Full Length Article

Effect of Elevated CO_2 Combined with Two Nitrogen Levels on Photosynthesis, Growth and Leaf Structure in CAM Orchid *Phalaenopsis*

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Abstract

In C_3 plants, elevated CO_2 will increase plant growth and physiological performance through its effect on photosynthesis, but nitrogen (N) usability is a key factor to limit the growth of plants and augment the response to the long-term elevated CO_2. To detect whether higher N availability holds back the photosynthetic down-regulation in CAM plant, the effects of photosynthetic physiology, leaf structure and growth of *Phalaenopsis* in the greenhouses under the combined conditions of two CO_2 concentrations (400 and 800 μmol·mol^{-1}) and two N levels (6 and 12 mmol·L^{-1}) was studied. The results showed that elevated CO_2 increased net CO_2 uptake rate of facilitated the accumulation of starch and soluble sugar in *Phalaenopsis* leaves, but no marked differences were discovered between two N levels. Meanwhile, long-term elevated CO_2 reduced the contents of N and soluble proteins, but N application had beneficial effect on the contents of soluble proteins and N in *Phalaenopsis* leaves under high CO_2 concentration conditions. The elevated CO_2 combined with N levels markedly increased the activities of photosynthetic enzyme. And elevated CO_2 significantly improved the thickness of *Phalaenopsis* leaves, LE (low N level and elevated CO_2 concentration) and HE (high N level and elevated CO_2 concentration) increased by 6% and 8% compared with LA (low N level and atmospheric CO_2 concentration) and HA (high N level and atmospheric CO_2 concentration), respectively; the number of starch grains each chloroplast profile was augmented ~60% by elevated CO_2, so the chloroplast profile area was added by 19% in LE and 11% in HE, compared with LA and HA. The elevated CO_2 significantly accelerated the biomass accumulation in *Phalaenopsis*, and the high N level further promoted this increase. Thus, increasing supply of N and CO_2 simultaneously was not very important in commercial production of CAM orchid *Phalaenopsis* during short-term elevated CO_2 exposure, but it is still necessary to properly increase the supply of N during short-term elevated CO_2 exposure. © 2019 Friends Science Publishers

Keywords: Elevated CO_2; Net CO_2 uptake rate; N level; Ultrastructure

Introduction

Elevated CO_2 will increase plant growth and physiological performance through its effect on photosynthesis, but the increases might differ greatly according to the pathway of carbon fixation and corresponding environmental conditions. In general, the biomass was augmented about 50% and 12% in C_3 and C_4 plants (Poorter and Navas, 2003; Prior *et al.*, 2005), respectively, and 35% in CAM plants (Drennan and Nobel, 2000). Under long-term elevated CO_2, the initial stimulation of net photosynthetic rate by elevated CO_2 may be diminished in most C_3 plants, it is frequently known as "down-regulation" or "photosynthetic acclimation" (Leakey *et al.*, 2009; Aranjuelo *et al.*, 2013). Most studies indicated that plants generally have decreased tissue concentration of N when growth under elevated CO_2 environments (Taub and Wang, 2008). Cotrufo *et al.* (1998) found that the dry mass concentration of N decreased by an average of 9% in roots

and 14% in aboveground tissues, and recent literature surveys have consistently found that the dry mass N concentration of plant grown at CO_2 elevated mean decreased by approximately 10-15% (Taub and Wang, 2008). The decreased tissue N concentrations of plants under elevated CO_2 conditions may be one of the reasons for photosynthetic acclimation. Although most studies have explained the account for this phenomenon, the definite mechanisms by which responsible for it are not certain, may be due to some dilution, the most often referred assumption and included biomass dilution and functional dilution (Gifford *et al.*, 2000). Decreased nitrogen (N) uptake decreased transpiration (Del Pozo *et al.*, 2007), less efficient of underground root architecture (Pritchard and Rogers, 2000), altered root uptake capacity and mycorrhizal status (Alberton *et al.*, 2005). Decreased N demand, reflected in increasing the use efficiency of plant N (Stitt and Krapp, 1999) and photosynthetic acclimation (Urban *et al.*, 2012). The elevated CO_2-mediated N loss,

influencing the plant loss N rate (Pang *et al.*, 2006) and individual drift in N concentration (Bernacchi *et al.*, 2007).

Compared with C_3 and C_4 plants, less studies have been elaborated the influences to photosynthesis and growth by elevated CO_2 than CAM plants which employ both phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphatecarboxylase / oxygenase (Rubisco) to uptake CO_2 over the day and night (Drennan and Nobel, 2000; Ceusters and Borland, 2011). Based on the limited literatures, most of the CAM species showed increased productivity under elevated CO_2 conditions (Ceusters and Borland, 2011), although the effect might differ greatly depending on the different CAM morphotypes (i.e., stem succulent, leaf succulent and epiphytes) and environmental factors (Zhu *et al.*, 1999; Monteiro *et al.*, 2009; Weiss *et al.*, 2010). For most CAM species, elevated CO_2 enhanced the net CO_2 uptake rate with an average increase of 35-40% (Ceusters and Borland, 2011; Drennan and Nobel, 2000). However, beyond the average value, different conclusions exist among species, ranging over enhanced nocturnal CO_2 uptake (Li *et al.*, 2002), increased daytime CO_2 uptake (Ceusters *et al.*, 2008) and accelerated CO_2 uptake both during day and night (Graham and Nobel, 1996; Zhu *et al.*, 1999). Furthermore, like most C_3 species, some changes occur in the biochemistry, morphology and anatomy of CAM plants under elevated CO_2 conditions, which may be conducive to the higher net CO_2 uptake rate and the augmented biomass productivity with rising atmospheric CO_2 concentration (Drennan and Nobel, 2000; Ceusters and Borland, 2011).

Rubisco is an important enzyme of photosynthesis, which accounts for 15% - 35% of total leaf N in C_3 plants (Makino and Osmond, 1991). Many C_3 species show decreases in Rubisco activities or content due to reduced leaf N contents in response to elevated CO_2, potentially resulting in the above-mentioned acclimatization to elevated CO_2 concentrations (Makino *et al.*, 2000; Urban *et al.*, 2012). Although studies have investigated the relation of Rubisco and N contents in leaf (Seneweera *et al.*, 2011), it is still unresolved about the specific physiological mechanism for the decrease of N and Rubisco content in leaves by CO_2 enrichment. However, it has still uncertain whether the CAM plants are potentially able to improve the growth to addition N during long-term exposure to higher CO_2 concentrations.

In comparison to C_3 and C_4 species, the data describing the enzymes are limited in CAM plants (Drennan and Nobel, 2000), it would appear that the carboxylation activities for the enzymes PEPCase and Rubisco decrease in response to elevated CO_2 conditions in most CAM species (Nobel *et al.*, 1994; Graham and Nobel, 1996), but Li *et al.* (2002) observed a much lower activity of Rubisco during the daytime and much higher PEPCase activity during the dark period in *Mokara* Yellow grown under higher atmospheric CO_2 conditions. Thus, to excavate the underlying mechanism of responding mechanism to elevated CO_2 in CAM plants, it is necessary to have an integrated study of the effects on photosynthesis, growth, metabolite levels, morphology and anatomy by elevated CO_2 in CAM plants.

The genus *Phalaenopsis*, a tropical epiphytic CAM orchid (Endo and Ikusima, 1989), is one of commercially important orchid as both cut flowers and pot plants around the world due to their long-lasting flowers and graceful appearance. The elevated CO_2 for *Phalaenopsis* has a good promotion effect on the growth and quality of *Phalaenopsis*, which are mainly manifested as enhanced photosynthetic capacity, accumulation of photosynthate, biomass increase (Li *et al.*, 2007), increase of number of stems and flowers and prolonged vase life of cut flowers (Xu *et al.*, 2015). In addition, the flower bud differentiation of *Phalaenopsis* will accelerate at 20°C under 700 $\mu mol \cdot mol^{-1}$ of CO_2 (Kataoka *et al.*, 2004). Yoon *et al.* (2009) found that the chlorophyll content of cultured seedlings was increased by elevated CO_2 in *Phalaenopsis*, making Rubisco activity increased by 26.4%, leading to the formation of complete photosynthetic mechanism and eventually increasing the survival rate after seedling transplantation. These researches provide certain theoretical basis for actual application of elevated CO_2 technology in *Phalaenopsis* production. However, on a long run, funder elevated CO_2, *Phalaenopsis* production is seldom studied and the mechanism of elevated CO_2 and N interaction on photosynthesis and growth of *Phalaenopsis* remains unclear. This work investigates the influence of increasing N supplying level on photosynthesis and growth of *Phalaenopsis* under elevated CO_2, in order to provide theoretical basis for enhancing the growth process and quality of *Phalaenopsis* in cultivation.

Materials and Methods

Plant Materials and Experimental Design

This experimental material was *Phalaenopsis* 'Neyshan Guniang', a type of 4-leaf seedling with uniform growth, which was cultivated in white transparent plastic flowerpot (with diameter of 3.5 inch) with water moss as substrate, in South China Normal University. The studies were carried out in two glasshouses (6 m × 4 m × 3 m) with shading rate of 50%, relative humidity of 85% and temperature of 28/26°C (day/night). Peters Professional No.1 fertilizer (Scotts, USA) was diluted by 3000 times, and then applied by 200 mL per each strain, and 10 d as a course using NH_4NO_3. The control method of CO_2 concentration referred to the method by Xu *et al.* (2014). Elevated CO_2 was 800 $\mu mol \cdot mol^{-1}$, which was conducted between 18:00 and 6:00 for every day. The CO_2 concentration for control greenhouse was set to 400 $\mu mol \cdot mol^{-1}$. So, the treatments were separated as LA, Low N level (6 mM) + Atmospheric CO_2 concentration (400 ± 30 $\mu mol \cdot mol^{-1}$); HA, High N level (12 mM) + Atmospheric CO_2 concentration (400 ± 30 $\mu mol \cdot mol^{-1}$); LE, Low N level (6 mM) + Elevated CO_2 (800 ± 50 $\mu mol \cdot mol^{-1}$); HE, High N level (12 mM) + Elevated CO_2 (800 ± 50 $\mu mol \cdot mol^{-1}$). Each treatment had 150 plants.

The intermediate part of the functional leaf was measured by gas exchange parameters at 30 and 120 d

during the experiments. The leaves were collected at corresponding time, subjected to liquid N flash freezing, and then stored at -80°C for determination of physiological and biochemical indexes.

Gas Exchange Measurement

The leaves were measured at 22:00-24:00 on 30 and 120 d during the experimental periods. Five plants were measured the parameters of net CO_2 uptake rate and stomatal conductance (Gs) randomly by a portable infrared gas analyzer (Li-6800, Lincoln, USA) for each treatment. The CO_2 conditions were carried out in each corresponding treatment environments (400 or 800 $\mu mol \cdot mol^{-1}$). The measurements were started until the values of net CO_2 uptake rate were fluctuated less than 0.1 for stabilization.

Determination of Physiological and Biochemical Indexes

The activities of Rubisco and PEPcase were measured on the basis of the method proposed by Yoon et al (2009). The content of total soluble sugar was measured by anthranone colorimetry (Morris, 1948). Starch content was measured by the method proposed by Zapata *et al.* (2004). The soluble protein content in leaf was measured by referring to the method of Bradford (1976). The total N content in leaf of *Phalaenopsis* was measured by half trace kjeldahl method. The chlorophyll content was measured using 80% acetone (Harmut and Lichtenthaler, 1987). The titrableacid content was measured using indicator titration method (Lin and Hsu, 2004). At 6:00, the middle part of upper fully expanded functional leaf was collected, subjected to liquid N flash freezing, and then stored at -80 ° C for determination of the titrableacid content.

Leaf Anatomical Structure

The microstructure of *Phalaenopsis* leaf was observed through traditional paraffin section method. At the morning after treatment for 120 d, the middle part of upper fully expanded functional leaf was collected, washed with distilled water and then $0.1 mol \cdot L^{-1}$ phosphoric acid buffer. By cutting along two sides of the middle main vein of leaf, a 1-2 mm square was obtained. After which, the square sample was fixed with 4% glutaraldehyde for 1 h using vacuum pump extraction, and rinsed with 0.1 M phosphate buffer 6 times (each lasting for 20 min). Subsequently, it was double fixed with 1% osmic acid and then rinsed with $0.1 mol \cdot L^{-1}$ phosphate buffer 6 times (each lasting for 10 min). Then dehydration treatments were carried out using alcohol in gradient of 30%, 50%, 70%, 80%, 90% and 100% twice (each lasting for15min). Next, the sample was successively subjected to epoxypropane (3:1) penetration for 1h, epoxypropane (1:1) penetration for 4h and epoxypropane (1:3) penetration for 1h, and then embedded with Epon-812 epoxy resin in an oven for polymerization. Finally, the

sample was sliced using LKB-5 ultra-microtome, and observed with JEM-100cx transmission electron microscope. Each observation covered 20-30 views, and the average value of all observation values was selected as the final result.

Growth Parameters

The newly grown leaves were marked during the treatment period. Those expanding leaves randomly been chosen to measure the leaf area in each treatment by a leaf area meter (LI-3100 C; Li-Cor Inc., USA). To determine fresh weight, whole plants were reaped, washed up, and weighed. Then these plants were putted in 80°C oven for 72 h and the underground parts and aboveground were weighed respectively to calculate the ratio of root/shoot. Five repeated tests were conducted for each treatment.

Statistical Analysis

The data analysis was executed according to two factor analyses of variances ANOVA (SPSS 20). In four treatments, CO_2 concentration was chosen as the first factor (400 $\mu mol \cdot mol^{-1}$ and 800 $\mu mol \cdot mol^{-1}$) and N as the second factor (6 m*M* and 15 m*M*) and three experimental replicates per treatment. Significant differences were calculated at 5% and 1%. When difference among the treatments reached a significant level on the basis of the ANOVA analysis, least significant differences were calculated utilizing Lees Significant Difference (LSD) ($P < 0.05$).

Results

Leaf Gas Exchange

Net CO_2 uptake rate was increased markedly in *Phalaenopsis* leaves by CO_2 but not significantly influenced by N (Fig. 1A). Elevated CO_2 increased the net CO_2 uptake rate of *Phalaenopsis* under two N supplying levels by about 50% at the 30th day of experiment. On 120 d, the net CO_2 uptake rate under HE was higher than under HA by 46%, but lower than under LA by 39%. Elevated CO_2 significantly reduced the Gs in *Phalaenopsis* leaves during the whole experiment process (Fig. 1B). The Gs value under LE was lowered by 34% than under HA, while Gs value under HE was lowered by 28% than under HA. At the 120th day of experiment, the Gs in HE was larger than in LE by 21%.

Enzyme Activity

Rubisco is a key enzyme for fixing CO_2 in C_3 plants, of which the activity is affected by multiple environmental factors. The activity of Rubisco was increased by elevated CO_2 (Fig. 2A). At the 30 d and 120 d of treatment, the Rubisco activity under LE was increased than under LA by 25% and 18%, respectively; however, activity under HE increased than under HA by 21% and 22%, respectively.
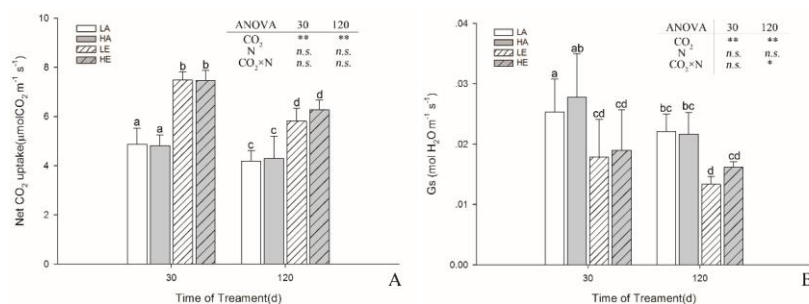
Fig. 1: Effect of elevated CO_2 combined with nitrogen levels on net CO_2 uptake rate (A) and Gs (B) in CAM orchid *Phalaenopsis* 'Ney Shan Gu Niang'. Bars represent Mean ± SD, n = 5 (with five plants per treatment). The different letters up the bars in represented the significantly different (*P* < 0.05) according to LSD test
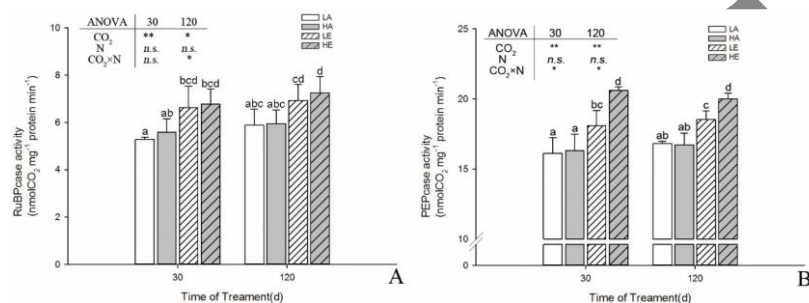

Fig. 2: Effects of elevated CO_2 combined with nitrogen levels on enzyme activities in leaves of *Phalaenopsis* 'Ney Shan Gu Niang'. (A) Rubisco activity; (B) PEPcase activity. Bars represent mean ± SD, n = 5 (with five plants per treatment). Bars with the same letter are not significantly different (*P* > 0.05) according to LSD test

Rubsico activity nearly had no response to the N supplying level. The effect of elevated CO_2 and N interaction reached significant level at the 120 d of the treatment.

PEPcase is a key enzyme to fix CO_2 for CAM plants. Elevated CO_2 significantly increased the PEPcase activity of *Phalaenopsis* leaves. The PEPcase activity under LE was increased than under LA by 10%, and the PEPcase activity under HE was increased under HA by 22%. N supplying level alone had no significant influence to PEPcase activity, only if it was combined applied with elevated CO_2.

Physiological and Biochemical Parameters

levated CO_2 significantly enhanced both content of soluble sugar and starch in *Phalaenopsis* leaves under two N levels (Table 1). At 120 d, the total soluble sugar under LE was increased than under LA by 27%, the total soluble sugar under HE was increased than under HA by 34%. In both N levels, the starch content was increased by about 45%. Under elevated CO_2 condition, the soluble protein content showed a decreasing trend. At 30 d, the soluble protein content under LE and HE was lowered than under LA and HA by 6% and 11%, respectively. At 120 d, the soluble protein content under LE and HE was lowered than under LA and HA by 21% and 15%, respectively. Elevated CO_2 and N supplying level had a significant influence on titratable acid content (Table 1). The titratable acid content

under LE was increased than under LA by 26%; the titratable acid content under HE was increased than under HA by 36%. Through measuring the N content in *Phalaenopsis* leaves, it was found that the elevated CO_2 increased the leaf N content by 8% at 30 d. At 120 d, the leaf N content under LE was decreased than under LA by 20%.

Leaf Microstructure

Through analyzing the microstructure of *Phalaenopsis* leaf, it was found that elevated CO_2 and N supplying level had no significant influence on the leaf microstructure (Fig. 3). This is probably due to no significant difference between spongy tissue and palisade tissue in CAM plants as compared with C_3 plants. Although elevated CO_2 significantly changed the photosynthetic characteristics of *Phalaenopsis*, the microstructure in *Phalaenopsis* leaf remained unchanged. However, elevated CO_2 improved the thickness of *Phalaenopsis* leaf (Fig. 3 and 4). The leaf thickness under LE and HE was increased than under LA and HA by 6% and 8%, respectively. The increase of leaf thickness may be one of main reasons accounting for the increase of net CO_2 uptake rate. On one hand, elevated CO_2 enhanced the net CO_2 uptake rate and growth rate of *Phalaenopsis* and increased the leaf thickness. On the other hand, the increase of leaf thickness increased the storage capacity of vacuoles, thus promoting the accumulation of titrable acid and enhancing the net CO_2 uptake rate at night.

Table 1: Effects of elevated CO$_2$ combined with two nitrogen levels on physiological and biochemical indices in leaves of *Phalaenopsis* 'Neyshan Guniang'

Time	Treatments	total soluble sugars (g·m^{-2})	Starch (g·m^{-2})	total soluble protein (g·m^{-2})	titratable acids (μmol·g^{-1})	Nitrogen (%)
30	LA	14.07±1.35 a	4.63±0.70 a	7.52±0.50 b	103.33±2.89 a	2.58±0.10 ac
	HA	14.89±0.71 ab	5.05±0.44 ab	7.86±0.41 b	104.33±6.03 a	2.57±0.07 ac
	LE	16.76±0.64 cd	6.39±0.45 b	7.07±0.36cb	127.67±2.52 b	2.80±0.19 c
	HE	16.33±1.37bc	6.26±0.97 b	7.02±0.33 c	138.33±2.89 c	2.80±0.19 c
120	LA	13.82±0.29 a	5.51±0.92 ab	8.29±0.44 a	122.33±2.52 b	2.45±0.10 a
	HA	13.79±1.16 a	5.78±0.78 ab	8.46±0.58 a	123.00±2.65 b	2.60±0.20 ac
	LE	17.58±0.60 cd	8.00±0.95 c	6.55±0.25 cd	157.67±2.52 d	1.96±0.06 b
	HE	18.45±0.93 d	8.44±0.85 c	7.20±0.57 b	171.67±2.89 e	2.55±0.12 ac
30	CO$_2$	*	**	*	**	*
	N	n.s.	n.s.	n.s.	*	n.s.
	CO$_2$*N	n.s.	n.s.	n.s.	n.s.	n.s.
120	CO$_2$	**	**	**	**	**
	N	n.s.	n.s.	n.s.	**	**
	CO$_2$*N	n.s.	n.s.	n.s.	**	*

The meaning of symbols used in ANOVA were: * Significant difference at 5%, ** Significant difference at 1%, the same below
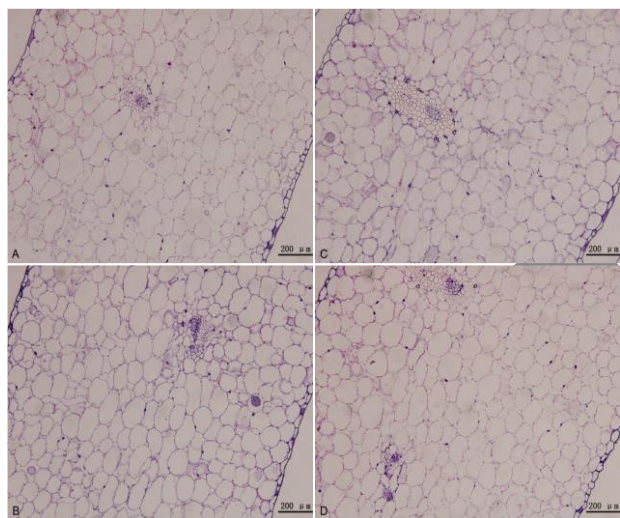

Fig. 3: Effects of elevated CO$_2$ combined with nitrogen levels on microstructure in leaves of *Phalaenopsis* 'Ney Shan Gu Niang' on 120 days. (A) LA;(B) HA; (C) LE; (D) HE。

Fig. 4: Effects of elevated CO$_2$ combined with nitrogen level on thickness in leaves of *Phalaenopsis* 'Ney Shan Gu Niang'. Bars represent mean ± SD, n = 5 (with five plants per treatment). Bars with the same letter are not significantly different ($P > 0.05$) according to LSD test

Leaf Ultrastructure

Under elevated CO$_2$ and N supplying level did not affect the membrane structure of chloroplast (Fig. 5). However, the number of starch grains in chloroplast was significantly increased by elevated CO$_2$ under two N supplying levels by up to 60% (Table 2). In contrast, the area of starch grain at high N supplying level was increased than at low N supplying level by up to 64% for both CO$_2$ concentration conditions. In addition, elevated CO$_2$ significantly increased the number and area of chloroplast. The area of chloroplast under LE and HE was increased than under LA and HA by 19% and 11%, respectively, while the number of chloroplasts increased by 36% and 18%, respectively. The N supplying level had significant influence on the width and area of chloroplast. The area of chloroplast under HA and HE was increased than under LA and LE by 45% and 36%, respectively.
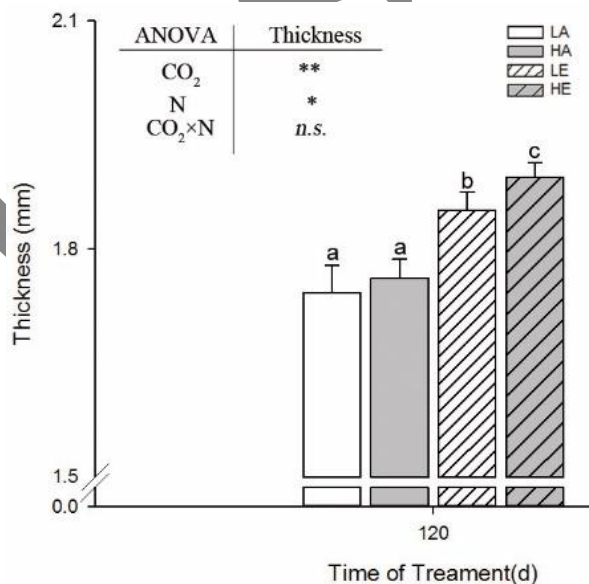
Growth and Biomass

The root/shoot ratio was increased by elevated CO$_2$, but elevated CO$_2$ and N supplying level had no significant influence on the biomass accumulation at 30 d (Table 3). At 120 d, the fresh and dry weights under HE were higher than under HA by 22% and 18%, respectively; while the fresh weight and dry weight under LE was higher than that under LA by 16% and 13%, respectively. The relative leaf area under LE and HE was increased than that under LA and HA by 14% and 15%, respectively; the root/stem ratio increased by 28% and 12%, respectively; but N supplying level had not significant influence on relative leaf area.

Table 2: Effects of elevated CO_2 combined with nitrogen level on Chloroplast ultrastructure of *Phalaenopsis* 'Ney Shan Gu Niang'

Treatment	starch grain			chloroplast		
	Area	Numbers	Length	Width	Area	Numbers
LA	0.21±0.03a	3.25±0.96a	7.30±1.92a	2.76±0.38a	12.64±1.27a	1.80±0.51a
HA	0.34±0.04b	3.50±0.58a	8.08±0.51a	3.24±0.10bc	18.36±1.17b	2.18±0.62ab
LE	0.22±0.06a	5.25±0.96b	7.65±0.88a	2.79±0.34ab	14.98±0.56c	2.45±0.17ab
HE	0.37±0.06b	5.75±1.50b	7.90±0.27a	3.42±0.27c	20.45±0.41d	2.57±0.26b
CO_2	n.s.	**	n.s.	n.s.	**	*
N	**	n.s.	n.s.	**	**	n.s.
CO_2*N	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3: The statistics on part growth indicators of *Phalaenopsis* 'Ney Shan Gu Niang'

Time (d)	Treatment	Fresh weight (g)	Dry weight (g)	Root-shoot ratio	Leaf area (cm^2)
30	LA	87.47±3.04a	5.67±0.11a	1.09±0.07bcd	75.63±1.56a
	HA	87.19±2.90a	5.61±0.07a	1.04±0.04abc	75.49±6.32a
	LE	90.62±3.48a	5.58±0.32a	1.13±0.05cde	76.54±4.16a
	HE	91.00±3.60a	5.79±0.14a	1.17±0.04de	76.09±4.28a
120	LA	121.69±2.20b	7.52±0.16b	0.96±0.13a	259.92±4.26b
	HA	122.64±2.41b	7.65±0.05b	0.97±0.06ab	265.17±5.10b
	LE	141.04±1.83c	8.49±0.28c	1.23±0.06de	295.50±6.50c
	HE	149.01±1.64d	9.06±0.16d	1.09±0.03e	304.29±9.81c
30	CO_2	n.s.	n.s.	*	n.s.
	N	n.s.	n.s.	n.s.	n.s.
	CO_2*N	n.s.	n.s.	n.s.	n.s.
120	CO_2	**	**	**	**
	N	**	*	n.s.	n.s.
	CO_2*N	*	n.s.	n.s.	n.s.

Remark: Leaf area was the total area of leaves newly emerged during treatment
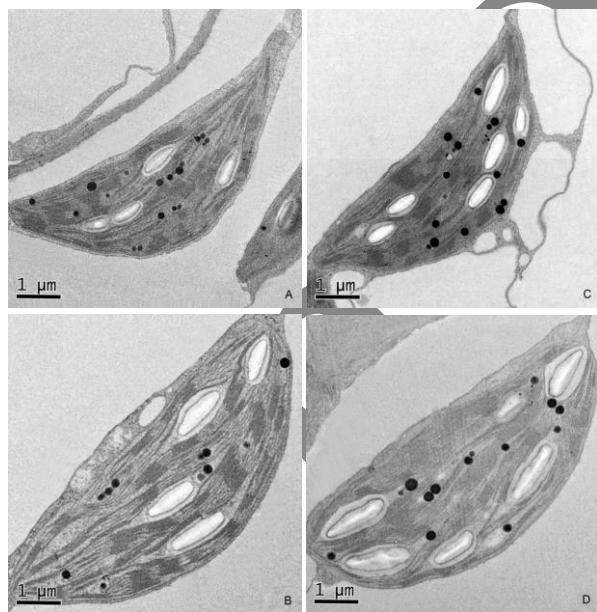

Fig. 5: Effects of elevated CO_2 combined with nitrogen levels on chloroplast ultrastructure in leaves of *Phalaenopsis* 'Ney Shan Gu Niang' on 120 days. (A) LA; (B) HA; (C) LE; (D) HE。

Discussion

According to previous researches, it can be known that the promotion effect on net CO_2 uptake rate by elevated CO_2 in CAM plants varies from species to species. The increase of net CO_2 uptake rate can be divided into net CO_2 uptake rate increase at night (Li *et al.*, 2002), daytime (Ceusters *et al.*, 2008) and at both night and daytime (Zhu *et al.*, 1999). This study only focuses on the elevated CO_2 to *Phalaenopsis* at night. In the whole experiment, the net CO_2 uptake rate was significantly increased by elevated CO_2 at night. Drennan and Nobel (2002) believed that there are completely different between instant and long-term of plants in response to the CO_2 concentrations. This is mainly because that the positive impact of elevated CO_2 can reach the best results only when the physical or any morphological changes (the thickness of green tissue increases) occur. At the late period (120 d) of the experiment, the net CO_2 uptake rate under LE was lower than under HE, but the difference was not significant. This means that the promotion influence of elevated CO_2 on net CO_2 uptake rate in *Phalaenopsis* will be decreased in some degree under long-term elevated CO_2 condition. Increasing N supplying level can inhibit such decrease of promotion effect of elevated CO_2 to certain extent, but the inhibition effect is not such significant than for C_3 plants.

For most of plants, long-term elevated CO_2 will lead to decrease of plant photosynthetic capacity. The reasons for such phenomenon can be mainly drawn from three aspects: (1) stomatal limitation, generally attributable to the lower stomatal conductance in leaves (Long *et al.*, 2004); (2) feedback inhibition, usually caused by the imbalance of source-sink (Qian *et al.*, 2012); (3) metabolic limitation, often resulting from a reduced ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) amount or carboxylation efficiency (Erice *et al.*, 2006).

In this work, the decrease of net CO$_2$ uptake rate of *Phalaenopsis* was accompanied with Gs decrease, increase of carbohydrates and improvement of Rubisco activity. This indicates that the decrease of net CO$_2$ uptake rate may be jointly resulted by stomatal limitation and metabolic limitation. The Gs is a key indicator affecting the response to CO$_2$ in plants, most of C$_3$ plants have significantly decreased in Gs by elevated CO$_2$. This is mainly due to that high CO$_2$ concentration enhances plant photosynthesis, increases the concentration of photosynthetic products in protective cells, and improves the cell water potential. On this basis, guard cells absorb water and expand, making the stoma closed (Ainsworth and Rogers, 2007). In this study, the decrease of Gs in *Phalaenopsis* leaves was accompanied by the increase of the content of soluble sugar and starch, which indicates that the decrease of Gs might be caused by the accumulation of carbohydrates.

The diurnal transformation and efficiency of carbohydrates have a critical role in classification stages of CAM plants and affect the CO$_2$ uptake at night (Borland and Taybi, 2004). Furthermore, due to the need of furnishing the PEP with a carbon skeleton, carbohydrates must be distributed to other organs to maintain the integrity of plant growth and photosynthesis. Thus, the mechanism of regulating these potential conflicts of interest for CAM plants remains unclear, but it is probably related to the synthesis, degradation and transport of carbohydrates (Häusler *et al.*, 2000). Studies show that the contents of soluble sugar and starch increase in different degrees under high CO$_2$ concentration (Vicente *et al.*, 2017). In this study, elevated CO$_2$ significantly enhanced the accumulation of carbohydrates. To account for why elevated CO$_2$ leads to increase of carbohydrate content of CAM plants, one possible reason is that the storage capacity of vacuole was significantly enhanced, which can dilute the concentration of solute in cells due to enhanced photosynthesis (Wang and Nobel, 1996). The other reason may be that the decrease of glycosyltransferase activity in CAM plant cell at elevated CO$_2$ conditions led to the decrease of hexokinase activity (Drennan and Nobel, 2000).

The tissue concentrations of N was decreased by elevated CO$_2$, and the decrease protein content in leaves could be resulted from the imbalance of source-sink due to the accumulation of carbohydrates, consequently caused to a feedback inhibition of Rubsico (Aranjuelo *et al.*, 2009), or caused by the redistribution of N occurs with the leaf growth (Fangmeier *et al.*, 2000). In this study, the decrease of soluble protein content was accompanied with the increase of carbohydrates. Therefore, it can be derived that the decrease of soluble protein content was probably caused by the redistribution of N. Under elevated CO$_2$ conditions, the most frequently mentioned hypothesis about the N content decrease is that caused by the dilution because of non-structural carbohydrates accumulation, which is typically explained as "Dilution Hypotheses" (Taub and Wang, 2008). Since the change of carbon-N metabolism led to an increase of the proportion of carbohydrates in biomass, the N-containing compound was diluted accordingly.

Any structural change caused by elevated CO$_2$ will affect the physiological property of plants. For all types of plants, the morphology and anatomy of photosynthetic tissues are important for light acquisition and gas exchange (Nobel *et al.*, 1994). There are not in abundance researches on the impact of elevated CO$_2$ in CAM plant leaf structure. According to most of these researches, elevated CO$_2$ increase the thickness of green tissues in CAM plants (Drennan and Nobel, 2000). For example, the cladode thickness of *O. ficus-indica* was increased by about 15% under doubled concentration of CO$_2$ (Nobel and Israel, 1994); the leaf thickness of *Agave deserti* and *A. comosus* was increased by about 11% (Graham and Nobel, 1996). In this study, elevated CO$_2$ had no marked influence on leaf microstructure in *Phalaenopsis*, but only increased its leaf thickness. Relevant researches show that under long-term elevated CO$_2$ conditions, the thickness of green tissue will increase, which is beneficial to enhance the capacity of vacuoles to store organic acids and thus enhancing the net CO$_2$ uptake rate in CAM plants (Graham and Nobel, 1996).

In recent years, studies about the effect on leaf microstructure by of elevated CO$_2$ are mainly focused on C$_3$ plants, but less on CAM plants. There is only one report on periphyte CAM orchid (Gouket *et al.*, 1999). Through observing the microstructure of *Phalaenopsis* leaf, it can be known that elevated CO$_2$ remarkably increased the number and area of the chloroplasts in mesophyll cells, which is in accordance with the results of research on arabidopsis (Teng *et al.*, 2006) and tobacco (Wang *et al.*, 2004). Although many studies have shown that the number of chloroplasts is increased by elevated CO$_2$, the regulating mechanism of chloroplast by applying elevated CO$_2$ in mesophyll cells remains unclear. It is probably due to the fact that elevated CO$_2$ is beneficial for the formation of chloroplasts, thus leading to the increase of number of chloroplasts (Wang *et al.*, 2004). The increase of chloroplast area of *Phalaenopsis* is probably due to the increase of starch content under elevated CO$_2$ condition. In this study, elevated CO$_2$ had no influence on the size of starch grain, but only increased the number of starch grains in chloroplast, which is in accordance with the results of biochemical measurement of starch content in *Phalaenopsis* leaves. Relevant studies showed that the accumulation of starch in chloroplast may represent a carbon storage mechanism, which is beneficial for enhancing the library capacity, and 75% of N in leaf participates in the synthesis of photosynthetic components in chloroplast (Li *et al.*, 2013), therefore the increase of N will inevitably increase the number and area of chloroplast. In this study, the N supplying level significantly increased the width and area of chloroplast of *Phalaenopsis* leaf. The area of chloroplast under HA and HE was increased than under LA and HA by 45% and 36%, respectively. In addition, the N supplying also increased the area of starch grain. Although, the

ultra-microstructure of *Phalaenopsis* leaf had significant change under single factor of elevated CO_2 or N supplying, the effect of combined application of elevated CO_2 and N supplying on leaf ultra-microstructure was not so significant. There have been no reports investigating the effect of combined application in elevated CO_2 and N supplying on leaf ultra-microstructure, and the response mechanism need to be further studied.

Compare with C_3 and C_4 plants, relatively little studies have studied the impacts of elevated CO_2 on growth characteristics in CAM plants. The influence of elevated CO_2 on CAM plants varies significantly upon plant types (i.e., stem succulents, leaf succulents and epiphytes) and environmental factors (Zhu *et al.*, 1999；Weiss *et al.*, 2010). Most researches show that elevated CO_2 can enhance the growth and improve the production of CAM plants (Drennan and Nobel, 2000; Ceusters and Borland, 2011). Some studies suggest that the increase of average plant biomass is highly consistent with the all-day net CO_2 uptake. For instance, the net CO_2 uptake rate of pineapple under doubled concentration of CO_2 is increased by 15% and the total biomass is increased by 23% (Zhu *et al.*, 1999). Through measurement of relevant growth indexes in this study, it can be known that elevated CO_2 remarkably increased the leaf area and thus enhanced the total biomass of fresh and dry weights in *Phalaenopsis*. In addition, increasing supply of N is beneficial for improving the area and extension of plant leaf, increasing the photosynthetic area and thus increasing the total biomass of plant.

Conclusion

The results showed that the increasing supply of N and CO_2 simultaneously was not very important in commercial production of CAM orchid *Phalaenopsis* during short-term elevated CO_2 exposure, but it is still necessary to properly increase the supply of N during short-term elevated CO_2 exposure.

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