Diurnal Photosynthesis and Metabolic Activity Year-round in Two *Dendrobium* Orchids Cultivars

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ABSTRACT

Dendrobium Sonia 'BOM Jo' and *Den*. 'Khao Sanan' are the most popular orchid cut-flower cultivars for exporting. Currently, orchid growers are facing climate variability that affect the quantity and quality of yields. Therefore, the objective of this research was to evaluate net CO_2 uptake and analyze organic acids and sugars contents year-round in two cultivars of *Dendrobium* hybrid. The experiment was conducted at a commercial nursery of Thaiorchids Company Limited, Damnoen Saduak, Ratchaburi Province, Thailand, with data collected every two months from June 2010 to April 2011. The two-year-old mature plants of *Den*. Sonia 'BOM Jo' and *Den*. 'Khao Sanan' with 4-5 pseudobulbs were measured for diurnal net CO_2 uptake, stomatal conductance (g_s), transpiration rate (E), organic acids and soluble sugars. The results showed that under commercial shade net house conditions, the diurnal net CO_2 uptake pattern of two cultivars was similar over the six measurement periods. Leaves exhibited a typical CAM photosynthesis, including dark CO_2 fixation (18:00-6:00), with the maximum value reached between 2:00–6:00, with 4−7 µmol m^{-2} s^{-1}. Net CO_2 uptake showed positive values in the early morning after sunrise (6:00–8:00) then, gradually decreased to zero in the daytime (8.00-10:00) until 18:00. Both *Den.* Sonia 'BOM Jo' and *Den*. 'Khao Sanan' varieties showed more than 80% of their total CO_2 uptake during the nighttime. Likewise, g_s exhibited a significant diurnal fluctuation in which the stomatal opening essentially occurred at night until early morning and remained closed throughout the day. E value generally followed g_s, with low values during the daytime, then gradually increased after sunset and reached two peaks after midnight and early morning. Only malic acid and sucrose were found in the leaves of the two *Dendrobium* cultivars, while citric acid, isocitric acid, glucose and fructose were not detected. The diurnal net CO_2 uptake and malic acid patterns indicated that both of the cultivars were strong CAM, despite year-round climate fluctuations. Net CO_2 uptake are intrinsically linked with the environment. Therefore, further studies should be conducted to determine enzyme activity and carbohydrate metabolism, with emphasis on the relationship between environment and photosynthetic parameters in CAM *Dendrobium* hybrids. All data have practical implications with regard to commercial cultivation in Thailand.

Keywords: CAM photosynthesis, CO_2 uptake, malic acid, orchid hybrid, sucrose

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INTRODUCTION

Orchids are the main export flowers of Thailand, with their total value ranked 2nd in the world after the Netherlands. Although orchid cultivation produces both cut orchid flowers and orchid pot plants, orchid cut flowers cover around 90% of the total orchid production area, most of them located in the central regions of Thailand. Growers can produce orchids at a competitive cost with a suitable climate and more than 40 years of orchid growing experience (Lekawatana, 2010). The most popular varieties for cultivation due to their high demand in the market are *Den.* Sonia 'BOM Jo' (*Den.* Caesar × *Den.* Tomie Drake) and *Den.* Khao Jiranand 'Khao Sanan' or *Den.* 'Khao Sanan' (*Den.* Walter Oumae × *Den*. White Doreen). They are fast growing, floriferous, and have a bright color and long vase life (Piluek and Wongpiyasatid, 2007). *Dendrobium* orchid cut flower production in Thailand typically involves plants grown on raised benches under 50−60% shade nets. Generally, *Dendrobium* flowers produce year-round, although they grow more during the rainy season than the dry, hot period (Lekawatana, 2010; Horticultural Science Society of Thailand, 2008).

Nowadays, it is thought that approximately 40% of tropical orchid species could exhibit some form of the crassulacean acid metabolism (CAM) pathway (Silvera *et al.*, 2010). Several researches reported that *Dendrobium* hybrids with thicker leaves perform through CAM photosynthesis (Sekizuka *et al.*, 1992; Khoo *et al*., 1997; He *et al.*, 1998). Additionally, these CAM pathways can operate in different modes; 'strong' to 'weak' in their leaves (Lüttge, 2004; Silvera *et al*., 2010; Borland *et al.*, 2011). For example, *Den*. *chrysotoxum* and *Den*. *nobile* were facultative CAM, while *Den*. *loddigesii* was strong CAM (Qiu *et al.*, 2015). The characteristic of the orchid photosynthesis pathway is based on the diurnal change in net CO_2 exchange rate, leaf titratable acidity, malic acid, leaf stomatal conductance and leaf carbon isotope values (Sekizuka *et al.*, 1992; Khoo *et al*., 1997; He *et al.*, 1998; Qiu *et al.*, 2015).

A few research studies, i.e. those on *Dendrobium* hybrid production under commercial shade net house conditions in Thailand, reported the diurnal CO_2 exchange rate in leaves showing a CAM photosynthesis pattern (Boonkorkaew *et al.*, 2003; Chuennakorn and Yingchajaval, 2010). However, there is no study regarding the variation in diurnal photosynthesis patterns in conjunction with metabolic activity monitoring year-round in *Dendrobium* orchid cut flowers under unique production conditions in Thailand. Furthermore, some researchers reported that acidity level of CAM orchids can vary with genus and species (Arditti, 1992; Chantrapradit, 2003) and that amounts of acids vary based on environmental factors (Knauft and Arditti, 1969; Pollet *et al*., 2011). Moreover, orchid growers are facing increasing fluctuations in climate that affect growth, flowering and subsequent production. Therefore, the objective of this research was to evaluate net CO_2 uptake and analyze metabolic activities (organic acids and sugars) year-round in most popular varieties of *Den.* Sonia 'BOM Jo' and *Den*. 'Khao Sanan'. Increased understanding of the photosynthesis pathway of these two *Dendrobium* hybrid cultivars is important for improving cultivation techniques.

MATERIALS AND METHODS

Plant Material and Environmental Conditions

The experiment was conducted over a year (June 2010 to April 2011) at a commercial orchid nursery of Thaiorchids Company Limited, Damnoen Saduak, Ratchaburi Province, Thailand. The two year old mature plants with 4−5 pseudobulbs of *Den.* Sonia 'BOM Jo' and *Den.* 'Khao Sanan' were taken from tissue culture and grown on a coconut husk box (4 plants per box), and raised benches 1 m × 40 m × 0.7 m. Plant density was approximately 20−24 plants per m^2 under a 50% shade net house expose to natural conditions. The environmental data were collected every 15 minutes. Air temperature and relative humidity were recorded by data logger (EasyLog EL-USB, USA), while light intensity was measured by line quantum sensors (LI-191, LI-COR,USA) with data loggers (Li-1400, LI-COR, USA).

At the experimental site year-round, the average temperature was 29.2−36.2°C (day), 21.6−27.1°C (night), and the difference between day and night was 5.5−9.1°C. The average relative humidity was 57.6−74.4% (day) and 86.6−91.4% (night). The day length was 12 ± 0.5 hours (6:00−18:00) and maximum light intensity ranged from 550−800 μmol $m^{-2}s^{-1}$. For growing conditions, irrigation was supplied daily each morning by a sprinkler system, except on rainy days. Foliar fertilizer composed of nitrogen, phosphorus and potassium (N-P-K) was applied at 4 g L^{-1} of 16-21-27 formula alternated with 4 g L^{-1} of 10-52-13 formula every other week according to good horticulture practices.

Sampling and Measurement

Sampling

The sampling was randomly done every 3−4 days, every other month over a year (June 2010 to April 2011). The photosynthetic parameters were taken from 10:00 a.m. to 10:00 a.m. the next day, performed every 2 hours on fully expanded leaves, from the middle part of the 3rd leaf to the front shoot. Five randomly selected plants (n = 5) were monitored. All determinations were replicated three times. Simultaneously, several leaf discs (0.1 g fresh weight each) were collected from the middle part of the testing leaf. Samples were frozen immediately in liquid nitrogen and stored at -80°C until metabolites analysis could be performed.

Measurement of Photosynthetic Parameters

Photosynthetic parameters, such as CO_2 uptake rate (CER), stomatal conductance (g_s) and transpiration rate (E) were measured on the fully expanded leaf (the 3rd leaf from shoot tip) using a portable photosynthesis system (LI-6400*XT*, LI-COR, USA). Each leaf portion (n = 5) was enclosed in a 2×3 cm leaf chamber (6400−08 Clear Chamber Bottom, LI-COR, USA). The Clear Chamber Bottom has a Propafilm® window similar to the standard chamber top. Measurements were recorded when the total coefficient of variation (CV) was less than 0.1% under shade net house exposed to natural conditions without controlled environment.

Metabolites Analysis

Organic acids

Organic acids (malic acid, citric acid and isocitric acid) were analyzed and determined by the Callaway *et al*. (1997) method. Leaf discs (1 g FW) were ground in liquid nitrogen with a pestle and added to 5 ml de-ionized water. The crude extract was transferred to test tubes, then boiled for 30 minutes, cooled to room temperature and centrifuged 10,000x g for 10 minutes. Organic acids were quantified using HPLC (Water e2695, Waters, Milford, MA, USA) and detected at 210 nm (Water, 2998, Waters, Milford, MA, USA). A Rezex-ROA-Organic Acid H^+(300 × 7.8 nm) cross-linked resin column from Phenomenex (USA) was used for analysis.

Soluble sugars

Soluble sugars (glucose, fructose and sucrose) were analyzed and determined by the Karkacier *et al.* (2003) method. In a pre-cooled mortar, 100 mg fresh weight tissue was ground with liquid nitrogen, extracted with 1 ml deionized water, vigorously shaken for 15 s, sonicated for 15 min and then centrifuged at 12,000 rpm for 15 min. The supernatant was filtered through a 0.45 μm syringe filter (model Verticlean^{Tm}, Vertical) and 0.1 ml of the filtrate was mixed with 0.9 ml of deionized water. The mixture was put through a 0.45 μm syringe filter and stored at -20°C. The chromatography system used was a Waters HPLC equipped with a Waters 600 Quat Pump and Waters 600 Controller, Waters 717 plus Auto sample and Waters 214 Refractive Index (RI) Detector. The column was MetaCarb 878 (7.8 × 300 mm, Varian, USA) and deionized water was used as the mobile phase at a flow rate of 0.4 ml min^{-1}. The sugar standards were obtained from Fluka (Fluka, Switzerland).

Statistical Analysis

Data was evaluated using ANOVA. For any significant different among treatments (6 months, namely June, August, October, December, February and April), further statistical analysis was done by the Duncan's new multiple range tests (*P < 0.05) using SAS version 9.1. Data were indicated as mean ± SE.

RESULTS AND DISCUSSION

CO_2 Uptake

Under a commercial shade net house, the diurnal net CO_2 uptake pattern of the two cultivars was similar over the six measurement periods. Leaves exhibited a typical CAM photosynthesis, including dark CO_2 fixation (18:00−6:00), and the maximum value was reached at 2:00−6:00, with 4−7 μmol CO_2 m^{-2} s^{-1}. It also had positive values in the early morning after sunrise (6:00−8:00) then, sharply decreased reaching values below zero in the late morning (8.00−10:00), then increased again but remained below zero until 18:00 (Figure 1A and 1B). Both *Den.* Sonia 'BOM Jo' (Figure 1A) and *Den*. 'Khao Sanan' (Figure 1B) showed more than 80% of their total CO_2 uptakes during the nighttime. Likewise, the stomatal conductance (g_s) exhibited a significant diurnal fluctuation wherein essentially all opening occurred at night until the early morning and remained closed throughout the day (Figure 1C and 1D). The transpiration rate (E) generally followed the pattern of $g_{S.}$, with a low value during the daytime, then gradually increasing after sunset and reaching two peaks after midnight and early morning (Figure 1E and 1F). The same pattern occurred in *Vanilla fragrans* (Nelson and Sage, 2008), identified as strong obligate CAM, in which plants were completely dependent on night and early morning for net CO_2 gain and had no CO_2 assimilation in the late afternoon (16:00−18:00). The CO_2 uptake in the early morning was a minor contribution as compared to that in the nighttime (Nelson and Sage, 2008). In contrast, *Den. loddigesii* plants, when grown under well-watered conditions, experienced a CAM-cycling pattern showing net CO_2 uptake only between 06:00−08:00, while it had negative values during most of the day (10:00−18:00) and nighttime (Qiu *et al.*, 2015). Additionally, the values of net CO_2 uptake of the two cultivars showed differences during the late morning (8:00−10:00). In December, February and April, the net CO_2 uptakes of *Den.* Sonia 'BOM Jo' were positive. Similarly, for *Den.* 'Khao Sanan', the values of net CO_2 uptake were more than zero in October, December and February while in June and August they had negative values (Figure 1A and 1B). This result indicated that CAM *Dendrobium* is intimately linked with the environment and can be perturbed by temperature, light level and water status (Dodd *et al.*, 2002).

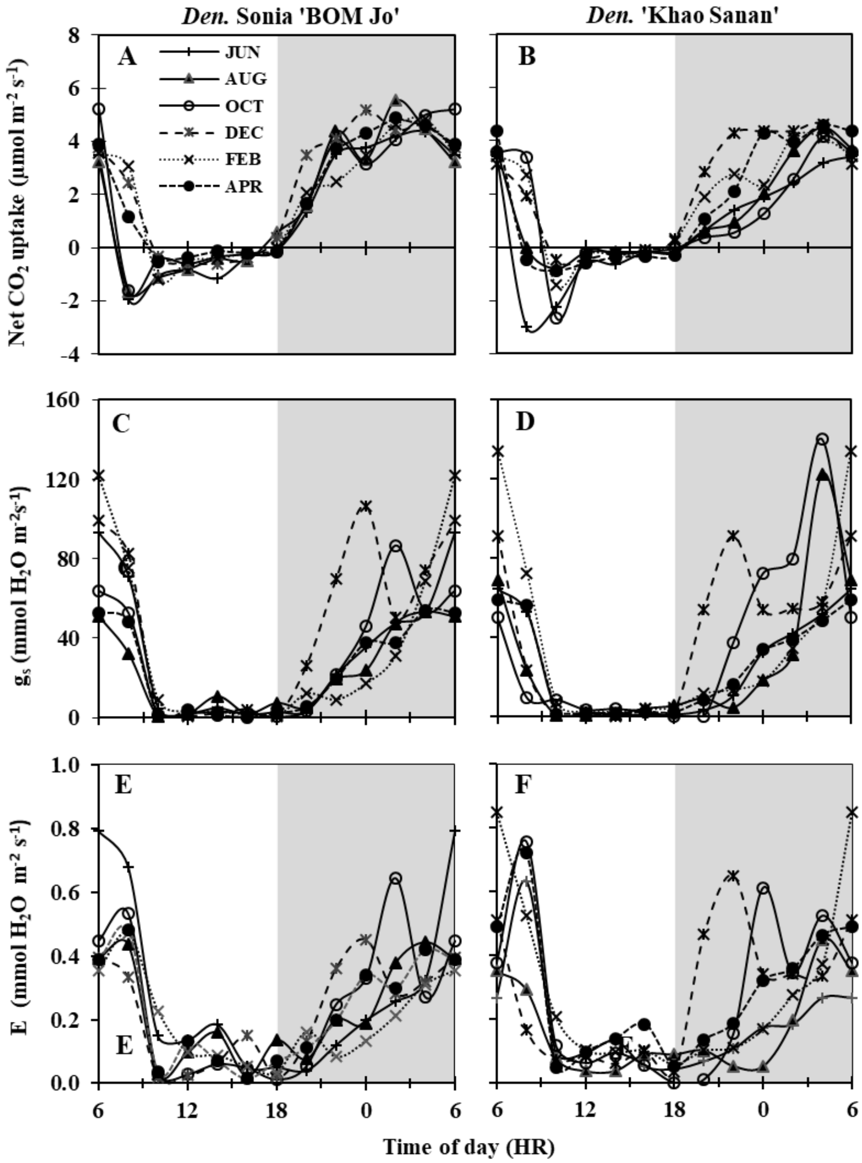

Figure 1 The diurnal patterns of net CO_2 uptake, stomatal conductance (g_s), and transpiration rate (E) of leaves *Den.* Sonia 'BOM Jo' (left panel; A, C, E) and *Den.* 'Khao Sanan' (right panel; B, D, F) year-round under a commercial shaded net house. Data was average of five individual plants. *Shaded portions* represent nighttime

Organic Acids

Malic acid was the major organic acid detected in leaves of the plant studied. The diurnal malic acid concentration of both *Den.* Sonia 'BOM Jo' (Figure 2A) and *Den*. 'Khao Sanan' (Figure 2B) suggested that both are CAM plants. Concentrations measured followed a typical CAM pattern, i.e. malic acid accumulation during the nighttime, with maximum values in the early morning (6:00−8:00); malic acid consumption during the daytime, with minimum values in the late afternoon (16:00−18:00). Figure 3A and 3B presented leaf malic acid analysis showing different intervals year-round. In leaves of *Den.* Sonia 'BOM Jo' (Figure 3A) malic acid built up to a maximum around 6:00, ranging between 18.2 to 26.5 mmol g^{-1} FW and declined to a minimum around 18:00, ranging between 5.0 to 8.8 mmol g^{-1} FW. Differences in malic acid contents at midnight, midday and dusk were significant (*$P < 0.05$) between months of monitoring and showed the highest value in December and lowest in June (Figure 3A). Leaves of *Den*. 'Khao Sanan' showed the same pattern as *Den.* Sonia 'BOM Jo' leaves, with maximum values of malic acid ranging between 19.4 to 28.1 mmol g^{-1} FW and minimum values ranging between 4.1 to 8.2 mmol g^{-1} FW, and malic acid content was significant (*$P < 0.05$) at dusk (Figure 3B). Several reports have indicated that CAM orchids including *Phalaenopsis* experienced citric accumulation (Chen *et al.*, 2008; Pollet *et al.*, 2011). This contrasts with the results of this study, in which the two *Dendrobium* cultivars demonstrated malic acid accumulation and fluctuation in the leaves while citric and isocitric acids were not detected. This distinction can be attributed to different growth characteristic patterns (monopodial in *Phalaenopsis* and sympodial in *Dendrobium*) and originating areas of the orchids (tropical and subtropical, respectively).

Soluble Sugars

Sucrose was the major free soluble sugar in the leaves of both *Den.* Sonia 'BOM Jo' (Figure 2C) and *Den.* 'Khao Sanan' (Figure 2D). The diel values of sucrose were approximately 2.0−5.0 mmol g^{-1} FW and fluctuations occurred year-round. The daily sucrose level showed differences between the two cultivars, with a higher sucrose content in *Den.* Sonia 'BOM Jo' than in *Den.* 'Khao Sanan' (Figure 2C and 2D). As seen in Figure 3C, sucrose content in leaves of *Den.* Sonia 'BOM Jo' showed significant (*$P < 0.05$) differences in the monitoring at dawn, midnight and dusk between months. August, October and December had the maximum values (3−5 mmol g^{-1} FW) while June and April had the minimum values (2−3 mmol g^{-1} FW). In summer (June and April) and the rainy season (August and October), sucrose content increased during the daytime, and reached a maximum level around 12:00−14:00, which was sustained until the evening, and decreased during the nighttime. Remarkably, the sucrose level in December increased during the daytime while between the two local maxima, a sudden decrease and subsequent increase was observed. In addition, the sucrose content in December was higher as compared with that in summer and rainy months (Figure 2C and 3C). The sucrose level of *Den.* 'Khao Sanan' during the nighttime, early morning and at dusk did not show significant differences and followed a similar pattern year-round, while at midday (12:00) there were significant (*$P < 0.05$) differences between months of monitoring, with the highest level in June at about 3.4 mmol g^{-1} FW (Figure 2D and 3D). In CAM plants, conservation of carbon as a carbohydrate during the light period, can be divided into two main groups, i.e., group I and group II (Christopher and Holtum, 1996; Black *et al.*, 1996). Group I stores mainly starch and glucans in chloroplast, while group II accumulates soluble sugars or polysaccharide in the vacuole. Both polysaccharide (starch) and soluble hexoses (sugars) are used as the precursor for glycolytic PEP formation (Fahrendrof *et al.*, 1987). The results indicate that the two *Dendrobium* hybrid cultivars may store sucrose as a substrate for nocturnal reactions. Conversely, the major free soluble sugars in leaves of CAM *Phalaenopsis* orchids were glucose, fructose and sucrose (Chen *et al.*, 2008; Pollet *et al.*, 2011). Meanwhile, *Den.* Sonia 'Earsakul' had these three sugars in its flower buds, but only sucrose in its leaves (Yanawat, 2008). In this study, glucose and fructose were not detected in the leaves of none of the cultivars, *Den.* Sonia 'BOM Jo' and *Den.* 'Khao Sanan'.

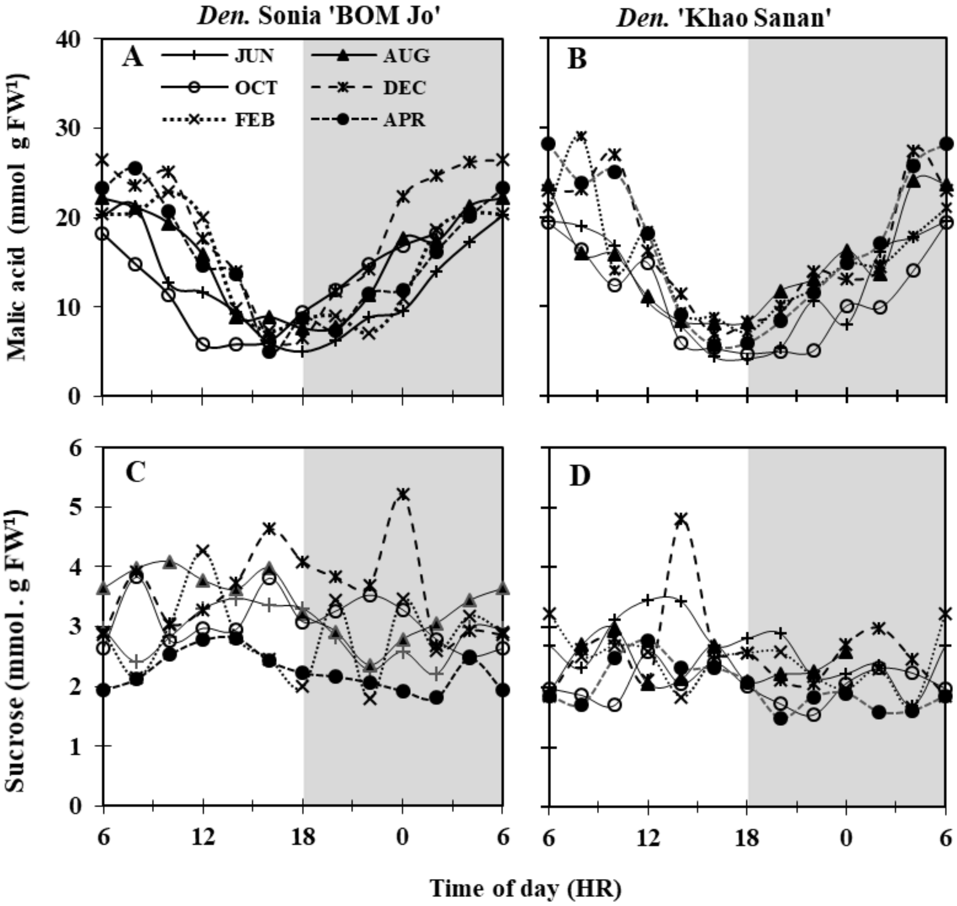

Figure 2 Diurnal pattern of Malic acid (A and B) and Sucrose (C and D) of leaves *Den.* Sonia 'BOM Jo' (left panel) and *Den.* 'Khao Sanan' (right panel) year-round under a commercial shaded net house. Data was average of five individual plants. *Shaded portions* represent nighttime

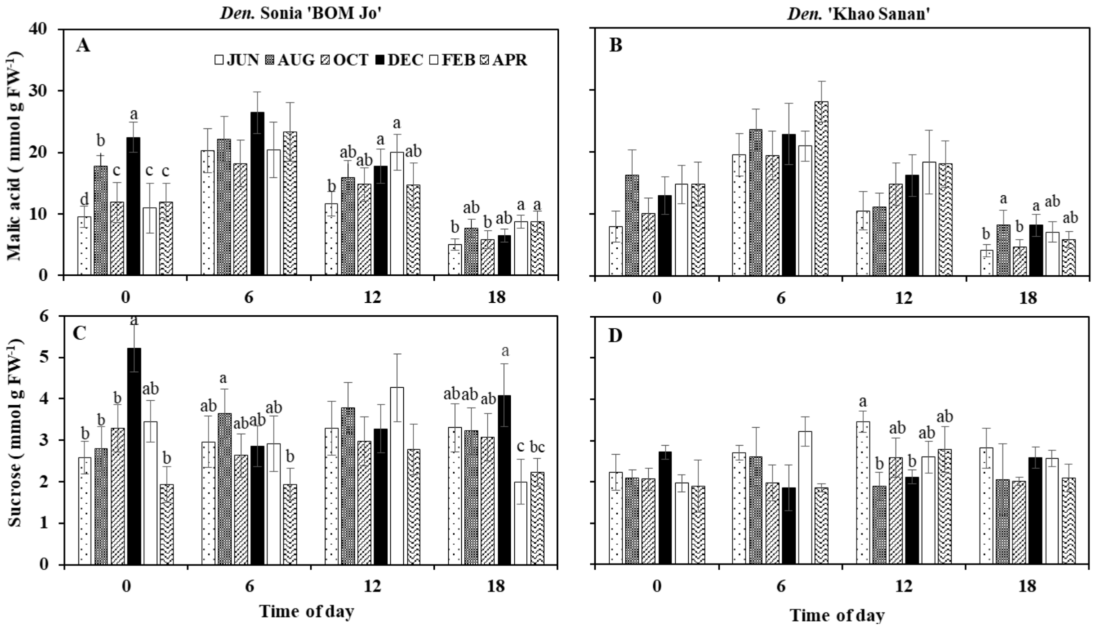

Figure 3 Malic acid (A and B) and Sucrose content (C and D) of leaves *Den.* Sonia ‘BOM Jo’ (left panel) and *Den.* ‘Khao Sanan’ (right panel) year-round under a commercial shaded net house. Data are means ± SE of five individual plants. The different letters indicate statistical significance (*P < 0.05)

Figure 4 shows the diurnal photosynthesis pattern in relation to metabolic analysis, i.e., CO_2 fixation through stomata during the nighttime until early morning in the two CAM *Dendrobium* cultivars. The receptor of CO_2 is phosphoenolpyruvate (PEP), derived from the breakdown of stored carbohydrates and resulting in the product malate, which is stored in the vacuole. During the day, malic acid is remobilized from the vacuole and decarboxylated. This decarboxylation generates high internal CO_2 concentration, which closes stomata in the leaf epidermis. The CO_2 released is re-fixed by ribulose-1,5-bisphosphate-carboxylase-oxygenase (RUBISCO) and assimilated via the Calvin cycle of photosynthesis (Osmond, 1978).

The data presented highlight strong implications about net CO_2 uptake duration during the late morning and year-round variation, both of which are intrinsically linked with the environment. Additionally, of the major of soluble sugars in leaves found, only sucrose differs from other CAM orchids. Therefore, further studies should be conducted to determine enzyme activity and carbohydrate metabolism, with emphasis on the relationship between environment and photosynthetic parameters in CAM *Dendrobium* hybrids. This information would increase understanding of the photosynthesis pathway and metabolic process of these hybrids. All data have practical implications with regard to commercial cultivation in Thailand.

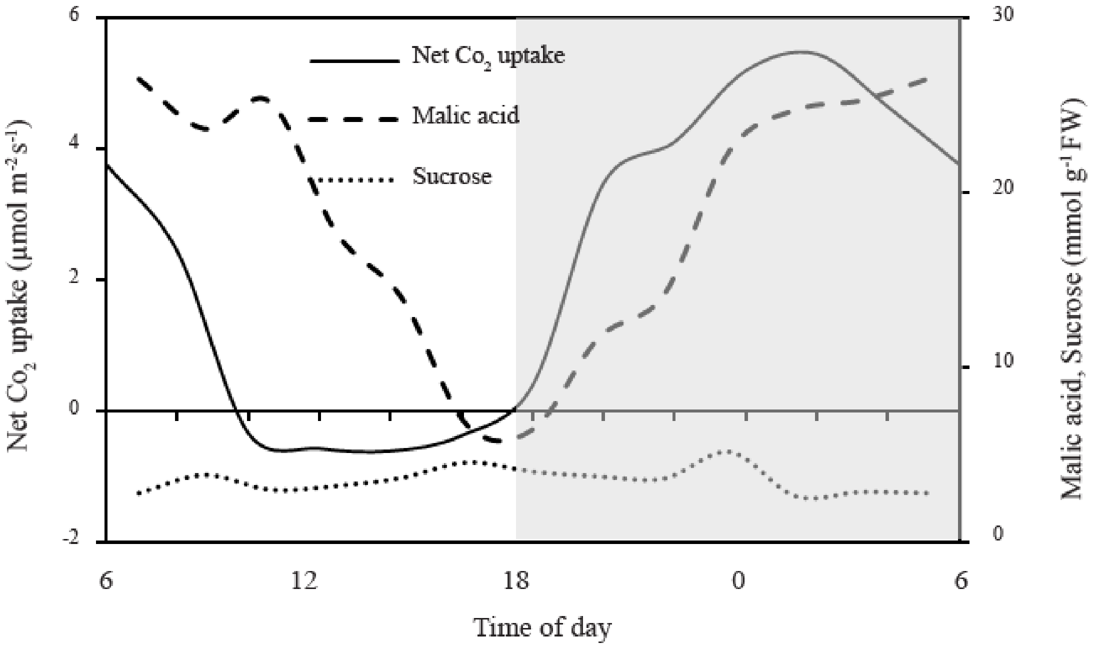

Figure 4 The relationship between diurnal net CO_2 uptake, malic acid and sucrose in leaves of two *Dendrobium* hybrid cultivars

CONCLUSIONS

Den. Sonia 'BOM Jo' and *Den.* Khao Sanan' leaves exhibited a typical CAM photosynthesis year-round in which the diurnal net CO_2 uptake, stomatal conductance, and transpiration rate patterns were similar over the six measurement periods. Only malic acid and sucrose were found in leaves of the two cultivars, while citric acid, isocitric acid, glucose and fructose were not detected. The relationship between the diurnal photosynthesis and malic acid indicated that both cultivars demonstrated a strong CAM photosynthesis pathway, despite year-round climate variability.

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