THESIS FOR THE DEGREE OF MASTER OF SCIENCE

CO_2 Uptake Behavior and Vegetative Growth of *Doritaenopsis* 'Mantefon' Orchids as Influenced by Light/Dark Cycle Manipulation

BY

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AUGUST 2017

MAJOR IN HORTICULTURAL SCIENCE AND
BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL
UNIVERSITY

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UNDER THE DIRECTION OF DR. KI SUN KIM SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

This study was performed to investigate how changing the period of light and dark influences the vegetation growth and the photosynthesis of *Doritaenopsis*. Clones of *Doritaenopsis* Queen Beer 'Mantefon' at 4-month-old stage were grown in a closed-plant factory system with four different light/dark cycles; 06/06 h, 08/08 h, 10/10 h, and 12/12 h. Temperature and relative humidity were set at 28°C and 80%, respectively, with a photosynthetic photon flux density of 160 ± 10 μmol· m-

$^{2}\cdot s^{-1}$. Repetitive measurements showed that the leaf length and the leaf width were the longest under 12/12 h closely followed by 10/10 h. The fresh weight and the dry weight of leaves and roots were the heaviest at 10/10 h treated samples. Different CO_2 uptake patterns were observed from different light/dark cycles. Under 10/10 h and 12/12 h treatments, the CO_2 uptake started at early dark period. When the light/dark cycles were shortened to 06/06 h and 08/08 h, the CO_2 uptake started at the middle of dark period. Total CO_2 uptake amounts were the highest under 12/12 h treatment followed by 10/10 h, 06/06 h, and 08/08 h treatments. Quantitative measurements showed that the vegetative growths under 10/10 h treatment were comparable with that of 12/12 h treatment. These studies indicated that manipulating light/dark can modify the photosynthesis patterns and vegetative growth of *Doritaenopsis* ‘Mantefon’, resulting in the reduction of the production period.

Keywords: CAM plants, Circadian rhythms, Orchid, vegetative growth

Student number: 2015-23012

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INTRODUCTION

Light/dark cycle generated by the Earth's rotation is a significant factor for the growth of plant. It influences the biomass accumulation, photosynthesis, seed number, and seed viability of plans closely related to plant physiology and agronomic. Thus offering a proper daylight condition is critical to the efficient plant vegetation growth. However, adaptation of light in plant is not a simple process but accompanies more sophisticated endogenous biological cycle adjustments known as 'circadian rhythms'. The term 'Circadian' originated from two Latin words which are 'CIRCA (about)' and 'DIES (day)' (McClung, 2006). This original word implies that circadian rhythm is related to the light and dark cycle of the day. The main functionality of circadian rhythms is that plants can persist under steady environmental conditions so they can anticipate their external alterations (Dodd et al., 2015). Hereby, circadian rhythms do a momentous role to enhance smooth growth of plant (Dodd et al., 2005).

The first scientific research on plant circadian rhythm was reported by de Marian who observed persistent daily leaf movements of heliotrope plant (1729). After a century, studies revealed that plants can have their own running rhythms which is shorter than 24 h (de Candolle, 1832; Pfeffer, 1915) and these endogenous rhythms were not operated by other exogenous factors. Then, the idea of heritable circadian rhythms by gene was suggested (Darwin and Darwin, 1880). The heritable circadian rhythms concept opened the era of circadian rhythms investigation by genetic analysis in 1970s (McClung, 2006). Until now, the main research stream of plant circadian rhythms focuses on genetic analysis and gene modification . On the other hand, only a few manuscripts reported physiological aspects of circadian rhythms. Withrow and Withrow (1949) reported that normal cycle (12/12 h) had advantages over short (6/6 h) and long (24/24 h) cycles for the growth of tomato. An *Arabidopsis* mutant with elongated circadian rhythms accumulated more biomass at a longer cycle (Green et al., 2002; Dodd et al., 2005). However, because solid mechanism of circadian rhythms feedback loop has not been well understood, it is necessary to approach from fundamental aspects of plant physiology.

Most of plants take CO_2 for their photosynthesis during the light period, however, some plants take CO_2 during the dark period while they do their photosynthesis during the light period with captured CO_2. This type of photosynthesis called CAM (Crassulacean Acid Metabolism) which has a distinguishable CO_2 uptake pattern that affects exogenous daylight cycle. One of the plants processing CAM is orchids. Orchidaceae has more CAM species than other plants. Because they usually are known to take CO_2 during the dark period, which is used for their photosynthesis, they have their own CO_2 uptake patterns.

Therefore, external light has an effect to regulate CAM photosynthesis (Kluge and Ting, 1978). From 12/12 h light/dark cycle, relatively short light period makes nocturnal stomata open and increase total CO_2 fixation during the dark period in *Kalanchoe blossfeldiana* (Queiroz, 1974). By contrast, a longer light period forwards daytime CO_2 uptake and inhibits fixation during the dark period, and finally results in limited growth (Nose et al., 1986; Sekizuka et al., 1995).

Orchids are distributed all regions of the world except Antarctica and are found growing in many different habitats and elevation gradients (Pridgeon, 2000). Despite the diversity of orchids in nature, only a small number of genera including *Phalaenopsis, Cymbidium*, *Dendrobium*, and *Oncidium* are cultivated in large quantities as commercial ornamental crops. In particular, *Phalaenopsis* hybrids that include *Doritaenopsis* species have become the most popular potted orchids because of their ease of scheduling to meet specific market dates, high wholesale value, and long post-harvest life (Wang and Lee, 1994).

As many orchidaceae plants do, *Doritaenopsis* also has a typical CAM process for its photosynthesis. Length of light period has been known to regulate CAM photosynthesis (Kluge and Ting, 1978). However, there has been little studies to investigate the effect of shortened light/dark cycle on the vegetative growth and CO_2 uptake of orchid plants

Therefore, the objective of this study was to determine the effect of shortened light/dark cycle on the CO_2 uptake and vegetative growth of immature *Doritaenopsis* Queen Beer ‘Mantefon’ in order to reduce the production period.

LITERATURE REVIEW

Circadian Rhythm

The 24-h daylight cycle is induced by the Earth's rotation. Biological influence of the 24-h cycle was excavated since 1700s. From several studies reported that plant biological cycles are adjusted depending on the external daylight cycle. This internal biological cycle is referred to as 'circadian rhythms'. The word 'circadian' originated from two Latin words which are 'CIRCA (about)' and 'DIES (day)' (McClung, 2006). Thus, the root of word 'circadian rhythms' is connected to the light and dark cycle of the day. The major role of circadian rhythms in plant is to adapt to the external environment in which the plant can continue its physiological functions (Dodd et al., 2015). Hereby, circadian rhythms manage and regulates the proper growth of plants (Dodd et al., 2005).

The heritable circadian rhythm concept is closely related to the development of genetic analysis in 1970s (McClung, 2006). Major research area of plant circadian rhythms focuses on genetic analysis and gene modification to control plant circadian rhythms and resulting biological features. Conversely, only a few researches have been conducted on the plant circadian rhythms control by the environmental condition manipulation. Preliminary work of Withrow and Withrow (1949) reported that the normal cycle (12/12 h) was beneficial for tomato growth when compared to the short (06/06 h) and long (24/24 h) cycles. A study of

Arabidopsis mutant reported that longer circadian rhythms could accumulate higher amount of biomass (Green et al., 2002; Dodd et al., 2005).

CAM photosynthesis

Crassulacean acid metabolism (CAM) is one of pathway to fix carbon in plants as an adaptation to arid area. The term Crassulacean Acid Metabolism was introduced in the 20C by observing *Bryophyllum calycinum*, a crassulacean plant that had a diurnal variation in leaf acid content. (Matiz, 2013) The most distinguishable characteristics of CAM plants is that the stomata in CAM plants remain shut during the day, get open during the dark period to collect CO_2. In details, CO_2 is absorbed during the dark period and fixed into malate or titratable acid (Phase I). At dawn, because the stomata remain open, CO_2 is fixed via enzymes. In the daytime, the stomata get closed, and organic acids which are stored during the dark period are decarboxylated, generating CO_2 for Calvin cycles. At dusk, stored organic acids become exhausted so the stomata re-open, allowing the CO_2 to be taken up right away via the Calvin-cycle (Lüttge, 2004). This process is effective to reduce photorespiration because main enzymes ratios for photosynthesis have been estimated larger than other plants which have different process (Osmond et al. 1999; Clanton C. Black, 2003).

Doritaenopsis

In 1950's, there were improvements in *Phalaenopsis* cultivation techniques to get high-quality, pink *Phalaenopsis* flowers. After then, diversification in the *Phalaenopsis* and other related species were bred. The first *Doritaenopsis* was incubated from the bigeneric hybridization of *Phalaenopsis equestris* and *Doritis pulcherrima*. After the birth of the first *Doritaenopsis*, many bigeneric hybrids of *Phalaenopsis* and *Doritis* had been born because *Doritis pulcherrima* could easily produce pink flowers. With the influence of *Doritis* predominating in their backgrounds, *Doritaenopsis* hybrids had a fame for the size of the inflorescences they produce. Subsequent breeding was focused on *Phalaenopsis*, to increase the size of flowers by strengthening the impact of *Phalaenopsis* (Batchelor, 1982).

Doritaenopsis also take up atmospheric CO_2 during the dark period, showing phases of CAM plants (Osmond, 1978).

MATERIALS AND METHODS

Plant and Growth Conditions

Clones of tissue cultured 4-month old *Doritaenopsis* Queen Beer 'Mantefon' were purchased from Sangmione Orchid Nursery (Taean, Korea) and transported to Seoul National University Farm (Suwon, Korea). The plants were transplanted into 10 cm pots filled with 100% sphagnum moss. The plants were irrigated every week with a water-soluble fertilizer (EC 0.8 $mS \cdot cm^{-1}$; Hyponex professional 20N-20P-20K, Hyponex Japan Corp., LTD., Osaka, Japan). Twelve uniform plants were chosen per each treatment for the experiment. The average leaf span was 4.3 cm measured from stretched leaves and the number of total leaves was 11 at the beginning of the experiment. Leaf span was measured by extending the longest leaves to a horizontal position, measuring the length from tip point of one leaf to another tip point of opposite leaf (Blanchard and Runkle, 2006).

Photoperiod and Temperature Treatments

Doritaenopsis Queen Beer 'Mantefon' clones of uniform size were grown in a closed-plant factory maintained at 28°C. Each compartment provided four different light/dark cycles: 06/06 h, 08/08 h, 10/10 h, and 12/12 h. A photosynthetic photon flux density (PPFD) of 160 ± 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was provided during the light period by warm-white LED lamps (GMG Korea Co., Ltd., Ansan, Korea). The PPFD was measured by using a spectrum solar electric quantum meter (Spectrum Technologies, Inc., Aurora, IL, USA) at the plant canopy. Twelve plants with 3-4 fully expanded leaves were placed in each compartment and grown for 17 weeks. Locations of plants were randomly rotated every week to maintain uniform light conditions.

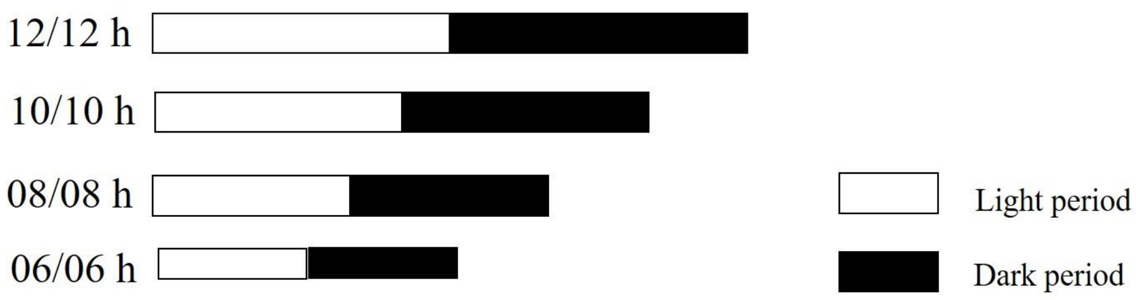

Fig. 1. Schematic diagram of four different light/dark cycle treatments.

Data Collection and Analysis

The number of new leaves (longer than 1.0 cm), the number of total leaves, leaf span, and leaf chlorophyll content were measured every 4 weeks. Length, width, and thickness of the uppermost matured leaf were also measured. The leaf chlorophyll content was measured with the uppermost matured leaf by using a chlorophyll meter (SPAD 502, Konica Minolta Sensing, Inc., Sakai, Osaka, Japan). Three plants were randomly collected from each light/dark cycle, the youngest mature leaves were used for photosynthetic gas exchange measurement for 24 h, and leaf conductance was measured at 5 weeks and 9 weeks after treatment with an infrared gas analyzer system (LI-6400, Li-Cor, Inc., Lincoln, NE, USA). External air was scrubbed of CO_2 and mixed with a supply of pure CO_2 to create a standard concentration of 400 $\mu mol \cdot mol^{-1}$ and flow rate was 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Each plant had been measured for 25 h and data was logged every 10 min for detecting unique photosynthetic patterns of CAM.

Statistical analysis for vegetative growth was performed by using SAS system for Windows version 9.3 (SAS Institute, Inc., Cary, NC, USA). Differences among the treatment means were assessed by Tukey's honestly significant difference test at $p < 0.05$. Regression and graph module analyses were performed by using Sigma Plot version 10.0 (Systat Software Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Effects of Light/Dark Cycle Manipulation on CO_2 Uptake Patterns

Under various light/dark cycle manipulations, 'Mantefon' exhibited CAM photosynthesis patterns (Fig. 2) and stomatal conductance patterns (Fig. 3). The patterns of CO_2 uptake in each light/dark cycle were similar to those in 2016 and 2017, where both experiments were processed with the same schematic time table (Fig. 1). The CO_2 uptake rate of 12/12 h cycle showed typical patterns of CAM plants (Fig. 2a). There was a gradual increase with no abrupt increase in the stomatal conductance after the dark period started. After 5 and 10 weeks of treatment, the net CO_2 uptake rate under 06/06 h treatment a showed sinusoidal pattern in which it started to increase from the midpoint of dark period and peaked at dark/light transition point (Fig. 2d). Then, it started to decrease and flattened at midpoint of light period.

From the first and second cycle, there was a fluctuation in the maximum CO_2 uptake rate value after 10 weeks of treatment. A similar pattern was observed from the stomatal conductance rate in 06/06 h treatment (Fig. 3d). The net CO_2 uptake rate in 08/08 h treatment started to increase linearly at light/dark transition point. Then, it dropped abruptly and flattened during the light period (Fig. 2c). The stomatal conductance in 08/08 h treatment also showed the similar trend, but started to increase from the mid dark period instead of light/dark transition point (Fig. 3c). The net CO_2 uptake in 10/10 h treatment started at light/dark transition points and peaked at the mid dark period. Then, it started to decrease and flattened at early light period (Fig. 2b).

In many cases, CAM is known as an adjustable metabolic system. Therefore, CAM plants quickly respond to the changes in environmental conditions (Franco et al., 1990; Haagkerwer et al., 1992; De Mattos and Lüttge, 2001; Dodd et al., 2002; Lüttge, 2004; Chen et al., 2008). As Fig. 2 showed, in 10/10 h treatment, CO_2 uptake patterns were similar to those in 12 /12 h treatment. In 10/10 h treatment CO_2 uptake began soon after dark period began. CO_2 uptake continued even after the light period began. However, when the light/dark cycle were shortened to 06/06 h (Fig. 2b) and 08/08 h (Fig. 2c), the start point of CO_2 uptake moved forward by 3 and 4 h, respectively, to the midpoint of dark period. In 06/06 h treatment, CO_2 uptake pattern showed different patterns compared with that in 12/12 h treatment (Fig. 2a,d). CAM plants usually close their stomata during the light period to minimize the loss of water and CO_2 in plants (Black, 2003).

CO_2 and water must be taken up by the open stomata during the dark period. Furthermore, the onset of darkness not only stops CO_2 uptake, but also starts the degradation of chloroplast starch (Dodd et al., 2002). The amount of starch in the chloroplasts falls through the dark period because breakdown products flow to the cytosol to sustain the export of sucrose to other organs (Taiz and Zeiger, 2006).

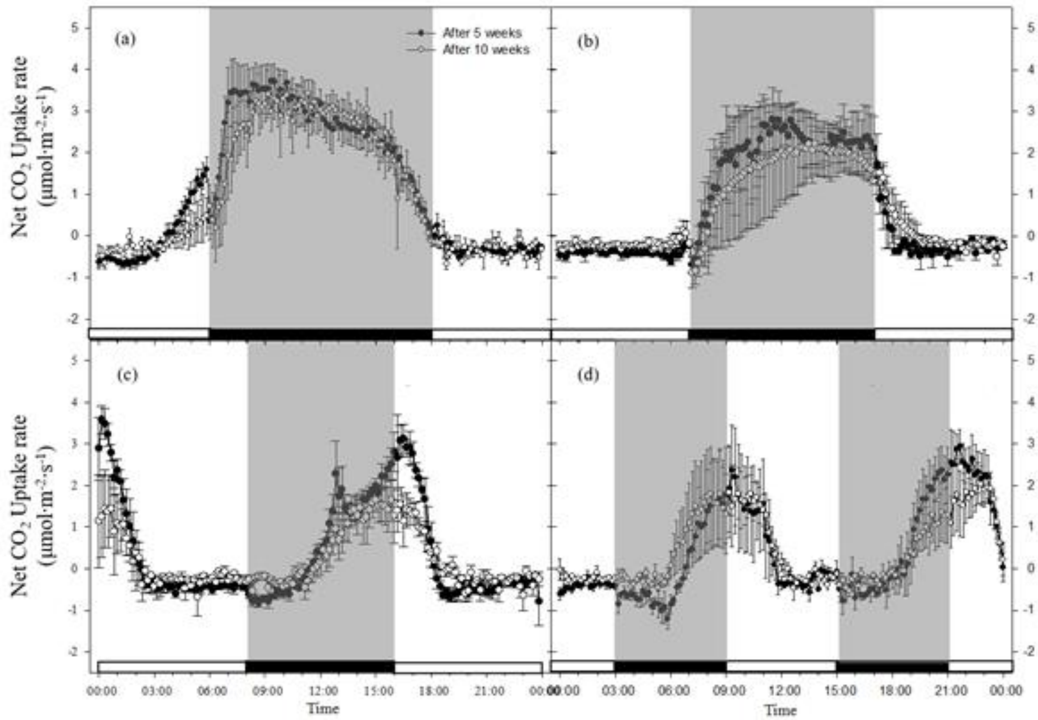

Fig. 2. The diurnal patterns of net CO_2 uptake rate of *Doritaenopsis* 'Mantefon' under 12/12 h (light/dark, a), 10/10 h (b), 08/08 h (c), and 06/06 h (d) treatments.

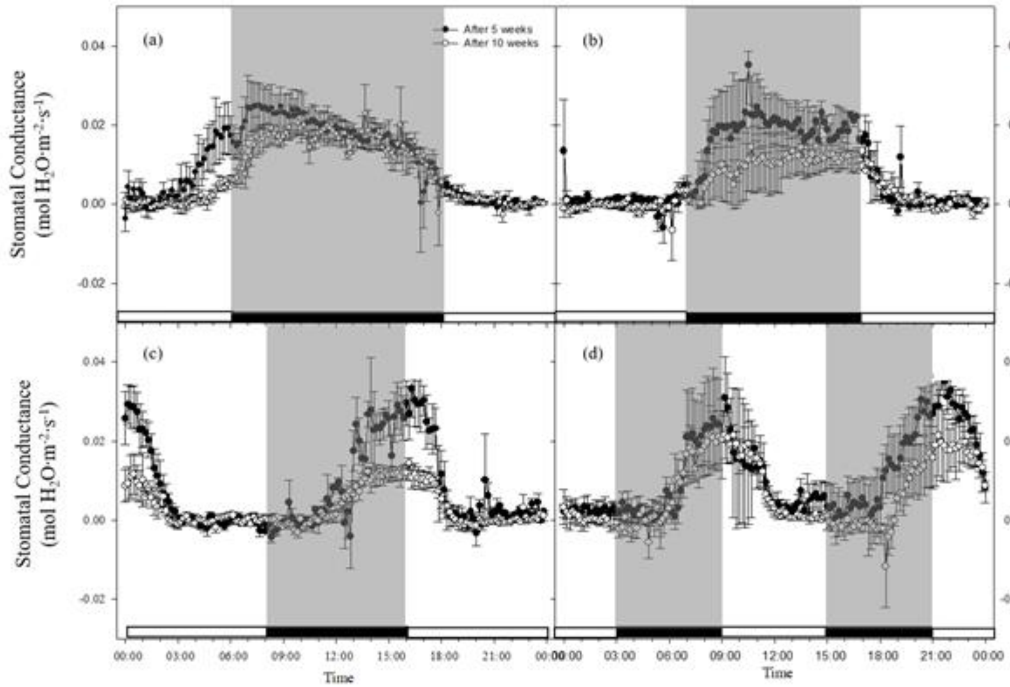

Fig. 3. The diurnal patterns of stomatal conductance of *Doritaenopsis* 'Mantefon' under 12/12 h (light/dark, a), 10/10 h (b), 08/08 h (c), and 06/06 h (d) treatments.

Doritaenopsis is a CAM plant (Cui et al., 2004) which fixes atmospheric CO_2 via open stomata during the dark period and does photosynthetic assimilation via the Calvin cycle during the light period (Chen et al., 2008). Because CAM plants possess a cyclical and reciprocal fluctuation of CO_2 uptake (Osmond, 1978), those patterns of CO_2 uptake affected by in vitro light/dark cycle affects photosynthetic characteristics in CAM. Altered light/dark cycle also affected the plant's own circadian rhythms because circadian rhythms are adjusted by external environmental stimuli throughout the light period (Greenham, 2015). Therefore, if light/dark cycles were shortened, circadian rhythms in plants also changed many physiological responses.

This kind of disparity in CO_2 uptake could appear obviously in the total amount of CO_2 uptake. Based on these patterns (Fig. 2), total amount of CO_2 uptake was calculated in their own light/dark cycle manipulation (Fig. 4). Except 06/06 h treatment, total CO_2 uptake during the dark period occupied the most of the diel amount. Both total amount and uptake amount during the dark period decreased as light/dark cycle length was shortened. Especially in 06/06 h treatment, CO_2 uptake amount during light period was larger than that of dark period. This aspect of total CO_2 amount seemed to stem from stomata opening during light period. The maximum CO_2 uptake amount during the dark period was found from 12/12 h treatment followed by 10/10 h, 08/08 h, and 06/06 h treatment (Fig. 4). Conversely, the maximum CO_2 uptake amount during light period was found from 06/06 h treatment followed by 08/08 h, 10/10 h, and 12/12 h treatment. Total CO_2 uptake amount was the highest in 12/12 h treatment and the lowest in 08/08 h treatment. Sekizuka et al (1995) reported that relationship between photoperiod and CAM does have relationship with energy and another control factor of CAM. In CAM plants, a short period of light would result in incomplete decarboxylation of malate which can inhibits stomata opening during the dark period, and therefore, CO_2 uptake lowers. (Lüttge, 2007). The similar results were observed in 'Mantefon' leaves in which 06/06 h treatment resulted low amount of CO_2 uptake during the dark period. Also 08/08 h treatment showed low amount of CO_2 uptake during the dark period compared with those of 10/10 h and 12/12 h treatment. These results indicated that CO_2 uptake of plants could be completely influenced by external stimuli.

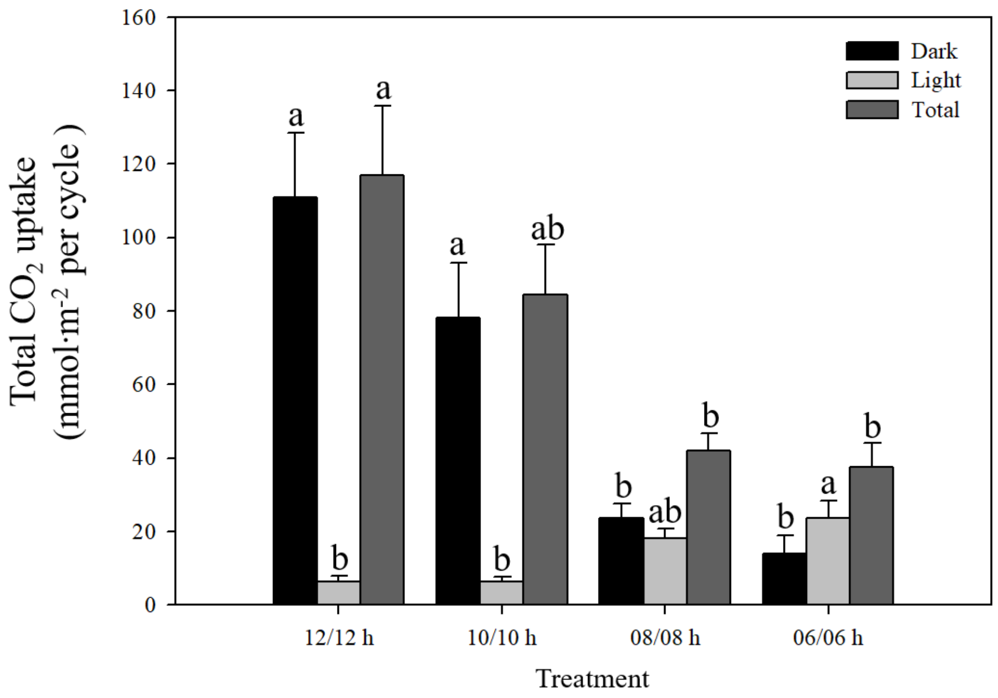

Fig. 4. Total CO2 uptake during the dark period (black), during the light period (light gray), and over a cycle of each treatment (dark gray) in the youngest mature leaf of *Doritaenopsis* 'Mantefon' in each treatment Results are means ± SE (n = 3); Means with the same letter are not significantly different at $p < 0.05$ by Tukey's honestly significant difference test.

Effects of Light/Dark Cycle Manipulation on Vegetative Growth

Overall growth showed differences to each other. (Table 1.) From the youngest mature leaf, no significant difference was found in leaf length. However, in leaf width and thickness, there were significant differences among treatments. 12/12 h and 10/10 h treatments showed wide and thick leaves than 06/06 h treatments. Especially in leaf thickness, 06/06 h treatments showed thin leaf than other treatments, with high leaf length/width ratio with no significance. There was no significant difference in the number of new leaves. However, as light/dark cycle treatment duration increased, the total number of leaves significantly increased. In fresh weight and dry weight, 06/06 h treatment showed the lowest values in both leaves and roots (Table 2). Contrary to the amount of CO_2 uptake, 10/10 h treatment resulted in the highest values in both leaf and root in dry weight (Fig. 6).

In an early study of the effects of photoperiod on plants vegetative growth, many researchers found that long light period promoted dry weight in many species of temperate grass (Heide et al., 1985, Hay, 1990, Solhaug, 1991). This study also showed that 12/12 h and 10/10 h treatments resulted in heavier dry weight than other two treatments. However, dry weight of 10/10 h treatment was higher than that of 12/12 h treatment. In some CAM plants, it was reported that short photoperiod could make the cell of plants get bigger so that the plants could absorb more CO_2 (Sipes, 1985). Sekizuka et al (1995) reported that hybrid of *Dendrobium* and *Phalaenopsis* had changes in its capacity for CO_2 uptake by photoperiod. Since we applied only four different light/dark cycle, more detailed research is needed to determine more appropriate light and dark period to promote vegetative growth more effectively.

Table 1. Effects of four different light/dark cycle on leaf characteristic of the youngest mature leaf and on leaf span after 17 weeks of treatment.

	Youngest mature leaf					
Treatment	Length (cm)	Width (cm)	Thickness (mm)	Ratio (length/width)	SPAD	Leaf span (cm)
12/12 h	11.16[z]	6.20 a	2.23 a	1.80	76.71	20.97
10/10 h	11.14	5.74 a	2.07 b	1.96	76.57	22.47
08/08 h	10.46	5.46 a	1.94 b	1.91	80.07	20.40
06/06 h	8.97	4.51 b	1.57 c	1.99	77.60	20.17
Significance	NS[y]	**	***	NS	NS	NS

[z]Mean separation within columns by Tukey's honestly significant difference test at $P < 0.05$
[y]NS, **, *** Nonsignificant or significant at $p < 0.01$ or 0.001, respectively.

Table 2. Effects of four different light/dark cycle on growth of the *Doritaenopsis* 'Mantefon' after 17 weeks of treatment.

Treatment	No. of new leaves	No. of total leaves	Fresh weight		Dry weight	
			shoot (g)	root (g)	shoot (g)	root (g)
12/12 h	3.14[z]	7.14	34.90 ab	20.03	2.10	1.43 ab
10/10 h	3.29	7.00	37.72 a	22.84	2.26	1.82 a
08/08 h	2.86	7.00	29.19 ab	19.43	1.90	1.60 ab
06/06 h	3.14	6.71	26.06 b	17.40	1.78	1.18 b
Significance	NS[y]	NS	*	NS	NS	*

[z]Mean separation within columns by Tukey's honestly significant difference test at $P < 0.05$.
[y]NS, * Non-significant or significant at $p < 0.05$, respectively.

Fig. 5. Effects of manipulated light/dark cycle on 4-month-old *Doritaenopsis* 'Mantefon' after 17 weeks of treatment.

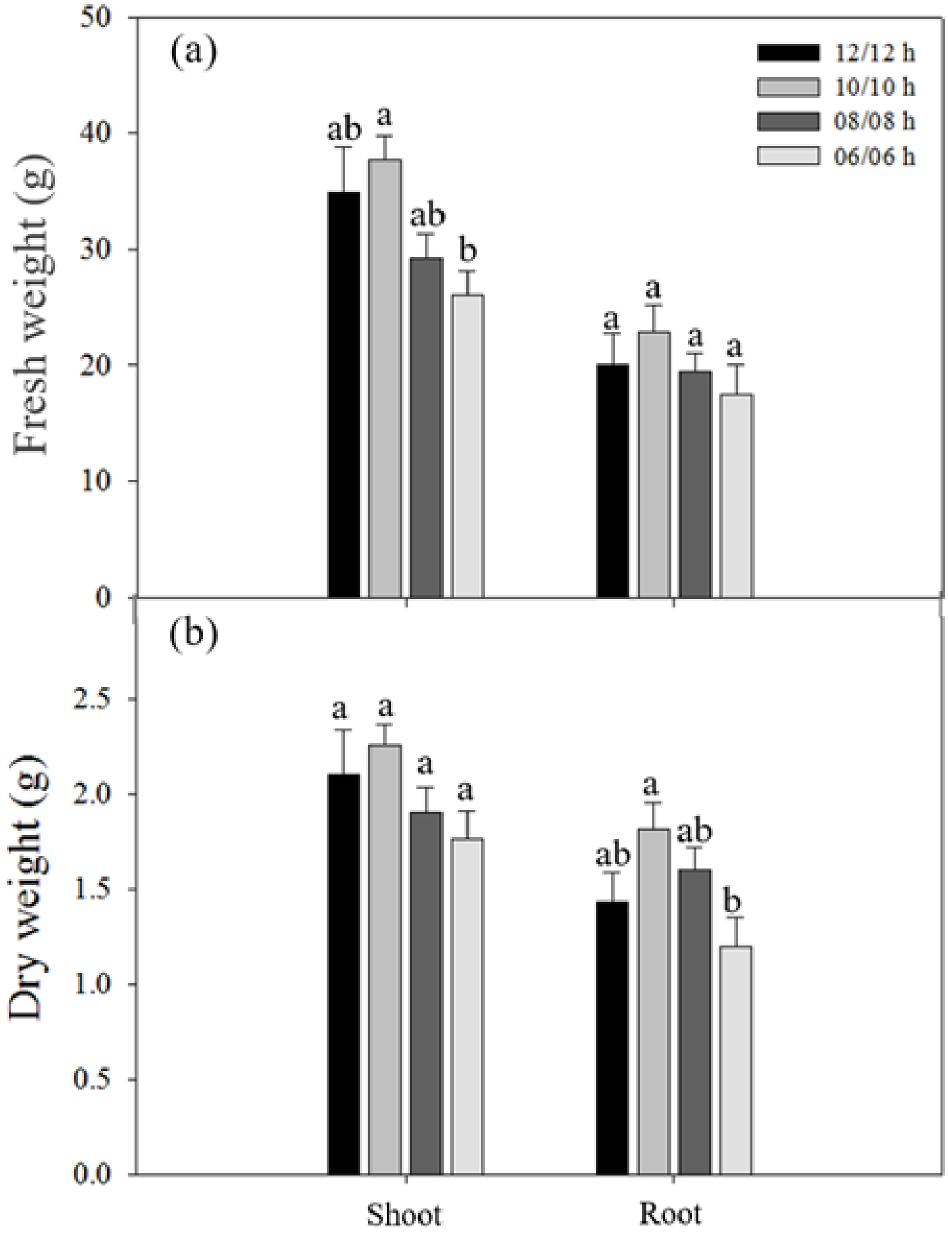

Fig. 6. Measured fresh weight (a) and dry weight (b) of *Doritaenopsis* 'Mantefon' after 17 weeks of treatment.

In conclusion, after manipulating light/dark cycle of *Doritaenopsis*, total CO_2 uptake amounts were the highest in 12/12 h treatment (control group), followed by 10/10 h, 06/06 h, and 08/08 h treatmenhts. In vegetative growth, only 10/10 h treatment closely followed 12/12 h treatment. The fresh weight and the dry weight of leaves and roots were the heaviest at 10/10 h treatment.

Because the deviation originated from the inherent characteristics of Crassulacean acid metabolism (CAM) photosynthesis pathway, detailed research is required to determine more effective ways to find appropriate light/dark cycle condition to enhance the vegetative growth of *Doritaenopsis*. As 10/10 h treatment resulted in the similar vegetative growth to 12/12 h treatment, more detailed studies are needed to determine the optimum length of light and dark cycle in order to reduce overall vegetative growth period.

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ABSTRACT IN KOREAN

본 연구에서는 명기와 암기를 조절하여 명암 주기를 기존의 24시간 보다 단축시켰을 때, 도리테놉시스의 영양 생장 및 광합성에 미치는 영향을 알아보고자 하였다. 식물 재료는 4개월 생 도리테놉시스 '만천홍' 품종을 사용하였으며, 명/암 주기는 각각 06/06 h, 08/08 h, 10/10 h, 12/12 h (대조구)를 처리하였다. 실험은 밀폐형 식물 공장에서 진행하였으며, 온도 및 상대습도는 각각 28°C, 80%, 모든 처리구의 광도는 160 ± 10 μmol·m^{-2}·s^{-1}를 유지하였다. 그 결과, 각각의 명암주기에서 각기 다른 이산화탄소의 흡수 패턴을 관찰할 수 있었다. 10/10 h, 12/12 h 처리구에서는 각각 암기가 시작되면서 바로 이산화탄소의 흡수가 시작됨을 보였으나, 그 보다 주기가 짧은 06/06 h, 08/08 h 처리구에서는 암기에서 이산화탄소의 흡수 시작점이 암기의 중간 지점으로 이동하는 것이 관찰되었다. 각각의 처리구가 갖는 한 주기 동안의 이산화탄소의 흡수량은 12/12 h 처리구에서 가장 높았다. 06/06 h 처리구는 명기 동안 이루어진 이산화탄소의 흡수량이 암기 동안 이루어진 흡수량 보다 많다는 특이점을 보였다. 4주 마다 반복적으로 영양생장을 측정한 결과, 12/12 h 처리구에서 가장 영양생장이 활발히 진행되었으며, 10/10 h 처

리구가 그 뒤를 이었다. 지상부와 근권부의 생체중과 건물중에 있어서는 10/10 h 처리구가 가장 큰 것으로 관찰되었다. 이에 따라, 10/10 h 처리구는 기존의 12/12 h 처리구와 비등한 영양 생장 효과를 가지고 있음이 확인되었다. 따라서 도리테놉시스의 생산 기간의 단축에 있어서, 명암 주기의 조절이 광합성 및 영양생장 기간을 단축시킬 수 있다는 가능성을 확인할 수 있었다.