m away from the natural populations (similar to the work of Zhang et al. [13]). In order to minimize the damage done to the transplanted plants within a chosen population, considerable soil was excavated—3–4 times more than the aboveground area and at a 30 cm depth. Therefore, most roots and buds could survive after transplantation. This study was conducted in 2016–2018, and the transplanted plants were properly managed in the nursery. At the third year after transplantation, the plant traits were measured.

Figure 1. Wild *Cypripedium calceolus* (**a**,**b**), *C. guttatum* (**c**,**d**), *C. macranthos*(**e**,**f**), and *C.* ×*ventricosum* (**g**,**h**) in Jixi.

2.3. Sample Collection

In early June 2018, the third year of transplantation, at least three populations of four species were selected from the full sun and understory sites, respectively. Within each population, at least three intact and well-developed shoots were chosen. For each shoot, firstly, we measured the leaf angle (i.e., the angle between the leaf's upper surface and erect shoot), the shoot height, the total number of shoot metameres, the length per metamere, and

the number of leaves. Then, we sampled total 20–30 fully expanded leaves and 20–30 root segments (5–10 mm in length) with root tips for each species.

Each fresh sample was divided into two subsamples: one was cleaned gently using deionized water and immediately fixed in formalin–aceto–alcohol solution (FAA, 90 mL of 50% ethanol, 5 mL of 100% glacial acetic acid and 5 mL of 37% methanal) for anatomical assessment [20]. The other was immediately put in a cooler with ice and transported to the laboratory within 4 h; the fresh leaf sample was used for stomatal and morphological analysis, and the root fresh sample was frozen for morphological analysis.

2.4. Soil Analysis

Additionally, soil cutting rings were used to measure the soil moisture contents of both sites. The surface fresh soil was also collected to define the physicochemical properties. Fresh soil samples were passed through a 2 mm sieve and divided into two subsamples. One subsample of fresh soil was extracted with 2 M KCL, and soil ammonium (NH_4^+-N) and nitrate (NO_3^--N) concentrations were determined with a flow-injection autoanalyzer (Auto Analyzer 3, SEAL Analytical GmbH, Norderstedt, Germany) [21]. Another subsample was air-dried, and passed through a 0.15 mm sieve, while total soil C and N concentrations were determined using an elemental analyzer (vario MACRO cube, Elementar Analysensysteme GmbH, Langenselbold, Germany) [21].

2.5. Morphological Trait Measurement

Stomatal density was measured by the nail polish impression method, as described in Franks et al. [22]. Transparent nail polish was uniformly applied to the leaf abaxial surface first, allowing it to harden. Then, clear cellophane tape was used to transfer the impression of the stomata to a microscope slide [22]. Stomatal intensity (i.e., the number of stomata per area, No. mm^{-2}) was calculated for each leaf as the mean of 8–10 fields at 100× magnification using a compound microscope (BX-51, Olympus Corporation, Tokyo, Japan). In total, 15–20 leaves were used for the stomatal measurement. Other leaves were used for leaf morphological analysis, which were scanned with an Epson scanner 10000XL (dpi = 400, Seiko Epson Corporation, Nagano-ken, Japan), then dried to constant weight (nearest = 0.0001 g) at 65 °C to find the leaf's dry mass. A total of 10–15 leaves per species were used for scanning and the following weighing. Leaf areas were processed using Image-J (National Institutes of Health, Bethesda, Maryland, MD, USA). Specific leaf area (SLA) was calculated as leaf area divided by leaf dry mass [23].

In total, 8–10 root segments with root tips were selected randomly. After washing with deionized water, all the roots were scanned with the Epson 10000XL 1.0 scanner, and dried to a constant weight (nearest = 0.0001 g) at 65 °C to obtain the root dry mass. Root length and root volume were analyzed with WinRhizo software (2004b, Regent Instruments Corporation, Quebec, QC, Canada). Finally, specific root length (SRL) was calculated as root length divided by root dry mass, and root tissue intensity (RTD) was calculated as root dry mass divided by root volume [24,25].

2.6. Anatomical Traits Measurement

To investigate the anatomical traits, 5–6 leaves and 5–6 root segments were chosen; specifically, a 1 × 0.5 cm rectangle was cut out from the leaf center and a root segment with a contact root tip of 5–10 mm length was also cut. Here, specimens were cross-sectioned by hand [26], and selected under an SZX7 dissecting microscope (Olympus, Tokyo, Japan). Only sections with complete structures and distinct tissues and cells in slides were chosen using the compound microscope (BX-51, Olympus Corporation, Tokyo, Japan) for subsequent analyses. For each species, leaf and root cross-sections were photographed using the Motic 3000 CCD camera (Motic, Xiamen, China). Leaf thickness (LT), mesophyll thickness per layer (MTL), root diameter (RD), cortex thickness (CT) and root stele diameter (RSD) were measured to the nearest 1 µm using Motic Images Advanced 3.2 software.

2.7. Leaf Photosynthesis Measurements

In early June 2018, all diurnal gas exchange rate measurements were made on the second fully expanded leaf counted basipetally from 07:00 to 18:00 on clear days. After equilibration with the local ambient conditions of each site, the photosynthetic rate (P_r), transpiration rate (E), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and atmospheric CO_2 concentration (Ca) were measured with a Li 6400 portable photosynthesis analysis system (Li-COR, Lincoln, NE). Water use efficiency was calculated as the ratio of P to E.

The photosynthetic responses to light were measured on fully expanded leaves under a constant leaf temperature (20 °C) and CO_2 concentration (350 mmol mol^{-1}), and the values of P_r were recorded at the following photosynthetic photon flux densities: 1200, 1000, 800, 600, 400, 200, 150, 100, 80, 60, 40, 20, and 0 μmol m^{-2} s^{-1}. After the initial measurement at 0 mmol m^{-2} s^{-1}, three plants were measured at each site. Data were fitted by a fixed non-rectangular hyperbola introduced by Ye [27] and Ye et al. [28]. Using this function, the maximum net photosynthesis rate (P_{nmax}), light compensation point (I_c) and light saturation point (I_{sat}), and dark respiration rate (R_d) were estimated by Photosyn Assistant software (v 1.1, Dundee Scientific, Dundee, UK).

After photosynthetic activity determination, the leaves were collected and brought back to the laboratory, and the contents of chlorophyll a and chlorophyll b (mg g^{-1}) were determined by using 98% ethanolic extract and reading its absorbance at 470 nm and 649 nm, according to the method described by Lichtthaler [29].

2.8. Data Analysis

The net rate of photosynthesis (Pn) was determined following Ye [27] and Ye et al. [28], $Pn = \alpha ((1 - \beta I)/(1 + \gamma I))I - Rd$, where I is the photosynthetically active radiation, R_d is the dark respiration rate, α is the initial slope of the light response curve of photosynthesis, and α, β and γ are the three coefficients.

For each species, means and SEs were calculated for morphology, anatomy and physiology, respectively. The effects of species and origin on the traits were tested using a two-way factorial analysis of variance (ANOVA). Fisher's least significant difference (LSD) test ($p = 0.05$) was used to identify the intra- and interspecific differences of each trait. A principal component analysis (PCA) was applied to the log-transformed data across the four species to determine major sources of variation across multiple traits and identify whether there were concerted above- and belowground organ trait adjustments to environmental changes. All statistical analyses were performed using SPSS software (2010, V. 19.0; SPSS Inc., Chicago, IL, USA). Data visualizations were made using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA) and ggplot2 package [30].

3. Results

3.1. Biological Characteristics

After transplantation, at the forest edge, besides the altered light intensity, the available nutrients also showed significant changes (Table 1). The biological characteristics of four *Cypripedium* species showed great inter- and intraspecific variations (Figure 1, Tables 2 and 3). Compared with the natural plants, all the transplanted ones were significantly shorter and thinner in their shoots, smaller in the leaf, and steeper in leaf angle ($p < 0.05$, Table 2). The dwarf shoots of transplanted *C. guttatum* and *C. macranthos* were accounted for by the shortened metamere length, the decreased metameres number for *C.* ×*ventricosum*, and the shortened and reduced metameres for *C. calceolus* (Table 2). The ratio of chlorophyll content, a to b, was higher in *C. calceolus* after transplantation ($p < 0.05$), but lower in other species ($p > 0.05$) (Table 2). ANOVA showed that nearly all the biological characteristics were significantly affected by species, origin, and their interaction (Table 3).

Table 1. Soil characteristics in undisturbed (understory) and disturbed (transplanted) sites, respectively.

Source of Variation	NH_4^+ (mg/kg)	NO_3^- (mg/kg)	Total N (g/kg)	Total C (g/kg)	C/N	Water Content (%)
Understory	5.85 ± 0.65 b	11.13 ± 0.86 b	7.14 ± 0.64 a	88.73 ± 6.41 a	12.42 ± 1.24 a	44.48 ± 2.25 a
Transplanted	12.41 ± 0.97 a	27.80 ± 1.86 a	7.53 ± 0.55 a	92.27 ± 7.84 a	12.25 ± 1.18 a	13.38 ± 4.39 b

Note: Mean ± SE. Different lower-case letters represent the significant differences of soil characteristics between understory and full sun sites according to Fisher's LSD.

Table 2. Inter- and intraspecific variations in plant growth parameters in four *Cypripedium* species.

Trait	Origin	Species			
		C. calceolus	*C. guttatum*	*C. macranthos*	*C. ×ventricosum*
Shoot height (cm)	W	45.69 ± 1.61 Aa	33.19 ± 0.43 Ab	46.43 ± 1.37 Aa	46.55 ± 1.45 Aa
	T	20.43 ± 1.04 Bb	27.66 ± 1.15 Ba	31.71 ± 1.09 Ba	30.52 ± 0.97 Ba
Stem diameter (mm)	W	6.10 ± 0.19 Ab	3.34 ± 0.07 Ac	7.69 ± 0.23 Aa	5.53 ± 0.23 Ab
	T	4.60 ± 0.15 Bab	2.80 ± 0.11 Bb	5.86 ± 0.25 Ba	6.42 ± 0.35 Ba
Number of metameres	W	5.00 ± 0.38 Aab	3.00 ± 0.00 Ab	5.71 ± 0.29 Aa	5.63 ± 0.26 Aa
	T	4.11 ± 0.20 Bb	3.00 ± 0.00 Ac	5.57 ± 0.17 Aa	4.38 ± 0.18 Bb
Leaf length (cm)	W	17.16 ± 0.33 Aa	12.56 ± 0.24 Ab	18.61 ± 0.49 Aa	16.60 ± 0.74 Aa
	T	10.49 ± 0.64 Ba	10.10 ± 0.23 Ba	12.51 ± 0.50 Ba	11.12 ± 0.44 Ba
Leaf width (cm)	W	6.65 ± 0.22 Ab	6.49 ± 0.22 Ab	8.17 ± 0.31 Aa	7.56 ± 0.33 Aab
	T	5.28 ± 0.30 Bb	5.21 ± 0.22 Ba	5.32 ± 0.12 Ba	4.92 ± 0.21 Bb
Leaf angle (°)	W	55.80 ± 1.41 Ab	45.90 ± 1.79 Aa	50.95 ± 2.14 Aa	54.78 ± 3.11 Ab
	T	32.67 ± 2.51 Ba	32.80 ± 0.83 Ba	32.10 ± 2.61 Ba	32.20 ± 2.41 Ba
Metamere length (cm)	W	35.67 ± 2.76 Ac	11.02 ± 2.12 Ad	63.59 ± 5.29 Aa	43.50 ± 5.29 Ab
	T	22.23 ± 2.08 Bb	8.76 ± 1.43 Bc	41.2 ± 5.87 Ba	39.80 ± 3.92 Aa
Ratio of chlorophyll concentration of, a to b	W	2.04 ± 0.06 Ba	2.08 ± 0.03 Aa	2.17 ± 0.12 Aa	1.84 ± 0.05 Ab
	T	2.23 ± 0.19 Aa	1.97 ± 0.11 Aa	2.02 ± 0.01 Aa	1.85 ± 0.02 Ab

Note: Mean ± SE. Different capital letters represent significant intraspecific differences in plant parameters between wild and transplanted plants according to Fisher's LSD. Different lower-case letters represent significant interspecific difference among four wild or transplanted populations according to Fisher's LSD. W and T represent the wild and transplanted plants, respectively.

Table 3. ANOVA of the effects of species, origin, and their interaction on plant growth and morphology traits.

Source of Variation	df	*p* Value							
		H	SDI	LL	LW	LA	ML	NM	Cab
Species	3	**<0.001**	**<0.001**	**<0.001**	**0.003**	0.118	0.553	**0.001**	**0.045**
Origin	1	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.014**	0.07	0.849
Species × Origin	3	**<0.001**	**<0.001**	**<0.001**	**0.001**	0.088	**0.017**	**0.001**	0.326

Note: Bold type means $p < 0.05$, and otherwise means $p > 0.05$. H: height; SDI: stem diameter; LL: leaf length; LW: leaf width; LA: leaf angle; ML: metamere length; NM: number of metameres; Cab: ratio of chlorophyll a to b concentration.

3.2. Morphology and Anatomy

The morphological and anatomical traits of leaf and root showed wide differences among different species (Figures 2 and 3). Generally, the leaves were thicker in the four transplanted species (Figure 2a), which is related to the thickening of the mesophyll thickness per layer (Figure 2c), not the mesophyll layer (Figure 2d). Additionally, after transplantation, SLA decreased significantly across the four *Cypripedium* ($p < 0.05$, Figure 2e), while the SDs were higher in *C. calceolus*, *C. guttatum* and *C. macranthos*, but lower in *C. ×ventricosum* ($p < 0.05$, Figure 2f). The results of ANOVA show that many leaf morphological and anatomical traits were significantly affected by species and origin, but not their interaction (Table 4).

Table 4. ANOVA of the effects of species, origin, and their interaction on the key morphological, anatomical and photosynthesis traits.

Source of Variation	df	*p* Value													
		LT	MT	MET	ML	SLA	SDE	RD	CT	RSD	SRL	P_{nmax}	I_{sat}	I_c	R_d
Species	3	**0.002**	**0.021**	0.184	0.654	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.108	**0.008**	0.073
Origin	1	**<0.001**	**<0.001**	**<0.001**	0.707	**<0.001**	**<0.001**	0.86	0.505	0.545	0.271	**0.002**	0.538	**0.001**	**0.009**
Species × Origin	3	0.238	0.152	0.26	0.834	**0.038**	**<0.001**	0.698	0.256	0.176	0.927	0.674	0.806	**0.002**	**0.001**

Note: Bold type means $p < 0.05$, and otherwise means $p > 0.05$. LT: leaf thickness; MT: mesophyll thickness; MET: mesophyll thickness per layer; ML: mesophyll layer; SLA: specific leaf area; RD: root diameter; CT: cortex thickness; RSD: root stele diameter; SRL: specific root length. P_{nmax}: maximum photosynthesis rate; I_{sat}: saturation irradiance; I_c: compensation irradiance; R_d: dark respiration rate.

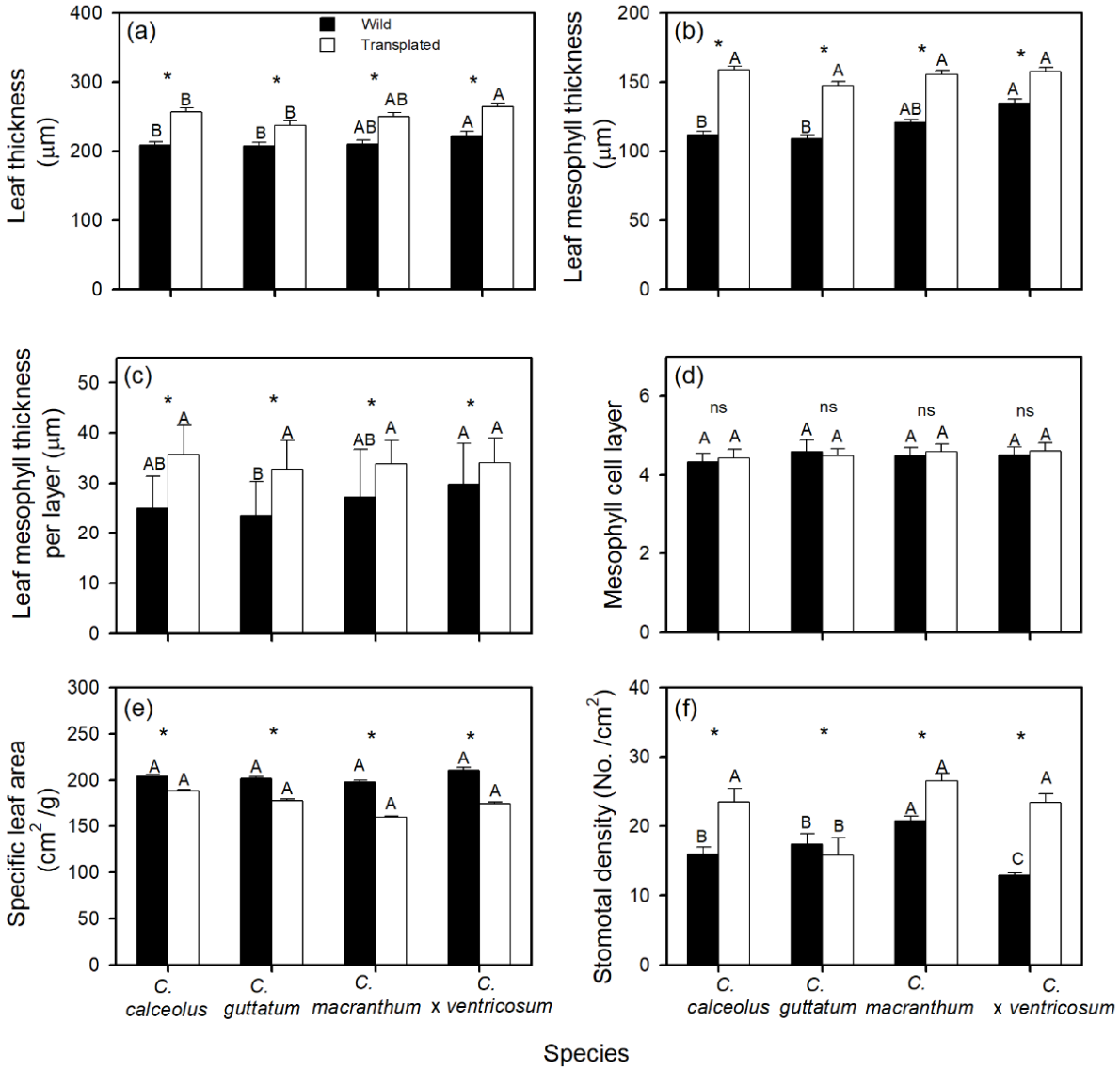

Figure 2. Leaf thickness (**a**), mesophyll thickness (**b**), mesophyll thickness per layer (**c**), and mesophyll cell layer (**d**), specific leaf area (**e**) and stomatal density (**f**) in four wild and transplanted *Cypripedium* species. The error bars represent 1SEM. * and ns indicate the difference between wild and nursery at the significant and insignificant levels, respectively. Different capital letters within same habitat represent a significant difference among different species according to Fisher's LSD.

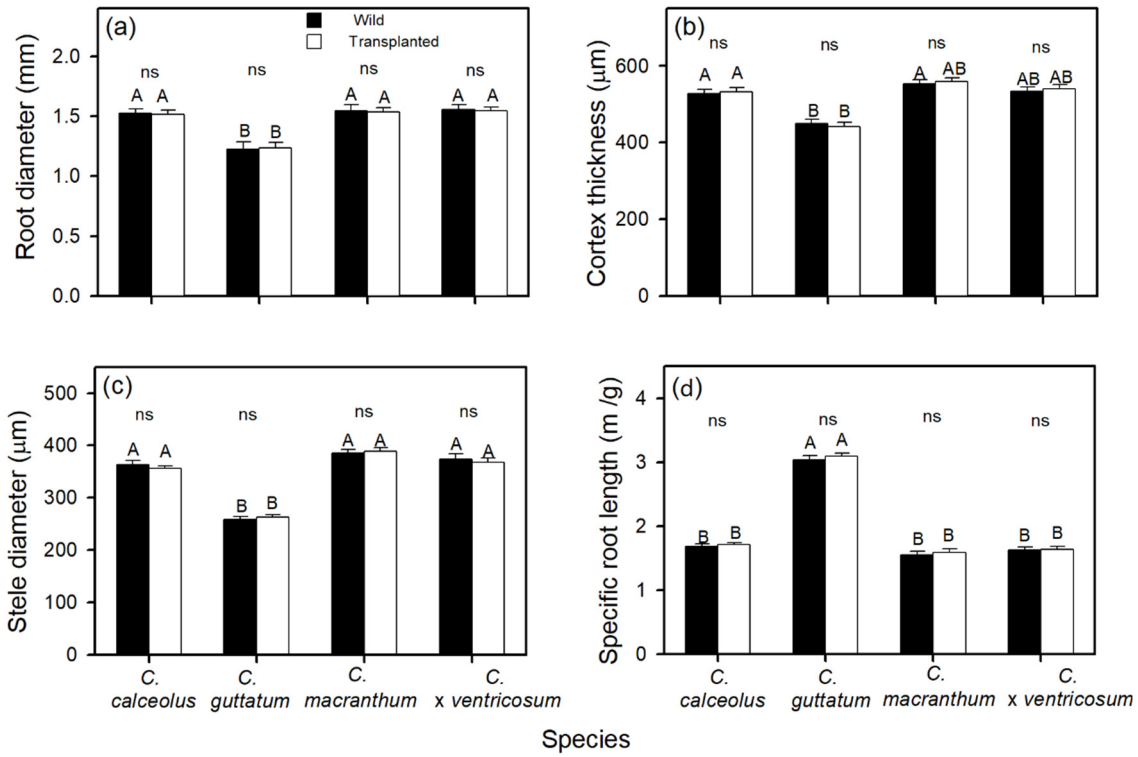

Figure 3. Root diameter (**a**), cortex thickness (**b**), stele diameter (**c**) and specific root length (**d**) in four wild and transplanted *Cypripedium* species. The error bars represent 1SEM. ns indicates the difference between wild and nursery at insignificant levels. Different capital letters within one habitat represent a significant difference among different species according to Fisher's LSD.

Unexpectedly, we did not detect any shift in root traits after transplantation (Figure 3). These root traits are significantly affected by species, but not origin, or the interaction of both (Table 4).

3.3. Photosynthetic Physiology

In both sites, the diurnal curves of photosynthetic rate were relatively stable, with no photosynthetic "noon break" in the four species, which consequently showed as one single peak (Figure 4a–d). The P_r of the transplanted *Cypripedium* was generally higher than that of the understory ones. For the understory plants, the maximum photosynthetic rate could be found at about 12:00, but it occurred at 10:00 for the transplanted ones (Figure 4a–d). Similar to the P_r curve, one peak was found in stomatal conductance (Figure 4i–l) and water use efficiency (Figure 4m–p), while relatively higher values were found for the transplanted ones. Additionally, great fluctuation could be found in the dark transpiration rate (Figure 4e–h) and the ratio of intercellular to atmospheric CO_2 concentration (Figure 4q–t), mainly focusing on the periods of 6:00–8:00 and 12:00–14:00.

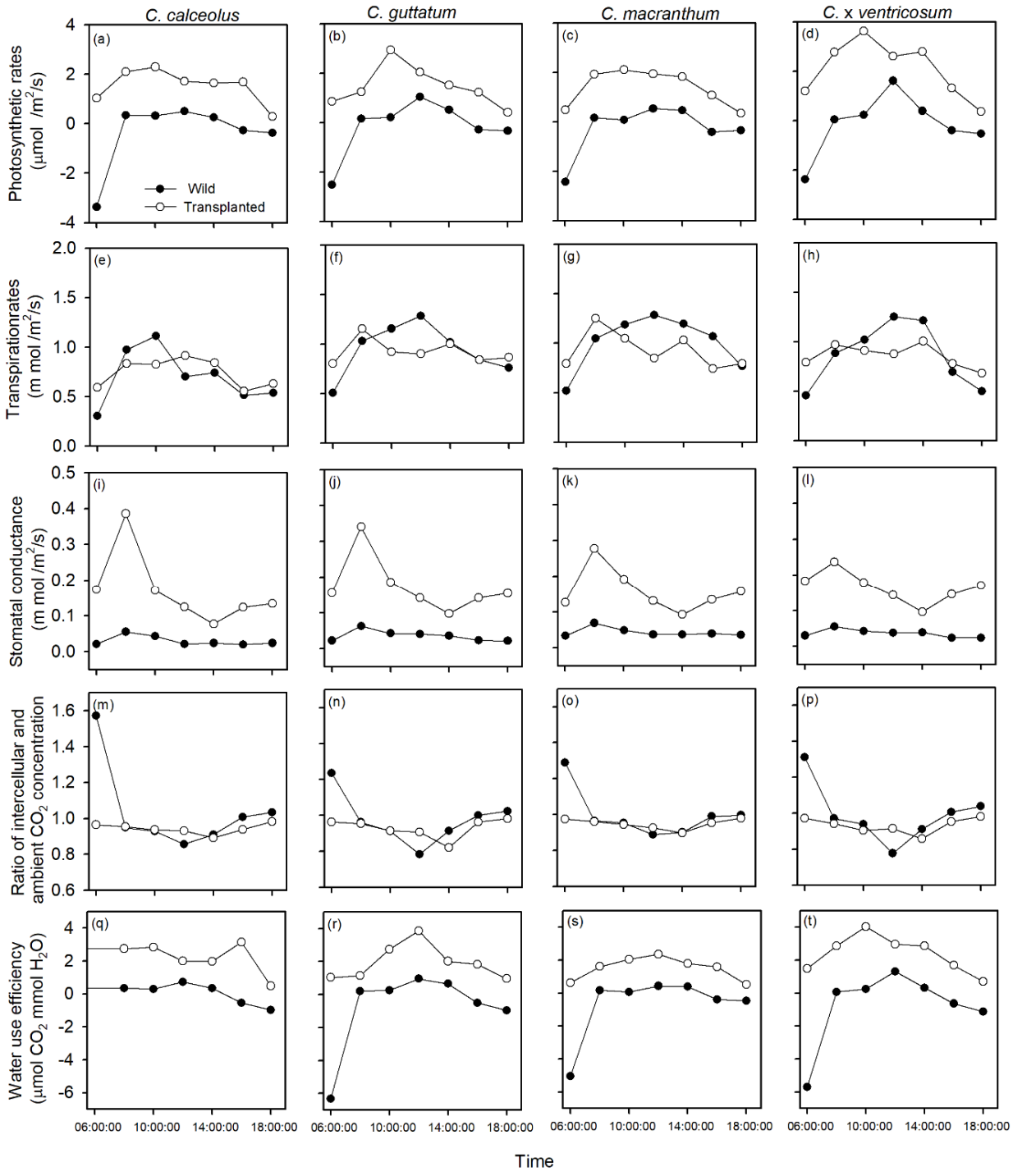

Figure 4. Diurnal changes in the photosynthetic rate (**a**–**d**), transpiration rate (**e**–**h**), stomatal conductivity (**i**–**l**), ratio of intercellular and ambient CO_2 concentration (**m**–**p**) and water use efficiency (**q**–**t**) of four wild and transplanted *Cypripedium* species, respectively.

The light response curves of P_r varied greatly across different species (Figure 5). According to the modified model of rectangular hyperbola, the P_{nmax} values of wild species were higher than those of transplanted ones—significantly so for *C. guttatum* and *C. macranthos* ($p < 0.05$), and insignificantly for *C. calceolus* and *C.* ×*ventricosum*, respectively ($p > 0.05$) (Table 5). Conversely, the I_s, I_c, and D_r of the transplanted plants were generally higher than the wild ones (Table 5).

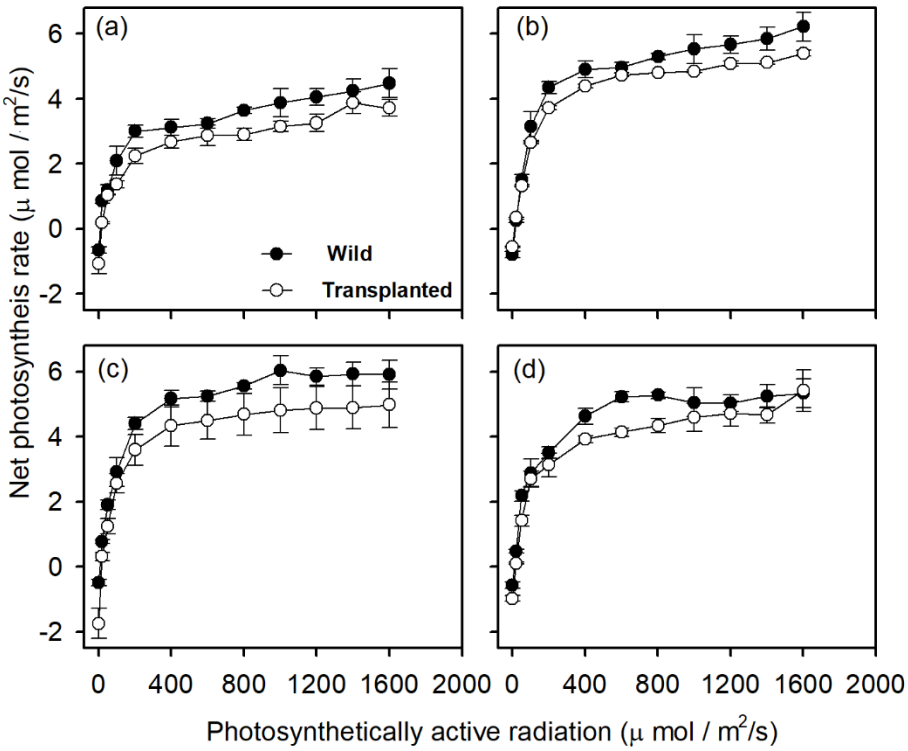

Figure 5. Light response curves of measured photosynthesis rates of wild and transplanted *Cypripedium calceolus* (**a**), *C. guttatum* (**b**), *C. macranthos* (**c**) and *C.* ×*ventricosum* (**d**), respectively.

Table 5. Results of light response curve given by a modified model of the rectangular hyperbola of four *Cypripedium* species.

Trait	Origin	Species			
		C. calceolus	***C. guttatum***	***C. macranthos***	***C.* ×*ventricosum***
P_{nmax} ($\mu mol(CO_2)\cdot m^{-2}\cdot s^{-1}$)	W	4.16 ± 0.10 Ad	6.19 ± 0.11 Ab	6.62 ± 0.48 Aa	5.47 ± 0.11 Ac
	T	3.50 ± 0.35 Ab	5.48 ± 0.11 Ba	5.72 ± 0.66 Ba	5.18 ± 0.54 Aa
I_{sat} ($\mu mol\cdot m^{-2}\cdot s^{-1}$)	W	1160.28 ± 98.73 Aa	1005.14 ± 69.75 Aab	898.35 ± 72.14 Bb	906.72 ± 76.08 Bb
	T	1205.36 ± 79.17 Aa	1129.01 ± 102.49 Aa	1105.76 ± 88.07 Ab	1209.48 ± 94.87 Aa
I_c ($\mu mol\cdot m^{-2}\cdot s^{-1}$)	W	6.84 ± 0.27 Bb	11.38 ± 1.86 Aa	6.95 ± 0.63 Bb	8.21 ± 0.64 Aab
	T	7.76 ± 0.19 Ab	10.98 ± 0.21 Ab	19.36 ± 1.52 Aa	12.92 ± 3.17 Ab
R_d ($\mu mol\cdot m^{-2}\cdot s^{-1}$)	W	0.39 ± 0.10 Bb	0.64 ± 0.02 Ba	0.16 ± 0.07 Bc	0.74 ± 0.05 Aa
	T	0.96 ± 0.18 Aab	1.05 ± 0.05 Ab	1.62 ± 0.32 Aa	0.64 ± 0.24 Ac

Note: Different capital letters represent a significant intraspecific difference in plant parameters between wild and transplanted plants. Different lower-case letters represent a significant interspecific difference between wild and transplantation. P_{nmax}: maximum net photosynthesis rate; I_{sat}: saturation irradiance; I_c: compensation irradiance; R_d: dark respiration rate. W and T represent the wild and transplantation plants, respectively.

3.4. Coordination of above- and Belowground Organs' Trait Shifts

The leaf and root responded to altered environmental conditions independently. The PCA showed that the first two trait axes explained 37.2% and 30.2% of the total variations, respectively (Figure 6), with leaf functional traits occupying the first axis, including SLA, mesophyll thickness and I_s, and root functional traits occupying the second axis, including SRL and root stele diameter. Due to the different leaf functional trait shifts, the *Cypripedium* populations could be divided into two groups, i.e., wild and transplanted. This categorization is relatively unrelated to the root.

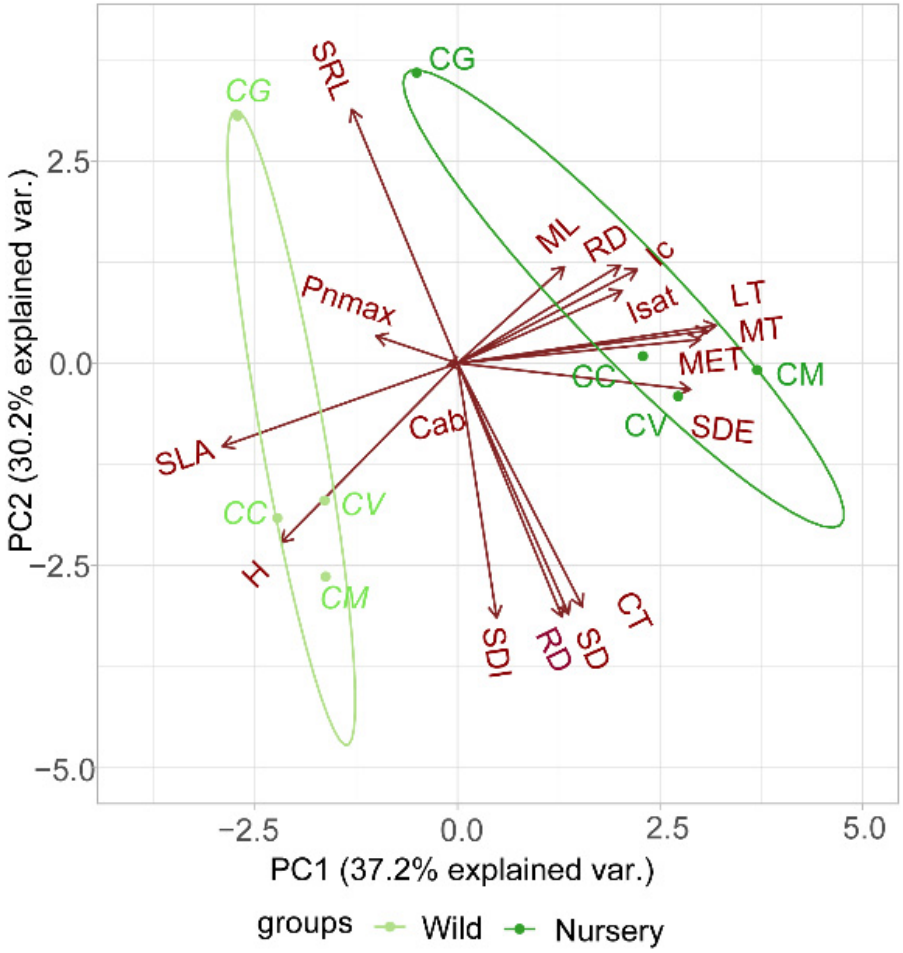

Figure 6. Principal component analysis (PCA) of functional traits of four wild and transplanted *Cypripedium* species. CC, CG, CM and CV represent *C. calceolus*, *C. guttatum*, *C. macranthos* and *C.* ×*ventricosum*, respectively. H: seedling height; SDI: stem diameter; LT: leaf thickness; MT: mesophyll thickness; MET: mesophyll cell thickness per layer; ML: mesophyll cell layer; RD: root diameter; CT: cortex thickness; SD: stele diameter; SLA: specific leaf area; SDE: stomatal density; SRL: specific root length; Cab: ratio of chlorophyll concentration, a to b. Pnmax: maximum photosynthesis rate; Isat: saturation irradiance; Ic: compensation irradiance; Rd: dark respiration rate.

4. Discussion

The morphology, anatomy and physiology of above- and belowground organs in *Cypripedium* reacted differently to sun exposition. It was the leaf, rather than the root, that showed the most profound responses to habitat changes. To the best of our knowledge, this is the first report demonstrating the response patterns of both above- and belowground organs to changes in environmental conditions, which provides a reference for ex situ conservation in the future.

4.1. Leaf Responses to Habitat Changes

Increased light intensity caused by deforestation affected *Cypripedium* shoots profoundly. We found thinner leaves and decreased SLA in the transplanted *Cypripedium* species, similar to the *C. flavum* growing at the forest edge [31], and this pattern was also general for other herbs and woody plants under high light intensity [32–34]. In our study, a steeper leaf angle between the leaf and the erect stem in transplanted *Cypripedium* ramets may result in more efficient light extinction, avoiding sunburn and maintaining the leaf's normal physiological function [35,36]. Zheng et al. [12] also found shorter *C. tibeticum* shoots, and smaller leaves and flowers, at the forest edge with increased light intensity.

Similar shrinking shoots were also found in our study, and the bloom disappeared in *C. calceolus* and *C. guttatum* (Table S1) in the third year, which might be related to the decreased carbohydrate accumulation, as reduced by the declined net photosynthetic rate.

Although the transplanted plants were higher in P_r in comparison to the wild ones, they were lower in P_{nmax}, and both of these were affected by species or origin, or their interaction. These facts might be explained by the following. Firstly, full sun would induce a relatively higher air temperature than in the understory, resulting in a higher plant respiration rate, and consequently more carbohydrates would be consumed. In order to offset the consumption, I_c increased in all transplanted *Cypripedium* plants. Such a pattern has been confirmed in previous studies, e.g., *C. lichiangense*, *C. yunnanense* [11] and *C. tibeticum* [12]. Secondly, increased light intensity might induce a photoinhibition phenomenon under full light [13], causing a decreased net photosynthetic rate. Therefore, higher D_r and lower P_{nmax} led to a decrease in carbohydrate accumulation, and the accompanying recession of individuals and potentially the population.

Consequently, providing optimal light intensity is key for the ex situ conservation of *Cypripedium*. More detailed information about the growth status and response strategies of *Cypripedium* under different light intensities must be collected.

4.2. Root Responses to Habitat Changes

Unexpectedly, contrary to the leaf, the root was almost inflexible to the increased light intensity in four *Cypripedium* species, even though many physical and chemical properties of the soil changed greatly after transplantation. The possible reasons for this are as follows. First of all, keeping existing roots would be preferred for *Cypripedium*, while reducing newborn roots would decrease the cost of carbohydrates. Root and rhizomes, as well as the dormant buds, were the main storage areas for photosynthate, which is crucial for the maintenance and development of the *Cypripedium* population [9]. *Cypripedium* populations have a long lifespan [37–39], which is related to the long lifespan of the rhizome and root [40]. In *Cypripedium*, new shoots mainly originate from dormant buds [6], meaning root conservation is crucial to maintaining population stability. Therefore, a high root turnover, i.e., quick root mortality and propagation, would induce a huge carbohydrate cost [41]. Therefore, compared with constructing new annual shoots, maintaining existing roots was preferential for *Cypripedium*.

Secondly, the conservatism of *Cypripedium* roots might be another reason for their insensitivity to altered environments. The thick cortex of the *Cypripedium* root would serve as a buffer against external changes [42], such as those in nutrient types [43] or soil temperature [44], which was also proven in wood plants with thick roots, conferring a thick cortex [16].

In this study, no coordinated responses to increased light intensity were found between the leaf and root in *Cypripedium* after transplantation. Trait shifts to increased light intensity were organ-specific, similar to in sunflower (*Helianthus annuus* L.), the leaf of which responds to light intensity more profoundly than to soil nutrient availability [23]. It should also be noted that in the long term, increased light irradiance would cause changes in the quantity and quality of soil fungi and microbes, eventually triggering mycorrhizal colonization and root growth in *Cypripedium* [4,45–47]. With vegetation restoration and forest succession, especially shrub and pioneer tree species colonization, canopy closures would decrease light intensity, resulting in fluctuations in the leaves and roots in *Cypripedium* [10]. Therefore, further in-depth research is urgently needed.

5. Conclusions

Our results confirm that in the short term, it was the leaves and shoots, rather than the roots, that most profoundly responded to the increased light intensity induced by deforestation. Without the shading of canopy, transplanted plants suffered from increased light irradiance, while the leaf dark respiration rate and maximum net photosynthetic rate decreased sharply in comparison to natural plants. Besides direct habitat destruction, the

indirect growth limitations of individual *Cypripedium* plants caused by deforestation might be the main reason for the decline in the population. Our study would serve as a guide for the ex situ conservation of *Cypripedium*, emphasizing the significance of specific light availability for the reestablishment and expansion of *Cypripedium*. The preservation of the canopy is also essential to the in situ conservation of the wild *Cypripedium* population.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13020166/s1, Figure S1: Floral morphology of *Cypripedium macranthos*. LIL: Lip length; LIW: Lip width; LIH: Lip height; DSL: Dorsal sepal length; DSW: Doral sepal width; PL: Petal length; PW: Petal width; SL: Synsetal length; SW: Synsetal width; OL: Ovary length; OW: Ovary width; Table S1: Inter- and intraspecific variations of floral functional morphology in four *Cypripedium* species; Table S2: ANOVA of the effect of species, origin and their interaction on flower morphological traits.

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