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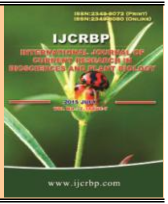

Original Research Article

Succulence Leaf of *Hoya* Species Influence the Photosynthesis Type and Drought Avoidance

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Abstract

Hoya is an epiphytic plant which has succulent and non-succulent leaves. Succulence is known as one of the characteristics of Crassulacean Acid Metabolism (CAM) plant which is adapted to drought conditions. The aim of this study was to measure the *Hoya*'s photosynthesis pathway with different succulent leaves under drought condition. This study used five *Hoya* species i.e., *H. verticillata* and *H. latifolia* (succulent), *H. bandaensis* (semi-succulent), *H. densifolia* and *H. multiflora* (non-succulent). The results showed that the succulence of leaf affected *Hoya*'s photosynthetic pathway. Under well-watered condition, *H. Verticillata*, *H. latifolia* and *H. bandaensis* showed pathway of CAM photosynthesis, while *H. densifolia* and *H. multiflora* showed intermediate activity of C3-CAM (CAM cycling). *H. verticillata*, *H. latifolia*, and *H. bandaensis* were able to avoid from drought, which were characterized by no change in photosynthetic pathway during drought condition. On the contrary, *H. densifolia* and *H. multiflora* has a weak CAM photosynthesis due to the decrease of photosynthesis during drought condition.

Keywords

CAM cycling
CAM Photosynthesis
Non-Succulent
Succulent *Hoya* species

Introduction

Hoya is an epiphytic genus from Apocynaceae, subfamily Asclepiadoidea. There are about 150-200 *Hoya* species in the world (Burton, 1992) and 50-60 species found in Indonesia (Rahayu, 2003). Distribution of *Hoya* started from the lowlands to uplands with common occurrence in lowlands (Rahayu, 2012). *Hoya* has morphology leaf thickness variations, from thick leaf (succulent) to thin leaf (non-succulent) (Rahayu, 2010). Anatomy of succulent *Hoya* leaf different with non-succulent *Hoya* leaves especially on the mesophyll thickness (Hafis et al., 2013; Hakim et al., 2013).

The main characteristic of succulence in the plant tissue is their ability to store water to support their physiological function when the external water is not available (Ogburn and Edwards, 2012). Furthermore, the succulent leaf tissue also have a role in storing and accumulating organic acids required for physiological function which perform a typical photosynthetic pathway, namely Crassulacean Acid Metabolism (CAM) (Kluge and Ting, 1978).

The advantages of plant with CAM metabolism has high water use efficiency (WUE), due to ability to avoid from drought (Lüttge, 2004). Therefore, CAM is a key adaptation of photosynthetic carbon fixation to limited water supply (Cushman and Borland 2002). The characteristic adaptation of CAM plant to limited water supply is nocturnal CO_2 fixation and daytime CO_2 re-assimilation (Cushman and Borland, 2002; Lambers et al., 2008) and stomata closed during the day and opened during the night (Taiz and Zeiger, 2010). One of criteria to determined CAM photosynthesis is presence daily organic acid fluctuation (Cushman and Bohnert, 1999). The study has been done in many CAM species showed that highly succulence correlated with increased nocturnal organic acid accumulation (Griffits et al., 2008).
Several studied showed that *Hoya* had CAM photosynthetic. Rayder and Ting (1983a) found that *H. carnosa* had CAM photosynthetic under well-watered, but it would shift from CAM to CAM-idling modification under water stress. The result of Lieth and Werger (1992) research showed that *H. austrialis* and *H. nicholsoniae* indication strong CAM photosynthesis. Yusnaeni (2002) also showed that *H. difersifolia, H. lacunosa, H. carnosa, H. macrophylla*, and *Hoya sp*. had CAM activity (Full CAM) under sun habitat and watering every week. If they were grown in 75% shade with watering every day or every week, they had physiological adaptation, tend to C3 (CAM cycling).

However, the previous studies on photosynthetic pathway did not correlated with the leaf succulence on the other species. Therefore, in this study we choosen five *Hoya* species with different leaf succulence i.e. *H. verticillata, H. latifolia, H. bandaensis, H. densifolia*, and *H. multiflora* under different watering to measure the *Hoya* photosynthetic pathway. Furthermore, five *Hoya* species would be analysed based on the leaf succulence correlate with its photosynthesis under drought condition.

Materials and methods

Plant material and growth conditions

Plants material were chosen based on the data of leaf anatomy with the different leaf succulence from previous study by Hafis et al. (2013) and Hakim et al. (2013). Plants material used stem cutting (2 nodes length). The selected plant material was collected from Bogor Botanical Garden collection. Two nodes of *Hoya* stem cuttings were planted in sandy planting medium during one half month in the greenhouse condition. After one half month, stem cuttings were transferred to planting medium contained a tree fern log and cocopeat (5:1 w/w). Stem cuttings were maintained for seven months before drought treatment. Measuring of growth rate was conducted every week during 7 months.

Experimental design

The experiment was designed using a randomized block design (RBD) factorial with two factors of treatment. The first factor was five *Hoya* species. The second factor was drought treatment, namely: watered every 2 days (control) and drought treatment for a period of 1 month.

Leaf succulence

Leaf succulence was measured when the plant seven months old with parameters of succulence were:

- Leaf thickness: it was measured with Micrometer.
- Saturated Water Content (Ogburn and Edwards, 2012):

$$\frac{\text{leaf mass at full hydration (g)} - \text{dry leaf mass (g)}}{\text{dry leaf mass (g)}}$$

- Relative Water Content (%) (Prochazkova, 2001):

$$\frac{\text{Fresh weight - dry weight}}{\text{turgid weight - dry weight}} \times 100$$

- Succulence Mesophyll (Kluge and Ting, 1978):

$$\frac{\text{Water content (g)}}{\text{Chlorophyll content (mg)}}$$

Organic acid content

Measurements of organic acids in the leaves can be determined by measuring the content of proton (H^+) (Medina et al. 1993). Measurement of H^+ was done by using the acidity titration method following Griffiths et al. (2008) with modifications. Titratable acidity was conducted to analysed changes of leaf acidity during 20 h per day. Mature leaf disc (dia. 2 cm) from well watered and drought were boiled in 4 ml aquadest for 20 min. Furthermore, the extract was cooled and titrated to pH 7 with 10 mM NaOH (Griffiths et al., 2008).

Physiological parameters

Observation of physiological parameters was consisted of photosynthesis rate, transpiration rate, stomatal conductance, and Chlorophyll content measured by Licor 6400 Xt Nebraska USA. Chlorophyll content was analysed by using the method of Arnon (1949).

Data analysis

Data was analysed by analysis of variance (ANOVA) and continued by DMRT/Duncan Test.

Results and discussion

Succulence of leaf *Hoya* species characteristics

Leaves of *H. verticillata* and *H. latifolia* (succulent) had thick leaves, higher Saturated Water Content (SWC), Relative Water Content (RWC) and Succulence Mesophyll (Sm) than *H. densifolia* and *H. multiflora* (non succulent) (Table 1). These results are well supported by previous studies on succulent *Hoya* species with the changes observed in morphology (Rahayu, 2003) and anatomy (Hafis, et al., 2013; Hakim et al., 2013). The increasing thickness leaves followed by an increase of SWC and RWC in succulent *Hoya* and semi succulent. *H. verticillata* had SWC and RWC significantly different (p<0.05) with *H. bandaensis*, *H. densifolia* and *H. multiflora*. *H. bandaensis* had leaf thickness lower than *H. verticillata* and *H. latifolia*, but higher than *H. densifolia* and *H. multiflora*. Therefore, *H. bandaensis* had Sm higher (p<0.05) than the other four *Hoya* species (Table 1).

Differences succulence leaf *Hoya* was due to differences in the anatomical structure of leaf. Hafis (2013) and Hakim (2013) reported that thickness of leaf, mesophyll, epidermis, and cuticle of succulent *Hoya* were thicker than Non succulent *Hoya*. The increasing of leaf thickness correlated with increasing of mesophyll cells of leaf and then followed by an increasing SWC (Nelson and Sage, 2008; Ogburn and Edwards, 2012). Saturated water content showed the water storage capacity in mesophyll. SWC of succulent *Hoya* was higher than non succulent *Hoya*.

Table 1. Leaf thickness, Saturated Water Content (SWC), Relative Water Content (RWC), and Succulence mesophyll (Sm) of five *Hoya* species.

Species	Leaf type	Leaf thickness (μm)	SWC (gH_2O/g dry mass)	RWC (%)	Sm (gH_2O/mg Chlorophyll)
H. verticillata	Succulent*	1220.56a	18.71a	95.29a	13.40b
H. latifolia	Succulent*	866.11b	17.16a	95.85a	7.83bc
H. bandaensis	Semi succulent	626.67c	13.98b	89.73b	23.17a
H. densifolia	Non succulent**	315.70d	8.91c	89.70b	3.43c
H. multiflora	Non succulent**	225.69d	6.40d	86.95b	1.01c

Data in the table represent means. Different letter in the same column represent significantly difference at p<0.05 (Duncan's test). Source comparison: (*) Rahayu (2010) and Hafis et al. (2013). (**) Rahayu (2010) and Hakim et al. (2013).

Sm in five *Hoya* species were significantly different and influenced by leaf succulence with the range of Sm between 1.01 until 23.17 gH_2O/mg chlorophyll. The Sm value indicated the level of leaf tissue succulence (Herrera et al., 2010; Deshmukh and Murumkar, 2013). Kluge and Ting (1987) reported that Sm value can used to showed CAM metabolism in plant species. The plant with CAM metabolism has Sm value was more than 1 gH_2O/mg chlorophyll The plant with CAM metabolism, Sm value was more than 1 gH_2O/mg chlorophyll (Jimenez et al., 1983; Herrera et al., 2010; Deshmukh and Murumkar, 2013).

Organic acid content

Organic acid content in *Hoya* leaf was measured by using H^+ content in leaf tissue. The result showed that two succulent *Hoya* species (*H. verticillata* and *H. latifolia*) and one species semi succulent *Hoya* (*H. bandaensis*) had H^+ content fluctuation during 20 h period (Fig. 1) which showed similar measurements with CAM photosynthetic pathway plants (Griffits et al., 2008; Desmukh and Murumkar, 2013). Medina et al. (1993) reported that H^+ content in the leaf indicated organic acid content which was 2 and 3 times of H^+ for malic acid and citric acid respectively. *H. verticillata* had H^+ content was found to be highest at 06.00 am under well-watered condition (125.01 mmol H^+cm^{-2}). The pattern of H^+ fluctuation in this study similar with CAM plant such as *Kalanchoe daigremontiana, K. Pinnata* (Griffits et al., 2008), *Tillandsia pohliana* (Freschi et al. 2010), and *Commelina nudiflora* (Deshmukh and Murumkar, 2013) where H^+ content high in early morning in those species. Under stress drought, H^+ content in *H. verticillata* and *H. bandaensis* decreased significantly (p<0.05). H^+ content of CAM species *Xerosicyos danguyi* (Cucurbitaceae) (Rayder and Ting, 1983b) and *Pyrrosia lanceolata* (Chiang et al., 2013) decreased when exposed in drought stress.

Fig. 1: Diurnal fluctuation of leaf-sap titratable acidity in five *Hoya* species under well-watered and drought condition.

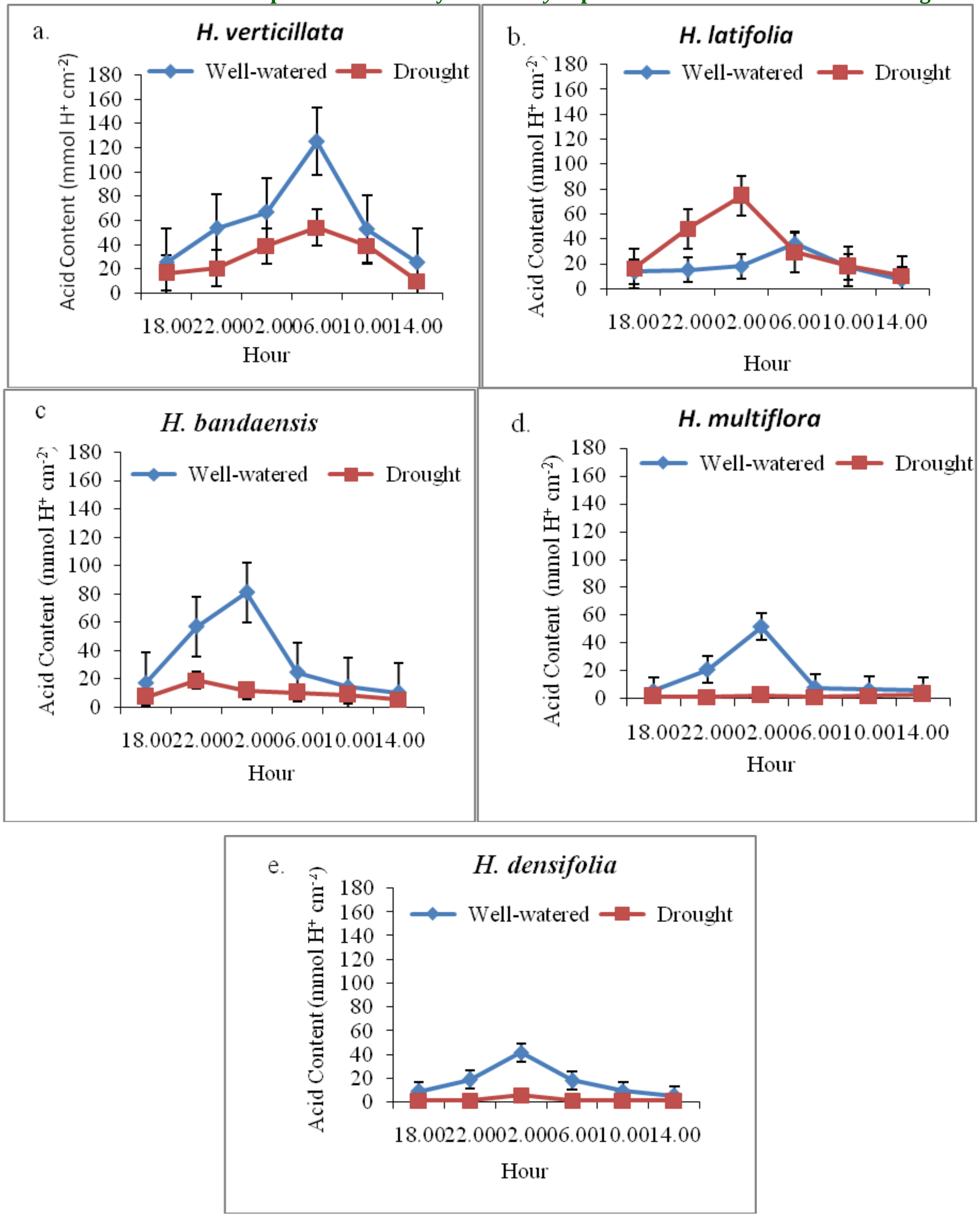


Table 2. Effect of water supply on physiological parameters of five *Hoya* species.

Species	Leaf type	Treatment	Photosynthesis rate (µmol CO_2 $m^{-2}s^{-1}$)	Stomatal conductance (mmol H_2O $m^{-2}s^{-1}$)	Intercellular CO_2 concentration (µmol CO_2 mol^{-1})	Transpiration rate (mmol H_2O m^{-2} s^{-1})
H. verticillata	Succulent	W	4.00ab	-4.53b	2708.85ab	-0.12b
		D	4.06ab	-2.59b	4290.02a	-0.08b
H. latifolia	Succulent	W	3.93ab	-2.18b	3612.62ab	-0.06b
		D	3.90ab	-3.93b	1771.60ab	-0.12b
H. bandaensis	Semi succulent	W	4.44ab	-4.63b	1927.98ab	-0.14ab
		D	4.43ab	-4.93b	1985.64ab	-0.15ab
H. densifolia	Non succulent	W	3.69ab	5.81ab	1218.10ab	0.35a
		D	1.55b	-2.06b	1145.82a	-0.03b
H. multiflora	Non succulent	W	6.00a	12.84a	-748.69c	0.20ab
		D	2.06b	-3.12b	2587.44ab	-0.08b

Data in the table represent means. Different letters in the same column represent significant difference at $p<0.05$ (Duncan's test).
W: Well-watered; D: Drought.

Table 3. Chlorophyll contents of five *Hoya* species at 8 MAP.

Species	Chlorophyll a (mg/L)	Chlorophyll b (mg/L)	Total chlorophyll (mg/L)
H. verticillata	0.85c	0.51b	1.43c
H. latifolia	1.12c	0.64b	1.76c
H. bandaensis	0.52c	0.125c	0.62d
H. densifolia	1.88b	0.88b	2.76b
H. multiflora	3.22a	1.37a	4.14a

Data in the table represent means. Different letter in the same column represent significant difference at $p<0.05$ (Duncan's test)

H^+ content of *H. densifolia* and *H. multiflora* increased in the night under well-watered, on the contrary, in the day, there was no H^+ content fluctuation both under well watered and drought condition. This result expected that two species of non succulent *Hoya* had photosynthetic intermediate C3-CAM (CAM-cycling). Allegations of CAM-cycling were supported by the high stomatal conductance indicating the opening of stomata in the daytime in non succulent *Hoya* (Table 2). Organic acid accumulation in night and stomata opened in daytime at Non succulent *Hoya* was similar with species intermediate C3-CAM (CAM cycling) like *Cissus quadrangularis* L. (Vitaceae) (Ting et al., 1983) and *Puya floccosa* (Herrera et al., 2010). Matiz et al. (2013) stated that CAM plants will accumulate organic acid at night and stomata open at daytime. Kluge and Ting (1978) stated that the characteristic of CAM cycling where stomata remain closed during the dark period but some nocturnal synthesis of organic acid fed by respiratory CO_2 occurs, and where stomata are open during the light period with uptake of atmospheric CO_2 and direct Calvin-cycle CO_2 reduction (C3-photosynthesis) in addition to assimilation of CO_2 remobilized from nocturnally stored organic acid.

This study reinforced that a degree of leaf succulence important for CAM metabolism as reported by Nelson et al. (2008) and Griffiths et al. (2008). Succulent *Hoya* and semi succulent showed the CAM metabolism under well watered and drought, but non succulent *Hoya* had not CAM metabolism in the same treatments.

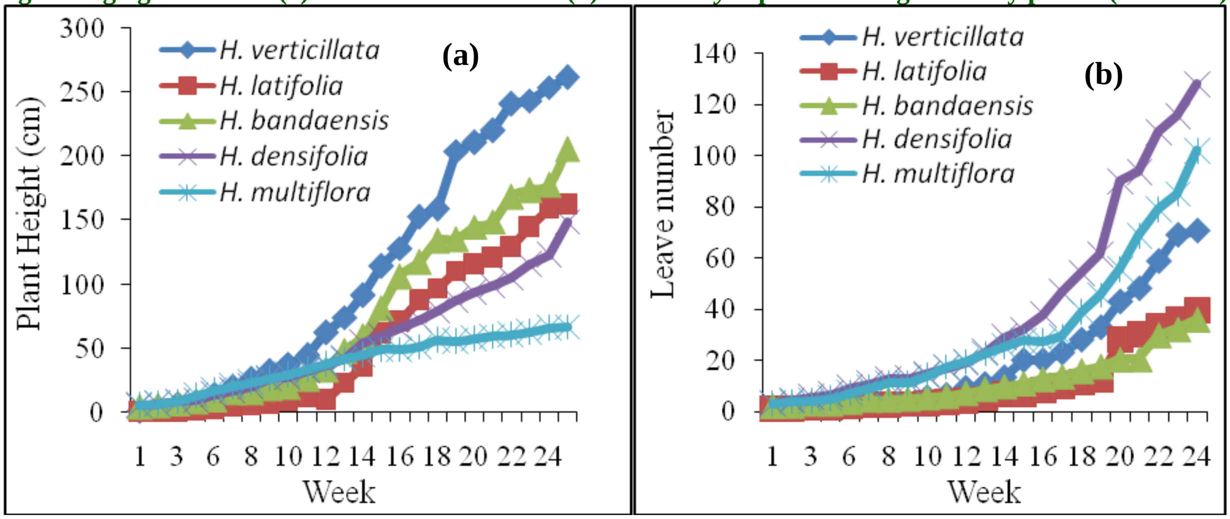
Growth rate

Growth rate in species succulent *Hoya* and semi succulent were lower as compared to non succulent *Hoya*, even the height of succulent and semi succulent *Hoya* was higher than non succulent *Hoya*. Leaf growth rate *H. densifolia*, *H. multiflora*, *H. verticillata*, *H. latifolia* and *H. bandaensis* were 6, 4, 3, 3, and 2 leaf $week^{-1}$ respectively (Fig. 2a), whereas high growth rate of *H. verticillata*, *H. bandaensis*, *H. latifolia*, *H. densifolia*, and *H. multiflora* were 11.3, 8.7, 7.0, 6.2, and 2.7 cm $week^{-1}$ respectively (Fig. 2b).

Different growth leave rate in five *Hoya* species probably correlated with different photosynthetic type. *H. verticillata*, *H. latifolia* and *H. bandaensis* were the CAM photosynthetic, whereas *H. densifolia* and *H. multiflora* were C3-CAM photosynthetic (CAM *cycling*). This fact supported by Black et al. (1996) that productivity of CAM plant lower than C3 plant due to the energy cost for CO_2 assimilation in the CAM plant higher than the C3 plant. High growth rate in succulent

Hoya more quickly than non succulent *Hoya* due to correlated with the habitus of five *Hoya* species. *H. verticillata*, *H. latifolia*, and *H. bandaensis* were climber plants. In climber plants, stem growth generally more quickly to support vertical shoot growth (Putz and Mooney, 1991).

Fig. 2: High growth rate (a) and number of leaves (b) of five *Hoya* species during the study period (25 weeks).



The effect of succulence to *Hoya*'s physiological parameters

The analysis data showed that there was no interaction between species *Hoya* with watering treatment on physiological parameters of *Hoya*, except in intercellular CO_2 concentration. The photosynthesis rate in succulent *Hoya* and semi-succulent when well watered were not significantly different ($p > 0.05$). The rate of transpiration under well water in *H. verticillata, H. latifolia* were significantly different with *H. densifolia* ($p < 0.05$), but the transpiration rate were negative. On the other side, stomatal conductance of *H. verticillata, H. latifolia, H. bandaensis*, and *H. densifolia* were significantly different ($p < 0.05$) with *H. multiflora* (Table 2). This condition showed that there was no transpiration detected and stomata closed when the measurement was carried out.

The rate of photosynthesis, intercellular CO_2 concentration, stomatal conductance and transpiration rate when the drought did not significantly different ($p > 0.05$) in semi-succulent *Hoya*. These results support our assumption that all three species of *Hoya* have a CAM pathway metabolic. The succulence on *Hoya* leaves influenced the low rate of photosynthesis compared to Non succulent *Hoya*. This is due to the low of mesophyll conductance to CO_2 and activity of Rubisco on succulent leaves (Griffits et al., 2008; Nelson and Sage, 2008). The lower mesophyll conductance is known to reduce the rate of photosynthesis in many species, such as the *Kalanchoe daegremontiana* (Grifitths et al., 2008), wild tomatoes (Muir et al., 2013) and Aizoaceae succulent (Ripley et al., 2013).

The leaf succulence reduced leaf stomatal conductance and the rate of transpiration in Succulent *Hoya* and semi-succulent (Table 2). The low of stomatal conductance and transpiration rate influenced succulent *Hoya* avoid to drought, even though the rate of photosynthesis still stable. In contrast, the rate of photosynthesis, stomatal conductance and transpiration rate at non-succulent *Hoya* in well-watered higher ($p < 0.05$) compared to succulent *Hoya* and semi-succulent. However, decreasing stomatal conductance and transpiration rate will be followed by decreased in the rate of photosynthesis when drought. Ogburn and Edwards (2010) reported that the succulent plants can be protected from drought, because it has a high hydraulic capacity as a consequence of the volume of water storage cells are large. In contrast to the non-succulent plants, have a low hydraulic capacity because it has a volume of cells or tissues are low to save water.

Chlorophyll content

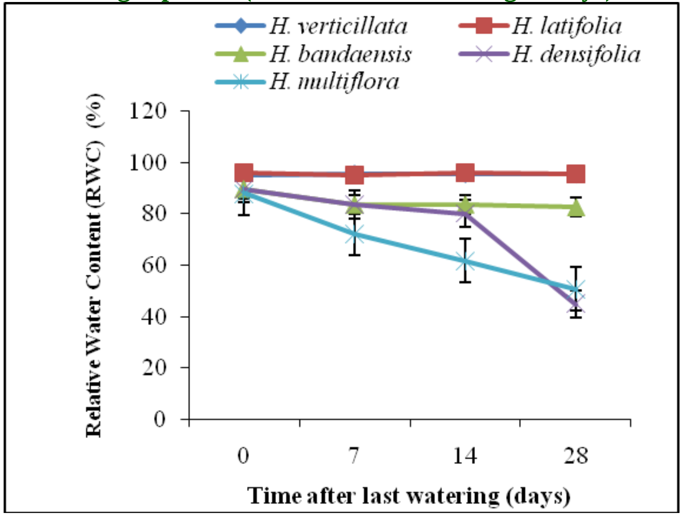
Succulent *Hoya* has chlorophyll a, b, and total chlorophyll content lower compared with non-succulent *Hoya* (Table 3). Chlorophyll b and total chlorophyll content in *H. bandaensis* (semi-succulent) was the lowest, while chlorophyll a, b, and total chlorophyll in *H. multiflora* (non-succulent) were the highest. Gibson

(1982) reported that the cells in the succulent tissue have a large vacuole for water storage that occupies 90% or more of the cell volume.

The effect of leaf succulence and drought RWC of leaf

Succulence of *Hoya*'s leaf affected RWC in some periods of drought (Fig. 3). RWC of *H. verticillata* and *H. latifolia* (succulent) tend to be stable up to 28 days after treatment. In contrast, RWC of *Hoya* non-succulent species decreased significantly (*p*<0.05), but the period for significantly decrease of RWC wary between species Decreasing RWC of *H. densifolia* 45% significantly different (*p*<0.05) was only occurred in periods of drought 28 days after treatment. RWC of *H. multiflora* decreased significantly (*p*<0.05) since in the 7 days after treatment. Lower RWC was observed in *H. multiflora* on 28 days after treatment by 37%. There was no decreasing of RWC in *H. bandaensis* until the end of the period of drought (28 days after treatment).

Fig. 3: Relative Water Content in five *Hoya* species during drought period (time after last watering in days).



Leaf RWC reflects the leaf water status. Leaf water status is closely related to several physiological variables such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and respiration (Kramer and Boyer, 1995). Lowering RWC of leaves causing a decreased of leaf turgor, resulting in withering, closed stomata and decreased the rate of photosynthesis (Alfredo and Setter 2000). In succulent *Hoya* with CAM photosynthetic pathway, water use efficiency (WUE) increased due to large water loss through the process of transpiration during the day can be minimized by regulation stomata are open during the night and close during the day (Luttge, 2004).

Conclusions

The succulence of leaf affected the *Hoya*'s photosynthetic pathway. Under well-watered, *H. verticillata* and *H. latifolia* (succulent) and *H. bandaensis* (semi-succulent) showed the CAM photosynthesis pathway, while *H. densifolia* and *H. multiflora* (non-succulent) showed the intermediate pathway C3-CAM (CAM cycling). *H. verticillata*, *H. latifolia* and *H. bandaensis* were able to drought avoidance which was characterized by there was no change in photosynthesis pathway when drought stress. In contrast, *H. densifolia* and *H. multiflora* has a weak of CAM photosynthesis and it decreased when drought stress.

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