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Identification of obligate C_3 photosynthesis in *Dendrobium*

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Abstract

Dendrobium is one of the three largest genera in the Orchidaceae and is distributed throughout various habitats. We investigated photosynthesis in seven *Dendrobium* species and cultivars by comparing their leaf $\delta^{13}C$ values, titratable acidity, and CO_2 exchange in well-watered and drought-stressed conditions. In addition, the leaf thickness and mesophyll succulence index (S_m) were measured in well-watered conditions. Our results indicate that *Dendrobium loddigesii* is a typical obligate (or constitutive) CAM plant because the leaf $\delta^{13}C$ values were −14.47 and −14.66‰ in both conditions, respectively. Others showed the leaf thickness of 0.31–0.89 mm and their $\delta^{13}C$ values ranged from −25.68 to −28.37‰. These are not the CAM plants but they could not be classified as obligate C_3 or C_3/CAM intermediate plants. *Dendrobium crepidatum* and *Dendrobium fimbriatum* were further identified as the obligate C_3 plants because the net CO_2 uptake was positive during daytime and negative during nighttime in both conditions. In contrast, *Dendrobium chrysotoxum*, *Dendrobium nobile*, and *D. nobile* 'V1' and 'V4', showed no positive net CO_2 uptake and low ΔH^+ values during nighttime under well-watered conditions, indicating C_3 photosynthesis. However, they showed the positive net CO_2 uptake and large ΔH^+ values during nighttime after drought-stress (21 or 28 days without H_2O), indicating CAM photosynthesis. Therefore, these four species and cultivars were identified as C_3/CAM intermediate (inducible or facultative) plants. In brief, obligate CAM, C_3/CAM intermediate, and obligate C_3 plant types all exist in the section of *Dendrobium*. To the best of our knowledge, this is the first report of the obligate C_3 plants in *Dendrobium*, and these diverse photosynthetic pathways may explain their varied environmental adaptations.

Additional key words: C_3/CAM intermediate plant; CO_2 exchange; crassulacean acid metabolism; titratable acidity; $\delta^{13}C$ value.

Introduction

The diverse photosynthetic pathways observed in plants are usually considered to be evolutionary adaptations to environmental variation (Ehleringer and Monson 1993, Ackerly *et al.* 2000, Caruso *et al.* 2005). C_3, C_4, and crassulacean acid metabolism (CAM) are three types of photosynthesis observed in vascular plants. The C_3 pathway was the first photosynthetic pathway that was biochemically characterized; C_4 and CAM photosynthetic pathways are evolutionarily derived from C_3 photosynthesis (Ehleringer and Monson 1993, Vaasen *et al.* 2006, Silvera 2010). CAM is a plastic photosynthetic adaptation to arid environments (Cushman 2001). In contrast to C_3 and C_4 metabolism, where CO_2 uptake occurs during daytime, CAM is characterized by CO_2 uptake at nighttime with depressed CO_2 uptake and stomatal closure during daytime. Several C_3 plants are able to switch between C_3 and CAM modes, which are known as C_3/CAM intermediate (inducible or facultative) plants. For example, *Mesembryanthemum crystallinum* (the ice plant) is an annual plant, which switches its photosynthetic mode in response to water and/or salt stress (Winter and Holtum 2007). In contrast, most C_3 plants use only C_3

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Abbreviations: CAM – crassulacean acid metabolism; Chl – chlorophyll; DM – dry mass; DS – drought-stressed; FM – fresh mass; ΔH^+ – titratable acidity accumulated during nighttime; LWC – leaf water content; S_m – mesophyll succulence index; WW – well-watered; $\delta^{13}C$ – carbone isotope ratio $^{13}C/^{12}C$.
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photosynthesis for CO_2 uptake throughout their life cycle, *e.g*., rice and wheat, and are classified as obligate C_3 plants. Plants that predominantly employ CAM photosynthesis to absorb CO_2 (Ting 1985), such as *Opuntia basilaris* (Hanscom and Ting 1978), are known as obligate CAM (or constitutive CAM) plants. CAM plants are widely distributed within the plant kingdom, *i.e*., 343 genera in 34 families (Holtum *et al.* 2007), but only few families, such as the Clusiaceae and Orchidaceae, include obligate C_3, C_3/CAM intermediate, and obligate CAM plants within a single family (Vaasen *et al.* 2006, Silvera 2010). Photosynthetic pathways have consistently demonstrated progress from C_3 ancestors to CAM photosynthesis (Ehleringer and Monson 1993, Vaasen *et al.* 2006, Silvera 2010). However, the genetic changes required for this progression (and reversion) remain unclear (Silvera 2010). Thus, it is important to study the multiple independent evolutionary origins of CAM in genera that contains the obligate C_3, C_3/CAM intermediate, and the obligate CAM plants. However, only a few genera, such as *Clusia* in the Clusiaceae and *Cymbidium* in the Orchidaceae, are known to contain the obligate C_3, C_3/CAM intermediate, and obligate CAM plants in a single genus (Vaasen *et al.* 2006, Silvera *et al.* 2010).

Orchids are model plants for studying the evolution of CAM. Orchidaceae is the largest family of flowering plants and CAM photosynthesis is widespread in this family (Winter *et al.* 1983, Silvera *et al*. 2005), *i.e*., approx. 9,000 species (Lüttge 2004, Silvera *et al.* 2010). *Dendrobium* is one of the three largest orchid genera, with approx. 1,500 wild species and several more hybrids (Cribb and Govaerts 2005, Lavarack *et al.* 2000, Schuiteman 2011). Most have high ornamental and/or medicinal value, such as *D. nobile*. Section *Dendrobium* is one of 11 *Dendrobium* sections, comprising 54 species that are mostly distributed in the Himalayas from India through to China as well as in Japan, with 27 species in China (Zhu *et al.* 2009). In the study of Gehrig *et al.* (2001), all *Dendrobium* species, which were investigated, were CAM plants. In *Dendrobium,* 25 wild species and some cultivars (mainly *Phalaenopsis* cane-type cultivars) are known to utilize exclusively the CAM photosynthetic pathway (*i.e*., obligate CAM plants) (Hew and Khoo 1980, Ando 1982, Fu and Hew 1982, Winter *et al.* 1983, He *et al.* 1998, Sayed 2001). However, several species in this genus appear to be very flexible with respect to their photosynthetic pathways; they switch between C_3 and CAM photosynthesis (Winter *et al.* 1983, Su *et al.* 2003, Ren 2008). However, no cases of obligate C_3 have been reported in *Dendrobium* in previous studies.

Several methods have been developed to identify the photosynthetic pathways in plants, such as leaf succulence or thickness and the leaf $\delta^{13}C$ value. In most species, there is a close correlation between greater leaf succulence or thickness and increasing magnitude of CAM (Silvera 2010), because CAM is typically associated with epiphytic or succulent life forms in tropical species. However, it is difficult to identify whether a plant is obligate C_3 based only on the leaf thickness. For example, a few plants with thinner leaves fix CO_2 using CAM, *e.g*., the leaf thickness of *D. bigibbum* is only 0.79 mm, but it yields −11.9‰ of $\delta^{13}C$, which is another characteristics of CAM plants (Winter *et al.* 1983).

C_3 and CAM plants can be distinguished by their leaf $\delta^{13}C$ value measurements ranging from −20 to −35‰ in C_3 and from −10 to −22‰ in CAM (O'Leary 1981, Sternberg *et al.* 1984, Farquhar *et al.* 1989). Twelve *Dendrobium* species (including *D. nobile*) were characterized as C_3 plants based on their leaf $\delta^{13}C$ values, which ranged from −20 to −35‰ (Winter *et al.* 1983). However, this analysis showed that *D. nobile* was the C_3 plant with the $\delta^{13}C$ value of 25.1‰, although the CO_2 exchange pattern could be induced to switch from the C_3 to the CAM mode by drought stress (Winter *et al.* 1983, Su *et al.* 2003, Ren 2008). Thus, *D. nobile* was shown to be the C_3/CAM intermediate plant. The $\delta^{13}C$ measurements are less time consuming and more convenient for large species surveys, but the $\delta^{13}C$ value alone cannot distinguish obligate C_3 and C_3/CAM intermediate species if CAM gives a small contribution to the total carbon gain of the C_3/CAM intermediate pathway (Borland *et al.* 1993, Holtum and Winter 1999, Winter and Holtum 2002).

Careful measurements of diel changes of titratable acidity or dark CO_2 uptake have been recently used to distinguish obligate C_3 and C_3/CAM intermediate species, although these two methods are highly time-consuming (Borland *et al.* 1993, Holtum and Winter 1999, Winter and Holtum 2002). In C_3/CAM intermediate plants, environmental stress (such as drought) can induce or enhance the expression of CAM; therefore, comparing diel changes of titratable acidity or dark CO_2 uptake under optimal conditions and environmental stress are useful for characterizing obligate C_3 and C_3/CAM intermediate plants.

In this study, based on leaf thickness differences, we selected five species from the section of *Dendrobium* (including *D. nobile*), one species from the section of *Chrysotoxae*, and two cultivars of *D. nobile* and identified their photosynthesis patterns by comparing the leaf thickness, mesophyll succulence index (S_m), $\delta^{13}C$ values, titratable acidity, and CO_2 exchange pattern. We observed a new type in *Dendrobium*, which was characterized by obligate C_3 photosynthesis. The discovery that three types of photosynthesis occur in plants within the single section of *Dendrobium* could help to understand the diversity of photosynthetic pathways and the evolutionary and ecological importance of CAM photosynthesis.

Materials and methods

Plant material and treatments: Seven *Dendrobium* species (cultivars) were selected as accessions in this study, *i.e.*, four species from the section of *Dendrobium* (including *D. nobile*), one species from the section of *Chrysotoxae* (*D. chrysotoxum*), and two *D. nobile* cultivars (Table 1). *D. nobile* and *D. chrysotoxum* were selected for the comparison because they are known to be the C_3/CAM intermediate plants. These species and cultivars were grown and maintained in the greenhouse at Huazhong Agricultural University, Wuhan, China. The potting medium used was moss and coconut blocks at the ratio of 3:1 (v/v); the diameter of the coconut block was 1.2 cm. All plants were watered with 1/2 HS nutrient solution (Hoagland and Snyder 1933) once a week from March to October during the growing season. From September to November, well-developed plants were selected when they developed more than four pairs of leaves; they were assigned to well-watered (WW) and drought-stressed (DS) treatments. The WW plants were placed into a climate chamber with light intensity at the level of leaves kept at about 250–350 μmol m^{-2} s^{-1} PPFD, a 55/75% (day/night) of relative air humidity, a 12/12 h (light/dark) photoperiod, and a temperature regime of 28/18°C (day/night) for at least two weeks. The plants were watered every other day. The volume of the climate chamber was 400 L. These conditions satisfied the requirements of the C_3/CAM intermediate plants to switch to the C_3 photosynthesis mode. The DS plants were placed into other climate chambers with light at about 250–350 μmol(photon) m^{-2} s^{-1}, 55/75% (day/night) humidity, and a 12/12 h (light/dark) photoperiod. The plants were kept without watering for at least 21 d to induce the CAM photosynthesis mode in the C_3/CAM intermediate plants. Normally, the potting medium contains water and is very difficult to dry, thus in order to dry plants easier, a temperature regime of 30/22°C (day/night) was applied. The middle leaves of the pseudobulbs were selected for measurements when they were fully expanded. All experiments were performed in triplicate.

Leaf succulence: The leaf succulence of the WW plants was determined by measuring the leaf thickness and the mesophyll succulence index (S_m). Six to ten leaves were measured with precision calipers to determine the leaf thickness. The leaf water content (LWC) was determined as the difference between the fresh mass (FM) and the mass after drying for 72 h at 75°C [dry mass (DM)], *i.e.*, LWC = (FM − DM)/FM. The total Chl content was spectrophometrically determined at wavelengths of 663 nm (Chl *a*) and 645 nm (Chl *b*) (*TU-1810, Beijing Purkinje General Instrument Co. Ltd.*, Beijing, China) (Hao *et al.* 2013) using 0.2-g leaf sections, which were collected at 18:00 h and weighed before cutting into smaller pieces. Chl was extracted from these samples for 12 h using 20 ml of acetone and ethyl alcohol (1:1, v/v). The formula used to calculate the Chl content was: Chl = (8.05 OD_{663} + 20.29 OD_{645}) 10^{-1}, where the results were expressed in mg g^{-1}(FM). S_m was calculated using the following formula: S_m = g(H_2O) mg^{-1}(Chl), *i.e.*, S_m = LWC/Chl content, which is the improved version of the method proposed by Kluge and Ting (1978).

Leaf carbon isotope ratio ($\delta^{13}C$): Leaf samples were harvested from the WW and DS-treated plants, frozen in liquid nitrogen, and stored at −70°C before measuring. Furthermore, the carbon isotope ratios ($^{13}C/^{12}C$) were determined using freezing-dried samples with their mass approx. 3.0 mg (Winter and Holtum 2002). Samples were analyzed using an organic elemental analyzer (*Delta V Advantage Isotope Ratio MS, Thermo Fisher Scientific Inc.,* Bremen, Germany) and compared with the PDB standard (*Belemnitella americana*). The stable carbon isotope composition was expressed in conventional delta (δ) notation as the $^{13}C/^{12}C$ ratio relative to the standard: $\delta^{13}C$ [‰] = [($\delta^{13}C$ sample)/($\delta^{13}C$ standard) – 1] × 1,000. The precision of the mass spectrometer used for the $\delta^{13}C$ analyses was 0.05‰.

Leaf titratable acidity: Leaf samples were harvested from the WW and DS plants at the end (18:00 h) and beginning (6:00 h) of the photoperiod, where 1.0 g of leaf samples were weighed, frozen in liquid nitrogen, and stored at −70°C before measuring their titratable acidity (Eastmond and Ross 1997). Samples were ground in a prechilled pestle and mortar with 5 ml of distilled water, and the crude extract was boiled for 20 min and then allowed to cool to room temperature. The extract was centrifuged at 5,000 × *g* for 15 min and 2 ml of the supernatant was diluted to 25 ml with distilled water before titration with 0.01 M KOH to the end point of pH 7.2, using bromothymol blue as an indicator. The titratable acidity was expressed in μmol(H^+) g^{-1}(FM). The titratable acidity accumulated during nighttime (ΔH^+) was calculated as: ΔH^+ = titratable acidity at dawn − titratable acidity at dusk.

Leaf CO_2 exchange: The net CO^2 uptake was measured every 2 h for 24 h using an infrared gas-exchange system equipped with a LED light source (*LI-6400-02B*) (*LI-6400, LI-COR Inc.,* Lincoln, NE, USA) (Pierce *et al.* 2002). Each leaf portion was enclosed in a chamber (2 cm × 3 cm), where the environmental conditions were controlled. A CO_2 cylinder was used to maintain a constant CO_2 concentration of 380 μmol mol^{-1}. The light intensity at the chamber was 300 μmol(photon) m^{-2} s^{-1} supplied by the LED, the photoperiod of 12/12 h (light/dark), and humidity of 55/75% (day/night) for both treatments. The temperature regime was 28/18°C (day/ night) for the WW plants and 30/22°C for the DS plants, respectively. On the

same time, the chamber of *LI-6400* was also placed during measurements in the bigger climate chamber (400 L), where the environmental conditions were also controlled as above. The net CO_2 uptake was analyzed for each treatment; the fully expanded leaves from the middle parts of the pseudobulbs were measured from six randomly selected plants.

Statistical analyses: A completely randomized design was used in the experiments and repeated three times. The means were compared by analysis of variance (*ANOVA*, three means) using *SAS v. 8.0* (*SAS Institute Inc.*, USA) Significant differences in leaf thickness and the mesophyll succulence index (S_m) among different species were analyzed by *ANOVA* with *Duncan*'s multiple range test; the *t*-test was used to test for differences in leaf $\delta^{13}C$ values between WW and DS plants and to test for differences between dawn and dusk of WW plants and that of DS plants within each species. The *t*-test was used to test for differences in ΔH^+ between WW and DS plants. The $P<0.05$ was considered as statistically significant.

Results

Leaf thickness and S_m: *D. loddigesii* showed the highest leaf thickness of 0.99 mm, which was significantly different from the other species or cultivars (Table 1). The leaf thickness of *D. fimbriatum* was the lowest, only 0.31 mm, although the leaf thickness of *D. crepidatum* was also low (0.38 mm). The leaf thickness of the other four species or cultivars (*i.e.*, *D. chrysotoxum, D. nobile,* and *D. nobile* 'V1' and 'V4') ranged from 0.58 mm to 0.89 mm. The highest S_m value was obtained with *D. loddigesii*, *i.e.*, 2.55 g(H_2O) mg^{-1}(Chl), which was significantly higher than those of the others. The lowest S_m value was obtained with *D. crepidatum*, *i.e.*, 0.53 g(H_2O) mg^{-1}(Chl). The S_m values of the other five species and cultivars ranged from 0.57 to 1.22 g(H_2O) mg^{-1}(Chl).

Leaf $\delta^{13}C$ values: The $\delta^{13}C$ values of *D. loddigesii* were within −15‰, *i.e.*, −14.47‰ and −14.66‰ under the WW and DS conditions, respectively (Table 2). This is a typical value for the obligate CAM plants. In the other species or cultivars, the leaf $\delta^{13}C$ values ranged from −25.68 to −28.37‰, which were lower than those of *D. loddigesii*. These values are typical for the C_3 plants, but we could not distinguish the obligate C_3 plants and the C_3/CAM intermediate plants based on this value alone. In addition, except *D. nobile*, no significant differences were in the $\delta^{13}C$ values of the WW and DS plants in other six species or cultivars.

Titratable acidity: For *D. loddigesii,* there was a significant difference between dawn and dusk in WW and DS conditions (Table 3). The highest ΔH^+ was obtained in *D. loddigesii*, *i.e.*, 355.5 and 425.9 μmol(H^+) g^{-1}(FM) in WW and DS conditions, respectively. For the other six species and cultivars, the ΔH^+ showed no significant difference between dawn and dusk in WW condition, which indicated that plants use C_3 photosynthetic pathway to uptake CO_2 under such condition. After DS for 21 d, the ΔH^+ was not significantly different between dawn and dusk in *D. crepidatum* and *D. fimbriatum*, but the ΔH^+ value of *D. fimbriatum* significantly increased to 48.0 μmol(H^+) g^{-1}(FM) compared with that of WW conditions. In contrast, for the other four species and cultivars, including *D. chrysotoxum*, *D. nobile*, and *D. nobile* 'V1' and 'V4', the ΔH^+ was significantly different between dawn and dusk in DS condition. In addition, the ΔH^+ values were 116.2, 184.8, 166.5, and 192.8 μmol(H^+) g^{-1}(FM), respectively, which clearly showed the significant increase compared with those of WW condition.

Leaf CO_2 exchange in plants with CAM photosynthesis: In the WW plants, net CO_2 uptake occurred only from 6:00 to 8:00 h, and the values were negative during most of the daytime (10:00 – 18:00 h) and nighttime (Fig. 1). After 14-d drought stress, there were significantly negative values during daytime and positive during nighttime. However, if the drought stress continued for another 14 d, the net CO_2 uptake was more or less constant at a low value (approx. 0 μmol m^{-2} s^{-1}) throughout the day,

Table 1. The leaf thickness and mesophyll succulence index (S_m) of seven *Dendrobium* species and cultivars in well-watered conditions. Results followed by *the same letter* within each species were not significantly different ($p<0.05$). The results represent the mean ± SE ($n = 3$).

Species or cultivar	Section	Thickness [mm]	S_m [g(H_2O) mg^{-1}(Chl)]
D. crepidatum Lindl.	*Dendrobium*	0.38 ± 0.02^{f}	0.53 ± 0.05^{d}
D. fimbriatum Hook.	*Dendrobium*	0.31 ± 0.01^{f}	0.58 ± 0.03^{d}
D. nobile Lindl.	*Dendrobium*	0.63 ± 0.03^{de}	1.22 ± 0.08^{b}
D. nobile 'V1'	cultivar	0.76 ± 0.02^{c}	1.10 ± 0.07^{bc}
D. nobile 'V4'	cultivar	0.58 ± 0.01^{e}	0.90 ± 0.09^{c}
D. chrysotoxum Lindl.	*Chrysotoxae*	0.89 ± 0.04^{b}	1.05 ± 0.02^{bc}
D. loddigesii Rolf.	*Dendrobium*	0.99 ± 0.04^{a}	2.55 ± 0.15^{a}

Table 2. Leaf $\delta^{13}C$ ($^{13}C/^{12}C$) values for seven species of *Dendrobium*. Results followed by *the same letter* within each species were not significantly different ($p<0.05$). The results represent the mean ± SE ($n = 3$).

Species or cultivar	Leaves/well-watered [‰]	Leaves/drought-stressed [‰]
D. crepidatum	−27.63 ± 0.27[a]	−28.37 ± 0.55[a]
D. fimbriatum	−27.62 ± 0.15[a]	−27.49 ± 0.38[a]
D. nobile	−27.07 ± 0.33[a]	−25.68 ± 0.21[b]
D. nobile 'V1'	−26.34 ± 0.73[a]	−26.73 ± 0.23[a]
D. nobile 'V4'	−28.09 ± 0.20[a]	−26.26 ± 0.06[a]
D. chrysotoxum	−27.55 ± 0.16[a]	−26.08 ± 0.76[a]
D. loddigesii	−14.47 ± 0.28[a]	−14.66 ± 0.43[a]

Table 3. Dawn and dusk analysis of the leaf titratable acidity in seven *Dendrobium* species and cultivars. Results followed by *the same small letter* of dawn and dusk under well-watered conditions within each species were not significantly different ($p<0.05$); results followed by *the same capital letter* of dawn and dusk under drought-stressed treatment within each species were not significantly different ($p<0.05$); results followed *by the same number of stars* of dawn-dusk between well-watered and drought-stressed treatment within each species were not significantly different ($p<0.05$).The results represent the mean ± SE ($n = 3$).

Species or cultivar	Well-watered [μmol(H^+) g^{-1}(FM)]			Drought-stressed [μmol(H^+) g^{-1}(FM)]		
	Dawn	Dusk	Dawn–Dusk	Dawn	Dusk	Dawn–Dusk
D. crepidatum	175.4 ± 19.2[a]	192.1 ± 50.5[a]	−16.7±31.2[*]	241.7 ± 50.5[A]	242.3 ± 46.8[A]	−0.6 ± 3.7[*]
D. fimbriatum	194.3 ± 15.2[a]	228.4 ± 16.3[a]	−34.1±1.1[**]	222.9 ± 14.5[A]	174.9 ± 15.9[A]	48.0 ± 11.4[*]
D. nobile	289.9 ± 57.6[a]	291.3 ± 20.7[a]	−1.3±36.9[**]	359.1 ± 41.4	174.3 ± 10.7[B]	184.8 ± 30.7[*]
D. nobile 'V1'	330.3 ± 43.2[a]	278.5 ± 11.4[a]	51.8±32.2[**]	331.2 ± 16.5[A]	164.7 ± 19.0[B]	166.5 ± 2.5[*]
D. nobile 'V4'	241.1 ± 30.6[a]	228.2 ± 21.5[a]	12.9±9.1[**]	425.7 ± 51.2[A]	232.9 ± 37.5[B]	192.8 ± 13.6[*]
D. chrysotoxum	311.5 ± 23.7[a]	275.8 ± 15.3[a]	35.9±8.4[**]	312.1 ± 13.5[A]	195.9 ± 27.9[B]	116.2 ± 14.3[*]
D. loddigesii	651.3 ± 36.3[a]	295.8 ± 36.0[b]	355.5±34.6[**]	705.5 ± 25.5[A]	279.6 ± 43.0[B]	425.9 ± 17.5[*]

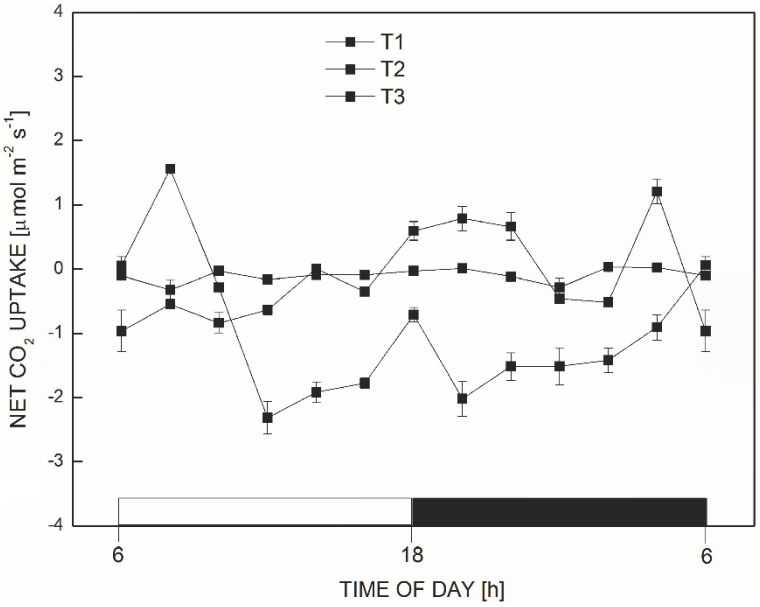

Fig. 1. Net CO_2 exchange by *Dendrobium loddigesii* in well-watered conditions (T1), 14 days without water (T2), and 28 days without water (T3). The results represent the means where $n = 6$. *Open bar* represents light period, *closed bar* represents dark period.

excluding 0.03 (2:00 h) and 0.02 μmol m^{-2} s^{-1} (4:00 h) in the evening (Fig. 1). Thus, *D. loddigesii* utilized the CAM photosynthesis mode under both conditions. In addition, the stomatal conductance of *D. loddigesii* in WW conditions, after 14 d and 28 d without H_2O showed values more or less about 0 mmol·m^{-2}·s^{-1}, except they were 0.045 and 0.085 mmol m^{-2} s^{-1} at 18:00 and 20:00 h after 14 days without H_2O (Fig. 1S, *supplement available online*).

Leaf CO_2 exchange of plants with C_3 photosynthesis: The net CO_2 uptake during daytime was positive in WW conditions (0.44 to 3.52 μmol m^{-2} s^{-1}), whereas no net CO_2 uptake occurred during nighttime (−0.5 to −2.92 μmol m^{-2} s^{-1}) (Fig. 2*A*). After 28 d without H_2O, the net CO_2 uptake during daytime decreased to a small positive value (approx. 0.5 μmol m^{-2} s^{-1}), but there was still no net CO_2 uptake during nighttime. The results were similar for

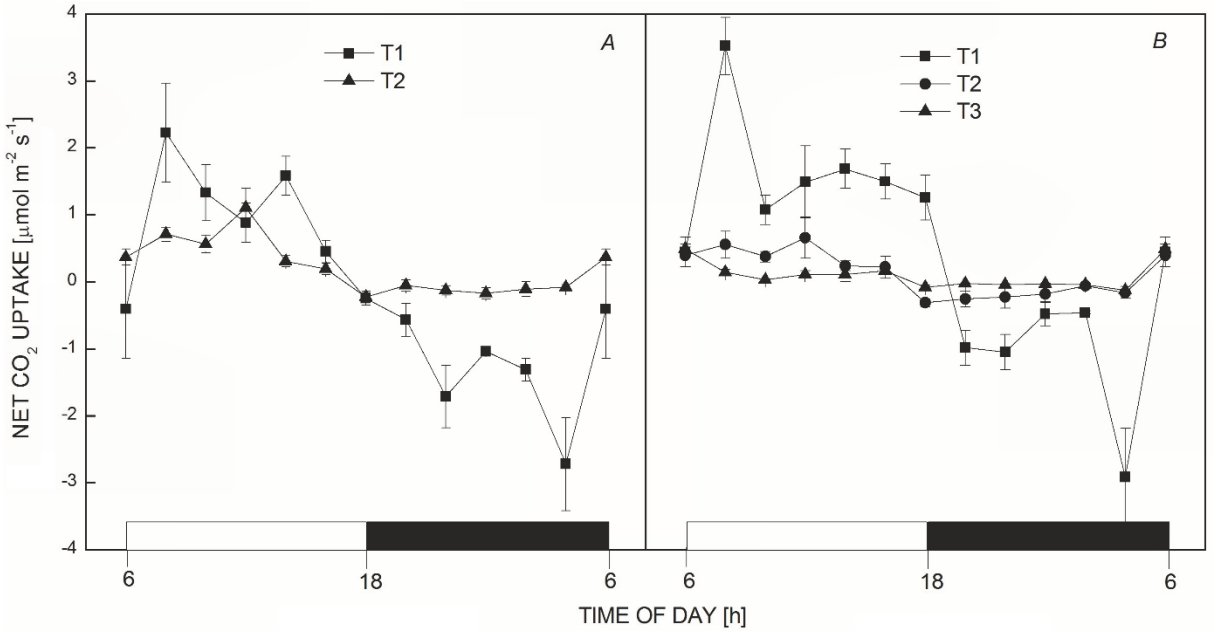

Fig. 2. Net CO_2 exchange by *Dendrobium crepidatum* (*A*) in well-watered conditions (T1) and 28 days without water (T2); and *Dendrobium fimbriatum* (*B*) in well-watered conditions (T1), 21 days without water (T2), and 28 days without water (T3). The results represent the means where $n = 6$. *Open bars* represent light periods, *closed bars* represent dark periods.

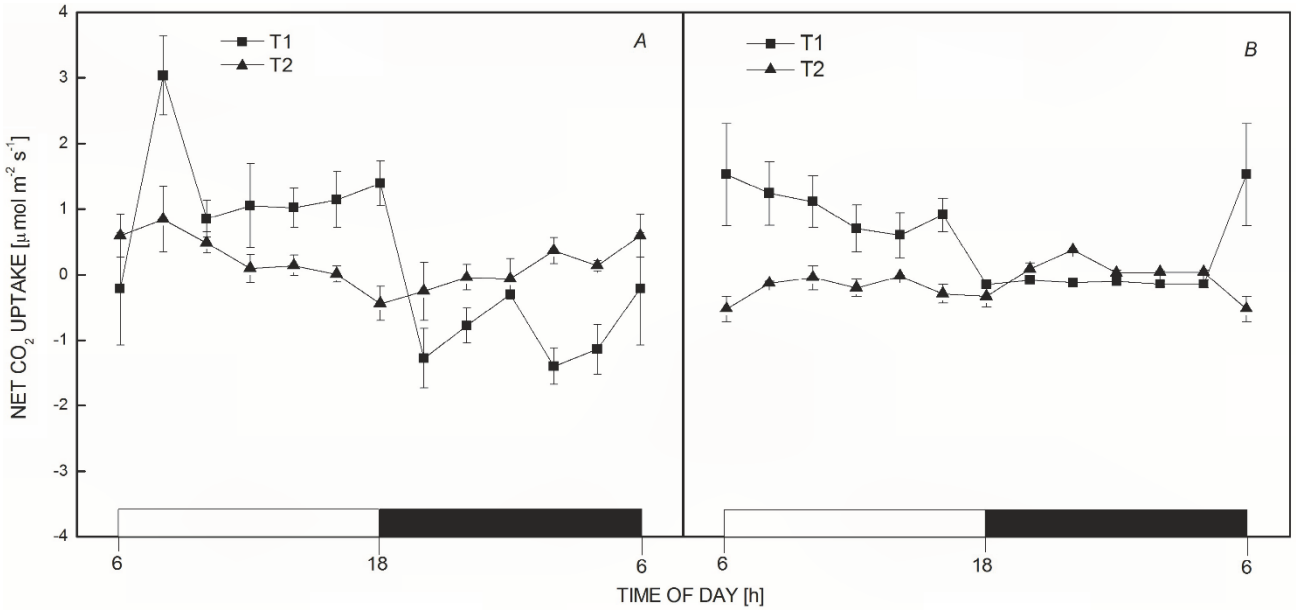

Fig. 3. Net CO_2 exchange by *Dendrobium nobile* (*A*) and *Dendrobium nobile* 'V1'(*B*) in well-watered conditions (T1) and 28 days without water (T2), respectively. The results represent the means where $n = 6$. *Open bars* represent light periods, *closed bars* represent dark periods.

D. fimbriatum (Fig. 2*B*). In addition, the leaf CO_2-exchange patterns of *D. fimbriatum* were measured after it was stressed for 35 d; the net CO_2 uptake decreased to a smaller positive value during daytime (from 0.032 to 0.489 µmol m^{-2} s^{-1}) and negative CO_2 uptake during nighttime (from −0.126 to −0.027 µmol m^{-2} s^{-1}) (Fig. 2*B*). Thus, there was no net CO_2 uptake by the two species during nighttime even under severe drought stress. The g_s of the two species was about 0–0.03 mmol m^{-2} s^{-1} (Fig. 2S, *supplement available online*).

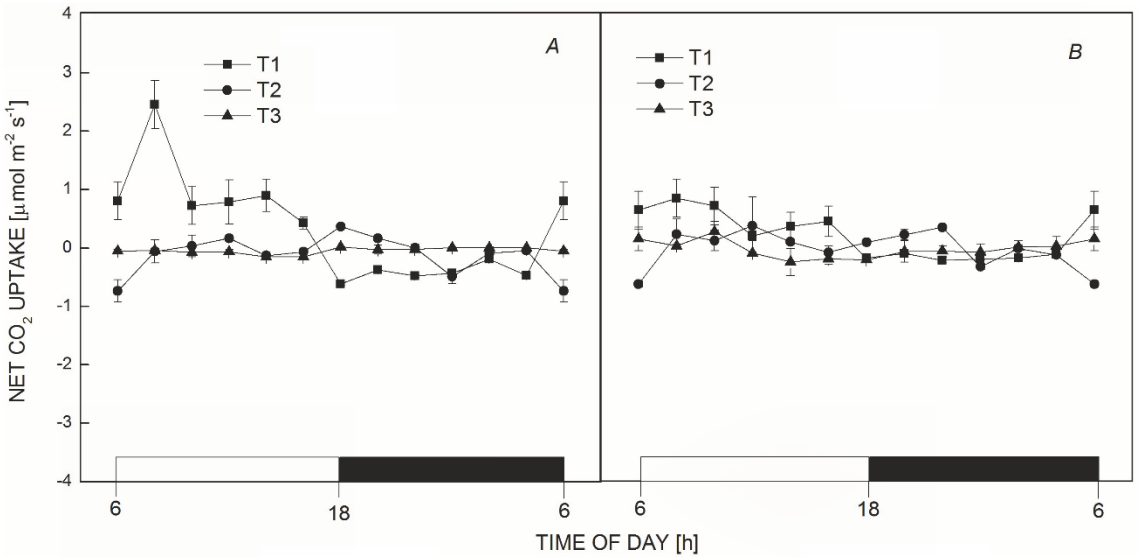

Fig. 4. Net CO_2 exchange by *Dendrobium nobile* 'V4' (*A*) and *Dendrobium chrysotoxum* (*B*) in well-watered conditions (T1), 21 days without water (T2), and 28 days without water (T3) , respectively. The results represent the means where $n = 6$. *Open bars* represent light periods, *closed bars* represent dark periods.

Leaf CO_2 exchange in plants with C_3/CAM intermediate photosynthesis: For *D. nobile, D. nobile* 'V1', *D. nobile* 'V4', and *D. chrysotoxum,* the net CO_2 uptake during daytime in WW condition was more or less constant with positive rates (Figs. 3, 4), whereas almost no nocturnal net CO_2 uptake was observed (except there was some positive at 18:00 h in *D. nobile*). For *D. nobile* 'V4' (Fig. 4*A*) and *D. chrysotoxum* (Fig. 4*B*), the net CO_2 uptake began to show positive values during nighttime after DS for 21 d. Nevertheless, *D. nobile* and *D. nobile* 'V1' began to switch from C_3 photosynthesis to the CAM photosynthesis mode after 28 d without water, with approx. 0 μmol m^{-2} s^{-1} throughout the day and only a small net CO_2 uptake during nighttime (Fig. 3). The daily CO_2 exchange patterns of *D. nobile* 'V4' (Fig. 4*A*) and *D. chrysotoxum* (Fig. 4*B*) in DS conditions (28 d without H_2O) are also shown in Fig. 4. The net CO_2 uptake was more or less constant at a low value (approx. 0 μmol m^{-2} s^{-1}) throughout the day, while the CO_2 exchange values were occasionally positive during nighttime and negative during daytime although they were very low; it was different from that of *D. fimbriatum* under drought stress for 35 d. In addition, the g_s of the four species or cultivars are shown in Figs. 3S, 4S (*supplements available online*).

Discussion

The results in this study demonstrated that obligate C_3 photosynthesis occurred in *Dendrobium*. For *D. crepidatum* and *D. fimbriatum*, net CO_2 uptake was positive during the day and negative during the night under the WW and DS conditions, which indicated they only use C_3 photosynthesis to fix CO_2. In addition, all of them yielded the $\delta^{13}C$ values lower than −27‰ under WW and DS conditions. Their leaf thickness was only 0.38 and 0.31 mm, respectively; and their S_m were 0.53 and 0.58 g(H_2O) mg^{-1}(Chl), which were also far below 1 g(H_2O) mg^{-1}(Chl). Thus, results were also consistent with other C_3 plants with lower leaf succulence and lower $\delta^{13}C$ values. Winter *et al*. (1983) and Silvera *et al*. (2005) investigated leaf thickness and $\delta^{13}C$ values of about 400 plants and found 105 plants with leaf thickness lower than 0.40 mm. All 105 plants did not yield the $\delta^{13}C$ values higher than −22‰, which indicated the plants with leaf thickness thinner than 0.40 mm that may only use C_3 photosynthesis to take up CO_2. However, the ΔH^+ of *D. fimbriatum* increased significantly under DS conditions, which might show that they use CAM photosynthesis to take up CO_2, but we did not found any significant change in ΔH^+ (dawn-dusk) of malic acid and citrate between WW and DS plants measured by HPLC (Liu 2014). Mao and Zhang (1985) also found obligate C_3 plant, *Oxalis corniculata*, with ΔH^+ of 120 μmol(H^+) g^{-1}(FM). Here, we were not sure what caused the increment of ΔH^+ values in *D. fimbriatum*. In 2001, Gehrig *et al*. discussed all *Dendrobium* species might be CAM plants for they have succulence organs. But we now know that not all succulent plants are CAM plants. And Ando (1982) identified some *D. nobile* hybrids as not being CAM plants. Thus, based on these analyses, we identified the two species only use C_3 photosynthesis no matter they were under stress condition or not.

Obligate CAM and C_3/CAM intermediate species were also found in the present study. *D. loddigesii* is the typical obligate (or constitutive) CAM plant because the leaf $\delta^{13}C$ values were −14.47 and −14.66‰ in both conditions. In

addition, the net CO_2 exchange pattern was significantly negative during daytime and positive during nighttime after DS treatment for 14 d without water (Fig. 1). However, we observed the net CO_2 uptake of *D. loddigesii* was positive only between 6:00 and 10:00 h in WW conditions, which could be classified as "CAM cycling". After 28-d drought stress, it maintained constant at a low value (approx. 0 μmol m^{-2} s^{-1}) throughout the day, though there were 0.03 (2:00 h) and 0.02 μmol m^{-2} s^{-1} (4:00 h) in the evening (Fig. 1); it could be classified as "CAM idling". The phenomenon of "CAM cycling" and "CAM idling" belongs to different permutations of CAM (Szarek *et al.* 1973, Osmond 1978, Ting 1985). It was reported that the obligate CAM plant, *Agave deserti,* could switch from a strong CAM type to a C_3 type pattern when plants were watered extensively (Hartsock and Nobel 1976). However, the day/night pattern of CO_2 exchange in the obligate CAM plant, *A. angustifolia*, did not shift towards the C_3 pattern completely when the supply of water was effectively unlimited (Winter *et al.* 2014). In contrast, *D. chrysotoxum*, *D. nobile*, and *D. nobile* 'V1' and 'V4' were identified as the C_3/CAM intermediate (inducible or facultative) plants, because there was no positive net CO_2 uptake during nighttime and low ΔH^+ values in WW conditions, but there was some degree of positive net CO_2 uptake during nighttime and large ΔH^+ values in DS conditions (21 or 28 d without water). However, the net CO_2 uptake of *D. nobile* 'V4' and *D. chrysotoxum* did not show any significant positive values during nighttime in DS condition (28 d without water); there was only 0.002–0.003 μmol m^{-2} s^{-1} (0:00–4:00 h) and 0.008–0.136 μmol m^{-2} s^{-1} (4:00–6:00 h), respectively. We also consider this the phenomenon of "CAM idling". In addition, Ando (1982) identified some *D. nobile* hybrids as not being CAM plants in WW environment. In this study, we further investigated and found that photosynthesis pathway of *D. nobile* 'V1' and 'V4' could be induced from the C_3 mode to the CAM mode by stress; thus, they belong to the C_3/CAM intermediate plants.

This comparative study of the single diverse section *Dendrobium* in one genus might be particularly useful for understanding the evolution and ecological importance of CAM. The molecular biology, biochemistry, and ecophysiology of CAM are well understood, but little is known about the evolutionary origins of CAM, particularly the genetic changes required for its evolution (Ehleringer and Monson 1993, Silvera *et al.* 2009). Thus, the section *Dendrobium,* where plants show three types of photosynthesis, suggests that the evolution towards CAM photosynthesis is still ongoing in the genus *Dendrobium.* This provides an opportunity to understand the adaptive and evolutionary significance of photosynthetic characteristics from an evolutionary view.

Many *Dendrobium* species are grown for their high ornamental or medicinal value. Understanding the photosynthetic pathways of plants is important for developing and improving cultivation techniques, and the results of the present study might be useful for local orchid growers who need to optimize the supply of water or light to plants. In addition, *Dendrobium* species have been the backbone of the cut-flower orchid business for several years. Since the first hybrid was reported in 1855, more than 8,000 novel *Dendrobium* cultivars have been bred *via* interspecific hybridization (Lavarack *et al.* 2000). However, several cultivars exhibit slow growth and/or they require high energy inputs. Most obligate C_3 plants grow more rapidly than obligate CAM plants, and C_3/CAM intermediate plants can use C_3 photosynthesis to improve their production in well-watered conditions, whereas they switch to CAM to survive in stressful environments. Thus, we can select obligate C_3 plants as parents to breed rapid-growing cultivars and use C_3/CAM intermediate or obligate CAM plants to breed new cultivars, more tolerant to adverse environments. Therefore, the results of this research might help select parents for cross-breeding of *Dendrobium* plants.

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