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ORIGINAL PAPER

Variation of photosynthetic capacity with leaf age in an alpine orchid, *Cypripedium flavum*

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Abstract Photosynthetic rate, chlorophyll fluorescence, leaf nitrogen and chlorophyll content of *Cypripedium flavum* were studied at different leaf ages. The photosynthetic capacity changed significantly with leaf age. Net photosynthesis and chlorophyll content peaked when leaf age was 60 days, decreasing at 30, 90 and 120 days. Stomatal conductance showed the highest value at 60 days, while mesophyll conductance decreased with increasing leaf age. Both leaf nitrogen content per unit area and leaf nitrogen content per unit mass decreased with increasing leaf age. The age-dependent variation in photosynthetic capacity could be linked to the changes in biochemical efficiency, leaf nitrogen content and CO_2 diffusion limitation.

Keywords *Cypripedium flavum* · Leaf age · Leaf nitrogen · Photosynthesis · CO_2 diffusion limitation

List of symbols

C_i	intercellular CO_2 concentration
C_c	chloroplast CO_2 concentration
Chl	chlorophyll content
F_v/F_m	maximum efficiency of PSII photochemistry
g_m	mesophyll conductance
g_s	stomatal conductance
J_{max}	light-saturated rate of electron transport
l_{g_m}	relative mesophyll conductance limitation
LMA	dry mass per unit leaf area
l_s	relative stomatal limitation
N_a	leaf N content per unit area
N_m	leaf N content per unit mass
A	photosynthetic rate
A_{max}	light-saturated photosynthetic rate
PNUE	photosynthetic nitrogen-use efficiency
PPFD	photosynthetic photon flux density
V_{cmax}	maximum RuBP saturated rate of carboxylation

Introduction

The genus *Cypripedium* comprises 49 species, which are distributed in the temperate and alpine zones of the Northern Hemisphere. *Cypripedium* species are long-lived orchids, which have suffered an alarming decline (Nicolė et al. 2005). Most of the species can survive for more than 30 years. Sexual reproduction often appears when a plant reaches 6–13 years (Kull 1999) of age. The slow growth and development of *Cypripedium* would limit their sizes of population and increase the threat of distinction due to the environmental disturbance. However, up till now, the reasons behind the slow growth and development of *Cypripedium* remain unclear.

The slow-growing species would delay the first flowering because of the slow resource accumulation, which can attribute to low photosynthetic capacity and short growing season, since the reproductive process may need considerable resource investment, such as carbon and nitrogen (Obeso 2002). For example, leaf removal and shading result in the decrease in plant size for several *Cypripedium* species (Primack and Stacy 1998; Shefferson et al. 2005). Under good light condition, the plants can obtain high photosynthetic capacity, which is beneficial for the flowering and fruit setting of *Cypripedium flavum* (Zhang et al. 2005). These observations imply that carbon accumulation

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during photosynthesis might limit the growth and reproduction of *Cypripedium*. In order to estimate the long-term carbon budget of leaf and of whole plant, the consideration of the effect of leaf age on photosynthetic capacity is necessary, since daily carbon gain is closely correlated with photosynthetic capacity (Reich et al. 1991; Gay and Thomas 1995). To the best of our knowledge, there is no published report on the variation in photosynthesis of alpine orchid in relation to leaf age.

Considerable amount of work has been done relating to photosynthesis and leaf age (Osmon and Milthorpe 1971; Sobrado 1994; Niinemets et al. 2005). However, the pattern of photosynthesis as a function of leaf age may vary among species (Bertamini and Nedunchezhian 2002; Hanba et al. 2001; Field and Mooney 1983; Schaffer et al. 1991), yet the physiological basis of age-related photosynthetic variation is still poorly understood (Ethier et al. 2006). The variation in photosynthetic capacity with leaf age is correlated with chlorophyll content, leaf N content, Rubisco activity and content, chloroplast ultrastructure, CO_2 diffusion conductance and leaf dry mass per unit area (Reich et al. 1991; Schaffer et al. 1991; Sobrado 1994; Niinemets et al. 2005). Furthermore, the variation of age-related photosynthetic capacity and photosynthetic nitrogen-use efficiency is related to leaf life span. The negative regression slopes of photosynthetic capacity against leaf age for species with short-leaf longevity are steeper than those with long-leaf longevity (Kitajima et al. 1997).

This paper examined the variations in photosynthetic rate, leaf nitrogen content and chlorophyll content of *Cypripedium flavum* relating to leaf age. The aims were to understand the relationships between leaf age and photosynthetic capacity, chlorophyll content, leaf nitrogen content and CO_2 diffusion conductance, and examine the similarity in the pattern of age-dependent photosynthesis between *Cypripedium flavum* and herbaceous plants.

Materials and methods

Study site and plant material

The study was conducted at Zhongdian experimental station of alpine plant in the Hengduan Mountains (E99°38.80′, N27°46.10′, altitude 3,240 m). From May to September in 2005, the mean monthly air temperature and total precipitation were 12.3°C and 430 mm, respectively. Compared with the climatic data during 1958–2000, the average air temperature was similar, while the precipitation was lower. Because the plants were grown in the greenhouse and watered regularly, rainfall had little impact on the physiology. Relative air humidity between the months of May and September during 1958–2000 averages 78.2% (data obtained from Zhongdian Meteorological Station).

Cypripedium flavum P.F. Hunt et Summerh is found in sparse woods or margin of forest at altitude of 1,800–3,700 m in Western China. It occurs on brown soil with abundant humic matter and pH 6.1–6.8. The plant can grow to 35–45 cm high with six to eight leaves produced from a rhizome. The growing period is about 140 days.

Fifty to sixty plants of *Cypripedium flavum* with flowering capacity were collected from the natural habitat (altitude 3,450 m, E99°50.10′, N27°47.76′) in March 2003. The plants were shaded by nylon netting to give 40–50% of full sunlight, and irrigated during the entire growing season. In 2005, the leaves emerged above ground on May 18, and flower appeared on June 14. Fruit setting occurred between July and September, and the plants became dormant again in early October.

Measurement of photosynthesis

Gas exchanges were measured using a LI-Cor 6400 portable photosynthesis system (LI-Cor, NE, USA) with 6400-40 fluorescence chamber on days 30 (June 18), 60 (July 18), 90 (August 17) and 120 (September 16) after leaf emergence, respectively. Photosynthetic and chlorophyll fluorescence measurements were conducted on the fourth leaf from the base of the plant synchronously. Before measurement, the leaf was kept in the dark for more than 10 h. After the minimal fluorescence (F_o) was determined by a weak modulated light. A 0.8-s saturating light of 6,000 μmol $m^{-2}s^{-1}$ was used on the dark-adapted leaf to determine the maximal fluorescence (F_m). Then the leaf was illuminated by an actinic light of 600 μmol $m^{-2}s^{-1}$ for 15 min. The photosynthetic light response curves [A–photosynthetic photon flux density (PPFD)] were made using an automated protocol built into the LI-Cor 6400. The program was configured to advance to next step if the sum of variation coefficients of CO_2, water vapor and flow rate was less than 0.3%, with minimum waiting time of 3 min. Each leaf was equilibrated to initial conditions by waiting at least for 10 min before executing the automated protocol. A-PPFD response curves of three leaves at various leaf ages were measured at 12 light intensities under controlled conditions: CO_2, 350 μmol mol^{-1}; leaf temperature, 20°C and leaf-to-air vapor pressure deficit, 1.0–1.5 kPa.

Photosynthetic CO_2 response curves (A–C_i) and A–PPFD curves were determined using the same leaves at leaf temperature of 20°C, PPFD of 600 μmol $m^{-2}s^{-1}$ and vapor pressure deficit of 1.0–1.5 kPa. After completion of the A–PPFD curve measurement, the leaf was induced at 600 μmol $m^{-2}s^{-1}$ PPFD and 350 μmol mol^{-1} CO_2 concentration for 20 min. A–C_i response measurements were started at ambient CO_2 concentration (350 µmol mol^{-1}), decreasing gradually to 0 µmol mol^{-1}, returned to ambient CO_2 concentration, and then increased to higher concentration. The photosynthetic rates and chlorophyll fluorescence were measured at different CO_2 concentrations using the automated protocol built into the LI-6400.

Calculation of photosynthetic parameters

The A–PPFD curves were fit by a non-rectangular hyperbola according to Prioul and Chartier (1977). Using this function, light-saturated photosynthetic rate (A_{max}) and dark respiration (R_d) were estimated by Photosyn Assistant software (Dundee Scientific, Scotland, UK).

Using A–C_i curves, the maximum carboxylation rate by Rubisco (V_{cmax}) and light-saturated electron transport (J_{max}) were calculated by Photosyn Assistant software that applied the biochemical model described by von Caemmerer and Farquhar (1981). Relative stomatal limitation (l_s) of photosynthesis was calculated from A–C_i curve by the method of Farquhar and Sharkey (1982).

The mesophyll conductance from sub-stomatal cavity to chloroplast was estimated according to variable electron transport rate method of Harley et al. (1992) as

$$g_m = \frac{A}{C_i - \frac{\Gamma*[J_{ETR}+8(A+R_d)]}{J_{ETR}-4(A+R_d)}} \quad (1)$$

where the rate of respiration (R_d) was calculated from A–PPFD curves. Γ* was the hypothetical CO_2 compensation point in the absence of R_d. The value of Γ* at 20°C was derived from the value at 25°C according to the method of Bernacchi et al. (2002). The mesophyll conductance g_m was calculated from the photosynthetic rate at C_i 100–350 µmol mol^{-1}, and the average value of g_m was determined for each leaf (Niinemets et al. 2005). Over this C_i range, the values of g_m were stable (Harley et al. 1992). The rate of photosynthetic electron transport (J_{ETR}) was obtained from chlorophyll fluorescence, and determined as

$$J_{ETR} = 0.5\Phi_{PSII}Q_{abs} \quad (2)$$

where 0.5 was a factor assuming an equal distribution of absorbed photons between PSI and PSII. Φ_{PSII}, the effective quantum yield, was calculated from the maximum fluorescence yield in light-adapted state. The absorbed light energy Q_{abs} was calculated as PPFD × leaf absorbance. Leaf absorbance was taken as 0.85.

The CO_2 concentration at carboxylation site, C_c, was calculated as

$$C_c = C_i - A/g_m \quad (3)$$

The photosynthetic rate under the actual g_m condition, A_{cc}, was estimated as (Ethier and Livingston 2004)

$$A_{cc} = \frac{(C_c - \Gamma*)V_{c\,max}}{C_c + K_c(1 + O/K_o)} - R_d \quad (4)$$

where K_c and K_o were the Michaelis constant for CO_2 and O_2, respectively. The values of K_c and K_o at 20°C were derived according to the method of Bernacchi et al. (2002). O was the O_2 concentration.

From the response of A–C_c, the limitation $\left(l_{g_m}\right)$ imposed on photosynthesis by diffusion from the sub-stomatal cavity to chloroplast was calculated as (Bernacchi et al. 2002)

$$l_{g_m} = \frac{(A_{cc} - A_{ci})}{A_{cc}} \quad (5)$$

Leaf physiological trait

The leaves from the sampled plants previously used in photosynthetic measurements were harvested. Leaf areas were measured using LI-3000A leaf-area meter (LI-Cor, NE, USA). Dry mass was determined after drying for 48 h at 70°C. Then, leaf N content was analyzed using an N analyzer (LecoFP-428, St-Joseph, USA). LMA was calculated as leaf dry mass per unit area (g m^{-2}). Chlorophyll was extracted by Moran and Porath (1980) method and chlorophyll content was calculated according to Inskeep and Bloom (1985).

Statistical analysis

Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). To estimate the differences among different leaf ages, the photosynthetic parameter and leaf trait were tested using one-way ANOVA and LSD multiple comparisons tests. The relationships between photosynthetic parameters and leaf traits were assessed using linear regression analysis.

Results and discussion

The photosynthetic capacity of *Cypripedium flavum* changed with leaf age (Fig. 1, Table 1). Net photosynthesis peaked when the leaf age was 60 days and then decreased at 120 days. The value of A_{max} at 120 days was less than 50% of A_{max} at 60 days. Most woody evergreen plants have relatively slow increase of photosynthetic rate after leaf emergence. Net photosynthesis reaches the maximum when leaves are fully mature, and then declines slowly (Sobrado 1994; Kitajima et al. 2002). For other species, especially herbaceous plants, photosynthetic rate peaks before leaves expand fully, and then declines rapidly

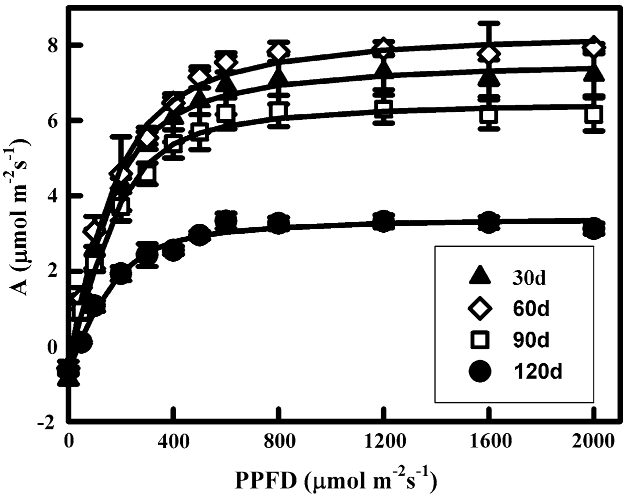

Fig. 1 Photosynthetic responses of *Cypripedium flavum* at various leaf ages to photosynthetic photon flux density (*PPFD*). *Error bars* represent ±1SE ($n = 3$)

(Reich 1984; Gay and Thomas 1995). The variation in photosynthetic rate of *Cypripedium flavum* in response to leaf age was similar to the former, but not to herbaceous plants.

The photosynthetic responses of *Cypripedium flavum* to PPFD were similar among different leaf ages (Fig. 1). There were no significant differences in photosynthetic saturation PPFD among leaf ages ($F = 2.682$, $P = 0.118$), but photosynthetic compensation PPFD of old leaf increased ($F = 9.053$, $P = 0.006$), this indicated that the old leaves had a lower use ability to the light of low intensity than that of young leaves. The photosynthetic CO_2 compensation point increased after leaf senescence (Fig. 2). The photosynthetic CO_2 compensation point of old leaves was significantly higher than that of young leaves ($F = 5.825$, $P = 0.021$).

The leaves at 60 days had the highest stomatal conductance (g_s), and the lowest stomatal conductance at 120 days. However, the mesophyll conductance (g_m) decreased with increasing leaf age (Table 1). Usually, both young leaves and older leaves have lower stomatal conductance (Henson et al. 1990), while g_s in another species decrease with increasing leaf age (Kitajima et al. 2002). However, there is no uniform trend in g_m with leaf age among species (Hanba et al. 2001). In *Alnus japonica*, the g_m increases with leaf age during leaf expansion and declines thereafter (Hanba et al. 2001), while the g_m of Mediterranean evergreen broad-leaved trees decrease with increasing leaf age (Niinemets et al. 2005). Decease in g_m is associated with increase in the fraction of cell wall (Niinemets et al. 2005). Lots of studies showed that the variation in photosynthesis with leaf age is linked to the change of g_s or g_m (Hanba et al. 2001; Hieke et al. 2002; Warren 2006), but g_m limits photosynthesis more strongly than g_s in the older leaves (Niinemets et al. 2005; Warren 2006). The A_{max} of *Cypripedium flavum* was also positively related to the g_s and g_m, but negatively to relative stomatal limitation and relative mesophyll limitation (Fig. 3). The CO_2 drawdown from sub-stomatal cavity to chloroplast increased with the decreasing g_m (Fig. 4). Apparently, CO_2 diffusion limitation had an important role in the variation of age-dependent photosynthesis of *Cypripedium flavum*.

Leaf N content per unit area and per unit mass decreased with increasing leaf age. Both N_a and N_m at 120 days

Table 1 Comparisons of photosynthetic parameters and leaf physiological traits of *Cypripedium flavum* at different leaf ages

	Leaf age				
	30 days	60 days	90 days	120 days	*P*-value
A_{max}	8.32 ± 0.56^{ab}	8.93 ± 0.24^{b}	6.98 ± 0.43^{c}	4.05 ± 0.16^{d}	<0.001
V_{cmax}	24.87 ± 0.92^{ab}	28.87 ± 1.26^{a}	23.90 ± 1.99^{b}	18.20 ± 1.18^{c}	0.004
J_{max}	92.87 ± 1.94^{a}	105.07 ± 2.48^{b}	84.40 ± 4.14^{a}	68.97 ± 4.74^{c}	0.001
g_s	75.27 ± 9.74^{a}	111.05 ± 5.74^{b}	61.70 ± 5.50^{a}	33.75 ± 2.03^{c}	<0.001
g_m	178.7 ± 49.1^{a}	84.5 ± 22.1^{ab}	76.6 ± 19.7^{b}	32.4 ± 11.8^{b}	0.041
l_s	28.65 ± 4.56^{ab}	21.86 ± 1.73^{a}	36.92 ± 5.07^{bc}	46.11 ± 4.69^{c}	0.018
l_{g_m}	7.82 ± 2.15^{a}	6.46 ± 2.25^{a}	15.42 ± 3.45^{ab}	19.49 ± 3.44^{b}	0.037
N_m	19.22 ± 0.67^{a}	19.34 ± 0.71^{a}	17.21 ± 0.99^{a}	11.75 ± 0.06^{b}	<0.001
N_a	1.24 ± 0.06^{a}	1.29 ± 0.14^{a}	1.15 ± 0.05^{a}	0.71 ± 0.02^{b}	0.003
PNUE	6.70 ± 0.19^{ab}	7.02 ± 0.50^{a}	6.04 ± 0.12^{bc}	5.67 ± 0.08^{c}	0.033
F_v/F_m	0.814 ± 0.003^{a}	0.818 ± 0.005^{a}	0.803 ± 0.004^{a}	0.725 ± 0.036^{b}	0.021
LMA	64.83 ± 1.09^{a}	66.70 ± 5.96^{a}	66.71 ± 2.52^{a}	60.82 ± 1.65^{a}	0.592
Chl *a*	2.31 ± 0.13^{a}	2.59 ± 0.10^{a}	1.74 ± 0.08^{b}	1.39 ± 0.10^{c}	<0.001
Chl *b*	0.88 ± 0.14^{a}	0.83 ± 0.03^{a}	0.57 ± 0.03^{b}	0.54 ± 0.03^{b}	0.024
Chl $a + b$	3.19 ± 0.26^{a}	3.42 ± 0.13^{a}	2.31 ± 0.11^{b}	1.93 ± 0.12^{b}	0.001
Chl $a{:}b$	2.72 ± 0.27^{ab}	3.11 ± 0.07^{a}	3.08 ± 0.01^{a}	2.57 ± 0.08^{b}	0.073

Different superscript letters within same row indicate statistically different mean values, $P < 0.05$, as determined by LSD test among species

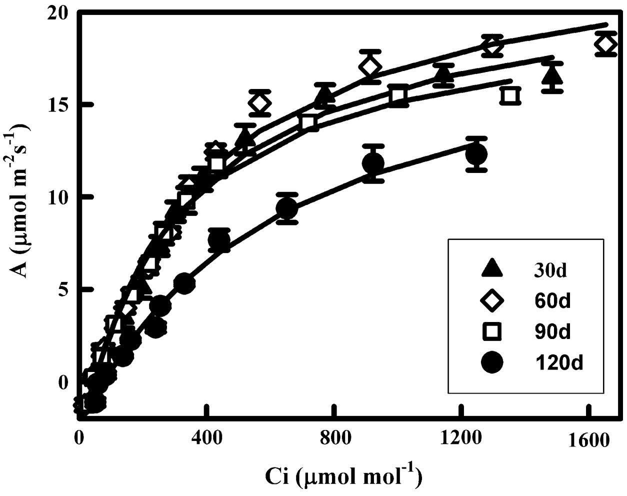

Fig. 2 Photosynthetic CO_2 response curves of *Cypripedium flavum* measured at various leaf ages. *Error bars* represent ±1SE ($n = 3$)

showed 30% reduction compared with those at 60 days (Table 1). Photosynthetic nitrogen-use efficiency (PNUE) was the highest at 60 days, and then declined thereafter. Previous studies suggested that the cause of decrease in photosynthesis with leaf age is partly biochemical, since the distribution and loss of leaf nitrogen is related to leaf age (Gay and Thomas 1995; Anten et al. 1998). The biochemical changes are often accompanied by changes in the maximum rate of carboxylation and light-saturated rate of electron transport (Warren 2006), so there is a strong relationship between leaf N content and photosynthetic capacity (Reich et al. 1991; Sobrado 1994). The decline in photosynthetic capacity after leaf expansion can be caused by the redistribution of resources, especially nitrogen, to younger leaves for optimization of whole plant photosynthetic income (Field and Mooney 1983; Kitajima et al. 2002). However, the redistribution of resources did not occur in *Cypripedium flavum* because all leaves were of the same age in a plant.

As shown in Table 1, both the maximum carboxylation rate by Rubisco (V_{cmax}) and light-saturated electron transport (J_{max}) were the highest at 60 days, the lowest at 120 days. There was a positive relationship between leaf nitrogen content and V_{cmax} or J_{max} (Fig. 5). V_{cmax} was related to the content and activity of Rubisco, while J_{max} was related to the regeneration of Rubisco. The content of Rubisco increases with leaf maturation (Hanba et al. 2001), and declines with leaf senescence (Mae et al. 1993). The change in photosynthetic rate with leaf age is related to the activities of ribulose 1,5-bisphosphate carboxylase (Suzuki et al. 1987; Shirke and Pathre 2004). Apparently, during the first 90 days after leaf emergence, the higher N content in *Cypripedium flavum* resulted in the higher Rubisco

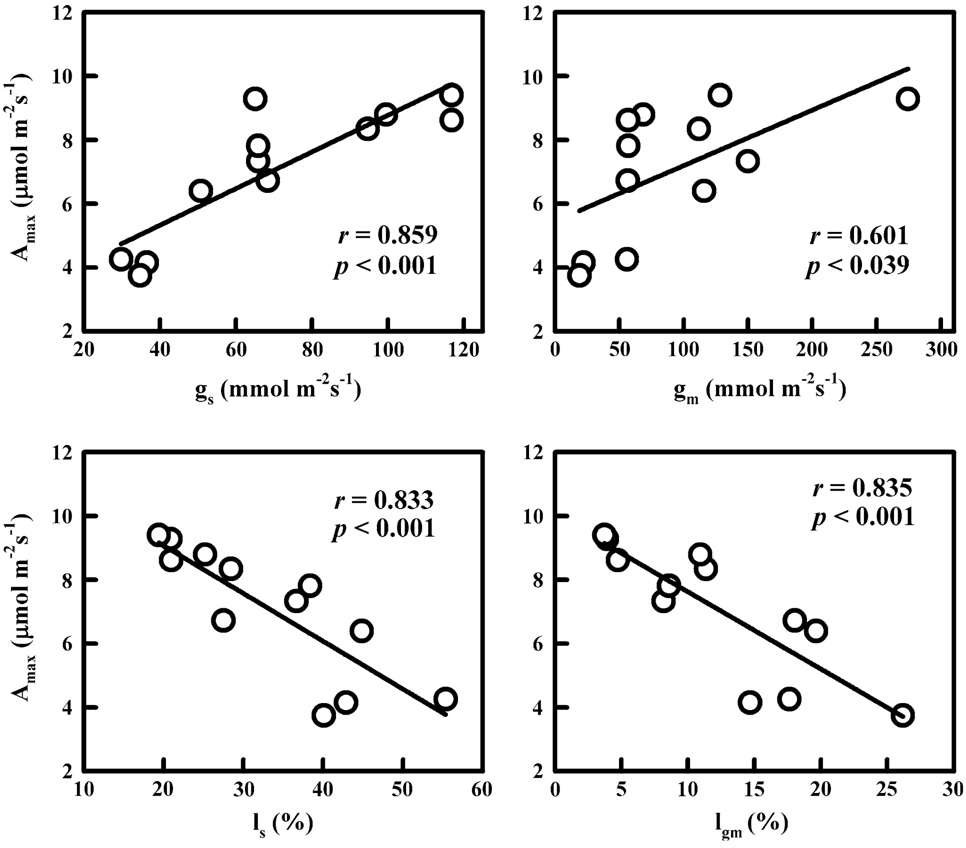

Fig. 3 Effects of stomatal conductance (g_s), mesophyll conductance (g_m), relative stomatal limitation (l_s) and relative mesophyll limitation (l_{g_m}) on photosynthetic rate of *Cypripedium flavum*

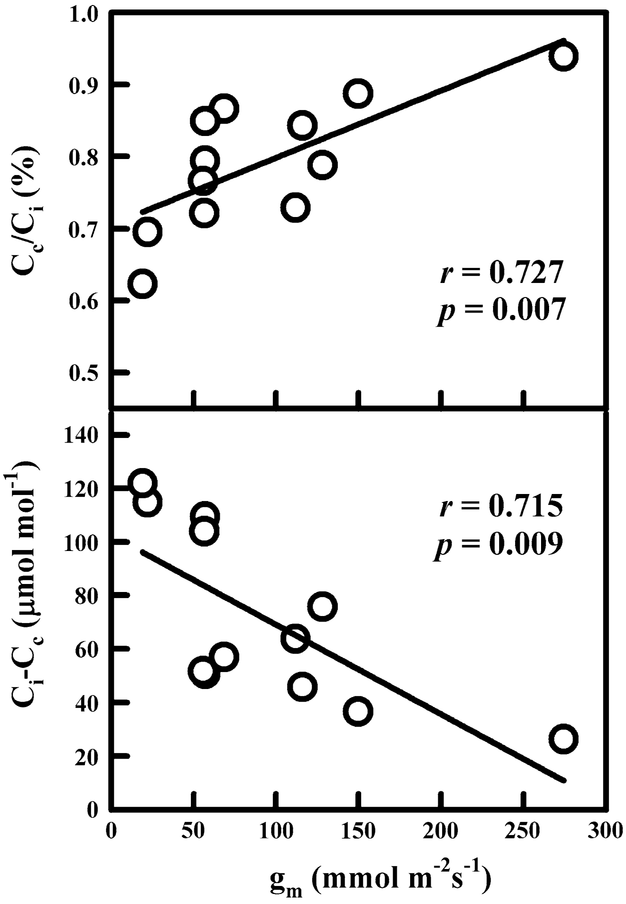

Fig. 4 Effect of mesophyll conductance (g_m) on the ratio of chloroplast CO_2 concentration (C_c) to intercellular CO_2 concentration (C_i) and difference in CO_2 concentration between sub-stomatal cavity and chloroplast

content, and thereby higher V_{cmax}, J_{max} and A_{max} (Fig. 6). The decrease of J_{max} and V_{cmax} in old leaves would be due to the decrease in apparent N investment in photosynthetic machinery. This decrease in apparent N investment in photosynthetic machinery is possibly the result of a larger fraction of nitrogen bound to cell walls (Niinemets et al. 2004). The marked reduction of Rubisco activity in old leaves is also correlated with the loss of small subunit and large subunit in RuBP carboxylase (Bertamini and Nedunchezhian 2002).

The PNUE of *Cypripedium flavum* was different across leaf ages (Table 1). Although some studies do not notice any change in PNUE with leaf age (Field and Mooney 1983), the decreasing PNUE with leaf age would be a general phenomenon (Sobrado 1994; Escudero and Mediavilla 2003; Niinemets et al. 2004). In *Cypripedium flavum*, 30-day-old leaves did not have the highest PNUE; the reason behind this would be that these leaves were not yet fully developed at this time (Reich et al. 1991). The decline in the ratio of RuBP carboxylase to total nitrogen and g_m with leaf age would be the major reason for low PNUE (Kitajima et al. 1997). Another reason for the low PNUE in old leaves is the decreased activation state of Rubisco and proportional down-regulation of electron transport toward the photosynthetic carbon reduction and photorespiration cycles in response to the reduction of CO_2 supply to the chloroplast stroma (Ethier et al. 2006).

There were no significant differences in leaf dry mass per unit area (LMA) among the considered leaf ages (Table 1). Chlorophyll content peaked at 60 days, and decreased rapidly after 90 days (Table 1). The chlorophyll content at 120 days was 56% of that of 60 days. The ratio of Chl *a* to Chl *b* was not significantly different during the 90 days after leaf emergence, but decreased when leaf age was 120 days. In avocado, Chlorophyll content increases until 42 days after bud-break (Schaffer et al. 1991). The chloroplast development of *Castanopsis sieboldii* proceeds more slowly than mesophyll cell expansion and continues well after the time of full leaf expansion, whereas in *Phaseolus vulgaris* these processes proceed synchronously and are completed by the time of full leaf area expansion. The development of chloroplast and Chl content has an impact on photosynthetic rate (Oláh and Masarovičová 1998; Miyazawa and Terashima 2001; Hieke et al. 2002). There was a positive relationship between chlorophyll content and photosynthetic rate in *Cypripedium flavum* (Fig. 7). During the first 30 days after leaf emergence, *Cypripedium flavum* had lower photosynthetic rate; it was likely that these leaves were not yet fully developed at that time (Reich et al. 1991). Jiang et al. (2006) further suggested that photosystem II develops gradually paralleled by an increase of photosynthesis during leaf development. The leaf senescence of *Cypripedium flavum* not only resulted in the decrease of Chl content, but also the reduction of maximal efficiency of PSII photochemistry (F_v/F_m) and photosynthetic capacity (Table 1). Loss of chlorophyll in older leaves might partly explain the decrease of apparent quantum efficiency and electron transport capacity (Gay and Thomas 1995). Another reason for the marked loss of PSII activity in old leaves would be the loss of polypeptides (Bertamini and Nedunchezhian 2002).

In conclusion, the photosynthetic capacity of *Cypripedium flavum* varied significantly with leaf age. Because the environmental changes were relatively small during the growth period, the differences in photosynthetic capacity of *Cypripedium flavum* at different leaf ages would be mainly caused by the changes in biochemical capacities linking to leaf N content, chlorophyll content and CO_2 transfer conductance. The results suggest that the effect of leaf age on photosynthesis should be taken into consideration when annual carbon income and growth of *Cypripedium flavum* are predicted.

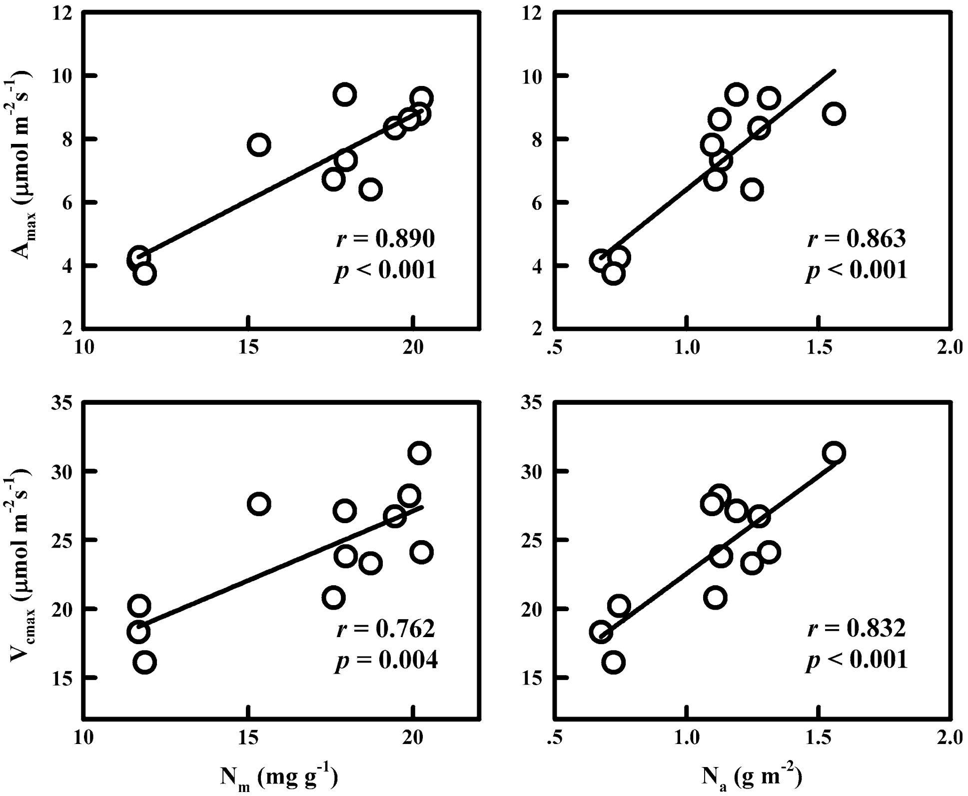

Fig. 5 The relationship between leaf N content and light saturated rate of photosynthesis (A_{max}), and maximum RuBP saturated rate of carboxylation (V_{cmax})

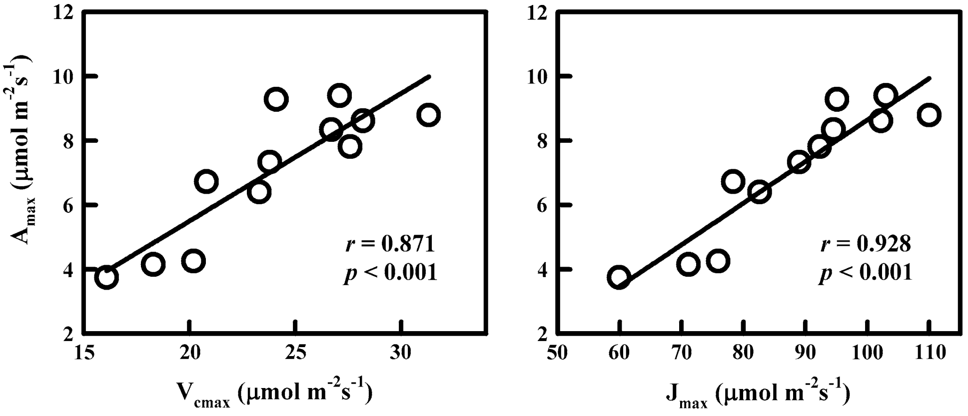

Fig. 6 Effects of maximum RuBP saturated rate of carboxylation (V_{cmax}) and light saturated rate of electron transport (J_{max}) on photosynthetic capacity of *Cypripedium flavum*

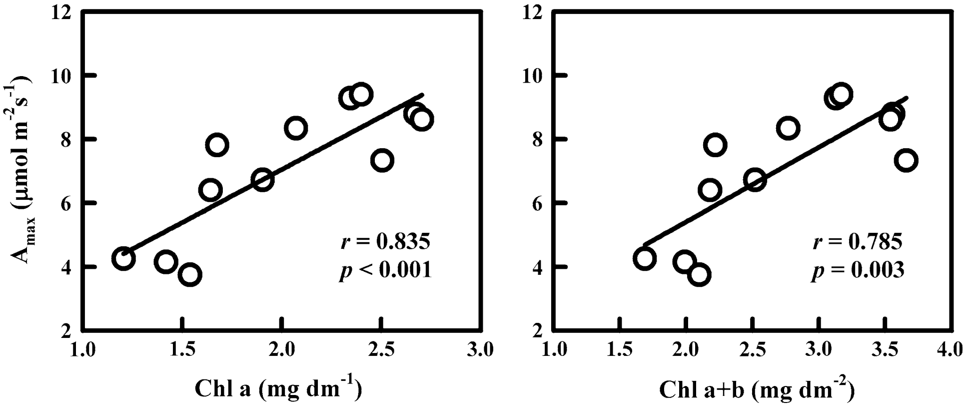

Fig. 7 The effect of chlorophyll *a* content (Chl *a*) and total chlorophyll content (Chl $a + b$) on photosynthetic capacity of *Cypripedium flavum*

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