Pak. J. Biotechnol. Vol. 15 (2) 505-511 (2018) ISSN PRINT: 1812-1837
www.pjbt.org ISSN Online: 2312-7791

# EFFECT OF DIFFERENT TEMPERATURE TREATMENTS ON PHYSIOLOGICAL TRANSFORMATION OF *IN VITRO PHALAENOPSIS* 'FORTUNE SALTZMAN' SEEDLINGS

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Article received 7.4.2018, Revised 6.6.2018, Accepted 14.6.2018

## ABSTRACT

*Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings were grown with 5 different day/night incubation temperatures (25/25ºC, 25/20ºC, 30/20ºC, 30/25ºC, and 35/25 º C). The seedlings used were clearly identified into three photosynthesis stages through $CO_2$ rhythm detecting. In Stage I (21 days after planting, DAP) it showed a typical C3 plant carbon fixation pattern but when plants continued to grow to Stage II (45DAP) they exhibited similar type C3-CAM plants. After 120DAP, plants showed significantly decreased $CO_2$ concentration at night, and showed a typical CAM plant carbon fixation pattern. The result showed that after 120 days, cultures with 30/20ºC and 30/25ºC incubation temperatures grew more stems and presented higher fresh weight and leaf lengths compared to the results of other treatments. Moreover, 30/25ºC treatment showed significantly higher responses in terms of dry weight, number of root, root length and number of leaf. The seedlings subjected to 30/25ºC treatment showed significantly higher levels of Rubisco enzyme activity than those subjected to the other treatments. Phosphoenolpyruvate carboxylase activities recorded during the night time in seedlings subjected to 30/25ºC incubation temperatures were also significant greater. Therefore, 30/25ºC treatment could advance growth of *Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings faster and can be recommended for commercial production.

Keywords; $CO_2$ rhythm, micropropagation, *Phalaenopsis,* physiological responses, temperature

## INTRODUCTION

*Phalaenopsis* is a monopodial and traditional horticultural epiphytic orchid species with high commercial value. *Phalaenopsis* originated from tropical and subtropical areas of the South Pacific Islands and Asia. In its native habitat, temperatures range throughout the year from 28 to 35ºC during the day and from 20 to 24ºC at night (Pridgeon, 2000). With the increasing market demand for orchids, studies to improve cultural techniques and precocious flowering of these plants have been developed. Temperature is one of the most important environmental factors regulating flower development in *Phalaenopsis*. Changes in temperature condition may affect several plants physiological, morphological and biochemical parameters of orchid tissue culture seedlings growth. To induce year-round flowering, many orchid growers in Taiwan and Japan have adopted a warm-night temperature (~28ºC) treatment to inhibit spike induction during autumn and winter, and a cool treatment (~20ºC) to stimulate spiking during summer (Blanchard and Runkle, 2006; Chen *et al*., 2008; Guo and Lee, 2006; Pollet *et al*., 2011; Sakanishi *et al*., 1980). Temperature is also a critical environmental factor affecting plant phenology. Temperatures constantly higher than 26 ºC promote the vegetative growth (i.e. juvenile phase) and inhibit flower induction; conversely, a lower thermal regime is required for flowering (i. e. flower transition and subsequent inflorescence development) (Lopez *et al*., 2007). Lootens and Heursel (1998) and Ota *et al*. (1991) reported that *Phalaenopsis* hybrid leaves have maximum net $CO_2$ uptake in a day time temperature of 20 to 25 ºC and night time temperature of 15 ºC. Thus, the objective of this study was to examine how different day/night temperatures influence photosynthesis, growth parameters and $CO_2$ rhythm changes of *Phalaenopsis* Fortune Saltzman tissue culture plantlets under different temperature conditions during growth periods.

## MATERIALS AND METHODS

**Plant material and growth conditions:** Tissue culture seedlings of *Phalaenopsis* Fortune Saltzman were ordered from Chi Yueh Company, Taiwan and were cultured on modified Hyponex medium (7N-6$P_2O_5$-19$K_2O$1 g $L^{-1}$ + 20N-20$P_2O_5$-20$K_2O$1 g $L^{-1}$). The medium was supplemented with 20g $L^{-1}$ sucrose, 2 g $L^{-1}$ peptone, 1 g $L^{-1}$ activated charcoal and 20g $L^{-1}$ potato. The pH was adjusted to 7.5 and 8 g$L^{-1}$of agar was added. Overall 10 seedlings/flasks were inoculated, and flasks were transferred to growth room at 25 ± 2°C under white florescent light with intensity 2000 lux for the growth and development (Parvez *et al.,* 2017). The seedling stages were checked using a CARBOCAP Carbon dioxide module

GMP222 connecting $CO_2$ probe (Vaisala, Finland) and LI-1,400 data logger mc (L1-COR, USA) to identity their physiological pattern. The measurement range is 0 to 10,000ppm of $CO_2$. Accuracy at 25ºC and 1013 hPa is ± (1.5 % of range + 2% of reading). After sample flasks were checked for $CO_2$ concentrations, seedling stages were verified for $CO_2$ concentrations over a period of $CO_2$ fixation. The seedlings were separated to three different stages according to Apinya (2012):

Stage I: Seedlings of 1-2cm in height with 1-2 leaves and 1-2 roots

Stage II: Seedlings of 3-4cm in height with 2-3 leaves and 2-3 roots

Stage III: Seedlings of 4-5cm in height with 3-4 leaves and 3-4 roots

The experiment was initiated using the seedling from stage I as the plant material

**Temperature treatment:** Stage I seedlings were cultured over a photoperiod of 12/12 h (day/night) in a growth chamber. Five different day/night temperature in the growth chamber were applied: 1) 25º /25ºC (Control), 2) 25ºC/20 ºC, 3) 30 ºC/ 20ºC, 4) 30ºC/25ºC and 5) 35ºC/25ºC

Fresh weight, dry weight, leaf length, root length, leaf quantity, root quantity and stem height values were determined at each stage to confirm the $CO_2$ rhythms and their physiological stages. The chlorophyll content analysis method used was modified from that presented by Kobza and Seemann (1989). Rubisco activity measurements were made according to Cheng and Fuchigami (2000) method with modifications. Phosphoenolpyruvate carboxylase (PEPC) measurements were recorded following Bradford (1976) method. Both measurements were also conducted twice at the beginning and end of the dark condition. Soluble sugars and starch contents were analyzed according to Yoshida *et al*., (1976) method. Finally, total nitrogen content was analyzed according to Kjeldahl (1883) method.

**Statistical analysis:** The Windows v.9.0 SAS program (SAS Institute Inc.) was used to analyze the data. When a significant ($p < 0.05$) difference was found for a measured parameter, means were separated using Tukey's HSD test (Zar, 1984).

## RESULTS AND DISCUSSION

**The effect of different day/night temperature on *Phalaenopsis* Fortune Saltzman tissue culture seedling growth:** After one month of culture, the seedlings of all treatments did not show significant differences in term of growth characters. However, after 5 months of culture, the seedlings subjected to 30/25ºC treatment showed significantly longer stem and leaves with higher fresh, dry weight, number of root and root length values (1.53cm, 3.88cm, 2.33g and 0.198g, 7.5, 5.96cm respectively) than those subjected to the other treatments. 30/20ºC treatment also showed significant higher values in term of leave length and root length (3.79cm and 5.34cm, respectively). For the 25/25ºC treatment (control), stem and leaf length and fresh and dry weight values reached 1.11cm, 2.75cm, 1.24g, and 0.078g respectively (Table 1). Our results showed that 30/25ºC temperature treatment promote *Phalaenopsis* 'Fortune Saltzman' seedling growth. In Taiwan, *Phalaenopsis* productions have been widely used 30/25ºC day/night temperature (Hung, 1998; Lee, 1988). There were several reports also showed that *Phalaenopsis* had the highest leaf production rate when grown under 30/25ºC (Lee and Lin, 1984, 1987; Lin, 1994; Lin and Lee, 1988; Wang 2005). Woei and Lee (2006) reported that 30/25ºC day/night temperature were optimal for *Phalaenopsis* leaf growth.

**Table 1:** The effect of different day/night temperature on growth of *Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings after 5 months of culture

| Treatment | Height (cm) | Leaf length (cm) | Fresh weight (g) | Dry weight (g) | Number of root | Root length (cm) |
|---|---|---|---|---|---|---|
| 25/25 | 1.11 ± 0.233 b | 2.75 ± 0.704 b | 1.24 ± 0.756 bc | 0.078 ± 0.001 e | 5.70 ± 1.059 b | 3.23 ± 1.129 b |
| 25/20 | 1.40 ± 0.337 ab | 3.38 ± 0.607 ab | 1.99 ± 0.863 ab | 0.095 ± 0.001 c | 5.60 ± 1.075 b | 4.27 ± 1.397 ab |
| 30/20 | 1.35 ± 0.292 ab | 3.79 ± 0.824 a | 2.00 ± 0.315 ab | 0.136 ± 0.001 b | 5.50 ± 1.080 b | 5.34 ± 0.675 a |
| 30/25 | 1.53 ± 0.408 a | 3.88 ± 0.763 a | 2.33 ± 0.698 a | 0.198 ± 0.001 a | 7.50 ± 1.649 a | 5.96 ± 1.808 a |
| 35/25 | 1.15 ± 0.310 ab | 2.77 ± 0.585 b | 1.06 ± 0.298 d | 0.080 ± 0.002 d | 4.80 ± 1.229 b | 3.06 ± 0.680 bc |

* Means followed by different letters within same column showed significant differences among treatments, based on ANOVA followed by Tukey's test at $p<0.05$. (n= 10)

**The effect of day/night temperature on chlorophyll, nitrogen, sugar and starch content in *Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings:** The seedlings grown under 25/20ºC and 30/25ºC showed higher levels of Chlorophyll a and total chlorophyll content than those grown under other treatments. After 1 month of cultureing, chlorophyll a, and total chlorophyll content

for 25/20 and 30/25ºC treatment reached 0.793 and 1.837, 0.821 and 1.959µg $ml^{-1}$, respectively (Table 2). After 5 months of culturing, chlorophyll a increased under 30/25 ºC, 30/20 ºC and 25/20 ºC treatments which reached 2.883, 2.778 and 2.235µg $ml^{-1}$, respectively. In term of total chlorophyll 30/25ºC and 30/20ºC showed significant higher total chlorophyll content than the control treatment (8.995 and 8.372 vs 4.835µg $ml^{-1}$) (Table 2). This showed that 25/20ºC, 30/20ºC and 30/25ºC significantly promoted the production of chlorophyll contents after 5 months of culture. After 1 month of culturing under 30/25ºC treatment, seedlings showed the significantly the highest nitrogen, sugar and starch contents (3.379, 3.45 and 3.940mg $g^{-1}$ respectively) of all of the seedlings studied (Table 3). After 5 months of culturing, nitrogen content increased under 30/20ºC and 30/25ºC which reached 4.413 and 4.610%, respectively while control treatment was 3.677%. The seedling under 30/25ºC treatment showed significantly higher sugar and starch content than control treatment after 5 months of culturing (3.071 and 3.375mg $g^{-1}$ vs 2.708 and 2.758mg $g^{-1}$, respectively) (Table 3). Chlorophyll a is an indicator of photosynthetic reactions that take place in chloroplasts and is proposed as a useful indicator of plant quality (Carvalho *et al.*, 2001, Serret *et al*., 2001, Borkowska 2006, Cassana *et al*., 2010, Oso´rio *et al*., 2013, Sa´ez *et al*., 2013). The chlorophyll and other pigments can be influenced by temperature changes (Carter and Knapp, 2001) and can indicate nitrogen utilization by plants (Bigot and Bocaud 1996, Von Wiren *et al.*, 1997).

**Table 2:** The effect of day/night temperature on chlorophyll content in *Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings after 1 month and 5 months of culture.

| Treatment | Chl a (µg $ml^{-1}$) | Chl b (µg $ml^{-1}$) | Total Chl (µg $ml^{-1}$) | Chl a (µg $ml^{-1}$) | Chl b (µg $ml^{-1}$) | Total Chl (µg $ml^{-1}$) |
|---|---|---|---|---|---|---|
| | After 1 month of culture | | | After 5 months of culture | | |
| 25/25 | 0.366 ± 0.127 c | 1.089 ± 0.520 a | 1.451 ± 0.646 ab | 0.580 ± 0.067 c | 4.256 ± 2.907 a | 4.835 ± 2.908 b |
| 25/20 | 0.793 ± 0.049 a | 1.044 ± 0.498 a | 1.837 ± 0.452 a | 2.235 ± 0.345 a | 5.124 ± 2.023 a | 7.359 ± 2.367 ab |
| 30/20 | 0.466 ± 0.128 bc | 0.726 ± 0.121 a | 1.192 ± 0.248 b | 2.778 ± 0.350 a | 5.594 ± 1.287 a | 8.372 ± 0.938 a |
| 30/25 | 0.821 ± 0.052 a | 1.138 ± 0.459 a | 1.959 ± 0.421 a | 2.883 ± 0.438 a | 6.112 ± 0.855 a | 8.995 ± 0.418 a |
| 35/25 | 0.498 ± 0.078 b | 0.738 ± 0.045 a | 1.236 ± 0.111 b | 1.447 ± 1.030 b | 4.855 ± 2.213 a | 6.302 ± 3.242 ab |

* Means followed by different letters within same column showed significant differences among treatments, based on ANOVA followed by Tukey's test at $p<0.05$. (n= 10)

**Table 3**: The effect of day/night temperature on nitrogen, sugar, and starch contents in *Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings after 1 month and 5 months of culture.

| Treatment | Nitrogen (%) | Sugar (mg $g^{-1}$) | Starch (mg $g^{-1}$) | Nitrogen (%) | Sugar (mg $g^{-1}$) | Starch (mg $g^{-1}$) |
|---|---|---|---|---|---|---|
| | After 1 month of culture | | | After 5 months of culture | | |
| 25/25 | 2.572 ± 0.001d | 2.863 ± 0.044b | 2.485 ± 0.012d | 3.677 ± 0.002b | 2.708 ± 0.002c | 2.758 ± 0.045d |
| 25/20 | 2.801 ± 0.002c | 2.916 ± 0.002b | 2.855 ± 0.006c | 3.917 ± 0.005b | 2.458 ± 0.007e | 3.015 ± 0.003c |
| 30/20 | 2.906 ± 0.002b | 3.056 ± 0.005ab | 3.645 ± 0.003b | 4.413 ± 0.004a | 2.915 ± 0.003b | 3.329 ± 0.003b |
| 30/25 | 3.379 ± 0.038a | 3.145 ± 0.395a | 3.940 ± 0.002a | 4.610 ± 0.046a | 3.071 ± 0.042a | 3.375 ± 0.005a |
| 35/25 | 2.783 ± 0.004c | 1.453 ± 0.002c | 2.346 ± 0.003e | 3.027 ± 0.518c | 2.503 ± 0.004d | 3.307 ± 0.003b |

* Means followed by different letters within same column showed significant differences among treatments, based on ANOVA followed by Tukey's test at $p<0.05$. (n= 10)

**The effect of day/night temperature on Rubisco and PEPC activity in *Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings:** In terms of photosynthetic enzyme activities, after 1 month of culturing, 30/25ºC condition showed significantly higher levels of Rubisco during the night and PEPC activity during the day and night (1 hour after light and dark conditions). The 30/25ºC condition generated Rubisco values of 25.354µmol $mg^{-1}$ $min^{-1}$ for the day time and 21.279µmol $mg^{-1}$ $min^{-1}$ for the night time. Rubisco activity of the 25/20ºC and 30/25ºC treatment generated significantly higher levels during the day which showed the result 24.721 and 25.354 µmol $mg^{-1}$ $min^{-1}$. While the control treatment generated Rubisco values of 21.363µmol $mg^{-1}$ $min^{-1}$ for the day time and 19.806µmol $mg^{-1}$ $min^{-1}$ for the night time. PEPC activity during the day and night in seedlings subjected to the 30/25ºC condition was significantly higher than that of other treatments after 5 months of culturing (12.553µmol $mg^{-1}$ $min^{-1}$ during the day and 12.590µmol $mg^{-1}$ $min^{-1}$ at nig-

ht) (Table 4). After 5 months of culturing, Rubisco activity was highest under the 30/25ºC treatment during the day and night.

**Table 4**. The effect of different day/night temperature on enzyme activity in *Phalaenopsis* 'Fortune Saltzman' tissue culture seedlings after 1 month and 5 months of culture

| Treatment | Rubisco activity at the beginning of darkness (µmol $mg^{-1}$ $min^{-1}$) | Rubisco activity at the end of darkness (µmol $mg^{-1}$ $min^{-1}$) | PEPC activity at the beginning of darkness (µmol $mg^{-1}$ $min^{-1}$) | PEPC activity at the end of darkness (µmol $mg^{-1}$ $min^{-1}$) | Rubisco activity at the beginning of darkness (µmol $mg^{-1}$ $min^{-1}$) | Rubisco activity at the end of darkness (µmol $mg^{-1}$ $min^{-1}$) | PEPC activity at the beginning of darkness (µmol $mg^{-1}$ $min^{-1}$) | PEPC activity at the end of darkness (µmol $mg^{-1}$ $min^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | After 1 month of culture | | | | After 5 months of culture | | | |
| 25/25 | 21.363 ± 0.549 b | 19.806 ± 0.868 cd | 5.430 ± 0.308 d | 4.893 ± 0.038 d | 47.231 ± 2.894 c | 30.493 ± 0.594 d | 6.820 ± 0.068 d | 7.750 ± 0.006 d |
| 25/20 | 24.721 ± 1.383 a | 20.468 ± 0.382 bc | 6.363 ± 0.009 c | 5.489 ± 0.283 c | 49.081 ± 0.297 c | 36.293 ± 0.756 c | 7.402 ± 0.198 c | 10.569 ± 0.282 c |
| 30/20 | 21.787 ± 0.461 b | 21.030 ± 0.210 ab | 10.224 ± 0.175 b | 11.549 ± 0.098 b | 64.693 ± 5.691 b | 46.229 ± 2.012 b | 9.128 ± 0.191 b | 14.173 ± 0.058 b |
| 30/25 | 25.354 ± 0.813 a | 21.279 ± 0.097 a | 12.553 ± 0.059 a | 12.590 ± 0.259 a | 90.197 ± 12.369 a | 59.139 ± 1.419 a | 17.966 ± 0.014 a | 22.483 ± 0.214 a |
| 35/25 | 18.360 ± 0.696 c | 19.747 ± 0.753 d | 4.571 ± 0.001 e | 4.781 ± 0.025 d | 43.767 ± 2.432 b | 28.198 ± 0.893 d | 5.555 ± 0.156 e | 7.443 ± 0.316 e |

* Means followed by different letters within same column showed significant differences among treatments, based on ANOVA followed by Tukey's test at $p<0.05$. (n= 10)

The 30/25ºC condition generated 90.197µmol $mg^{-1}$ $min^{-1}$ of Rubisco activity during the day and 59.139µmol $mg^{-1}$ $min^{-1}$ at night as compared to control treatment which generated 47.231µmol $mg^{-1}$ $min^{-1}$ of Rubisco activity during the day and 30.493µmol $mg^{-1}$ $min^{-1}$ at night. PEPC activity during the day and night in seedlings subjected to 30/25ºC condition was significantly higher than that of other treatments after 5 months of culturing (17.966µmol $mg^{-1}$ $min^{-1}$ during the day and 22.483 µmol $mg^{-1}$ $min^{-1}$ at night). The control treatment generated PEPC activity levels of 6.820µmol $mg^{-1}$ $min^{-1}$ for the day and 7.750µmol $mg^{-1}$ $min^{-1}$ at night (Table 4). Overall, Rubisco enzyme activity was higher during day and lower at night, serving as evidence of a C3 photosynthesis system. C3 plants convert $CO_2$ into a 3-carbon compound (PGA) with Rubisco during the day. On the other hand, CAM plants convert $CO_2$ into a 4-carbon intermediate (OAA) by using PEPC at night (Yamori *et al.,* 2014). These results similar to Gouk *et al.* (1997), who found that the highest levels of PEPC enzyme activity are achieved at night (20:00) in Mokara seedlings (CAM plant).

**$CO_2$ rhythm testing:** The $CO_2$ rhythm of *Phalaenopsis* tissue culture seedlings, all the treatment classification clearly exhibited three complete different patterns. Stage I (21DAP) showed a typical C3 $CO_2$ rhythm and had lowest $CO_2$ concentration (1000ppm) during the night and highest peak (1800ppm) at early morning of day time. At stage II (45DAP) the $CO_2$ concentration also occurred strongly (800ppm) at early morning and steeply dropped at night (50ppm). Both concentrations gradually dropped down to similar range of next stage (III). However, the $CO_2$ concentration at stage III (120DAP) showed highest peak at noon (600-800ppm) and dropped down to lowest point at night (50ppm). The sharp changes of $CO_2$ concentration exhibit a typical CAM plant physiological pattern. In this experiment after cultured for 6 months, the seedling from stage I begin to move into stage III $CO_2$ pattern. At this period, the $CO_2$ concentration showed a rise during the light period (from around 50ppm to 800ppm) and during dark it appeared a sharp drop and down low to original level. Which showed a more clearly CAM type (Fig 1). We showed that $CO_2$ fixation patterns of *Phalaenopsis* 'Fortune Saltzman' can shift from C3 to CAM pathways over 120 days of growth. *Phalaenopsis* 'Fortune Saltzman' *in vitro* seedlings also showed evidence of physiological pattern transformations from C3 to CAM during the growth period. At an early seedling stage (Stage I), $CO_2$ concentrations during the dark period were high and decreased during the light period while the seedlings were still very young. When the seedlings matured, they showed high concentrations of $CO_2$ during the light period and low concentrations during the dark period. Huang (2006) and Apinya (2012) conducted experiments which also showed evidence of physiological pattern transformations in different *Phalaenopsis* spp. or in a variety of seedlings

from C3 to CAM during growth. The *Chrysanthemum* $CO_2$ rhythm of C3 to a *Phalaenopsis* species began with 3000ppm during a light period, then gradually decreased to less than 500ppm. However, when the dark period started, it increased to 3000ppm and exhibited a typical C3 plant form. This result reflects Woei and Lee's (2006) finding that *Phalaenopsis* seedlings develop CAM metabolism during maturation and utilize the C3 pathway when leaves are less than 20 days of age.

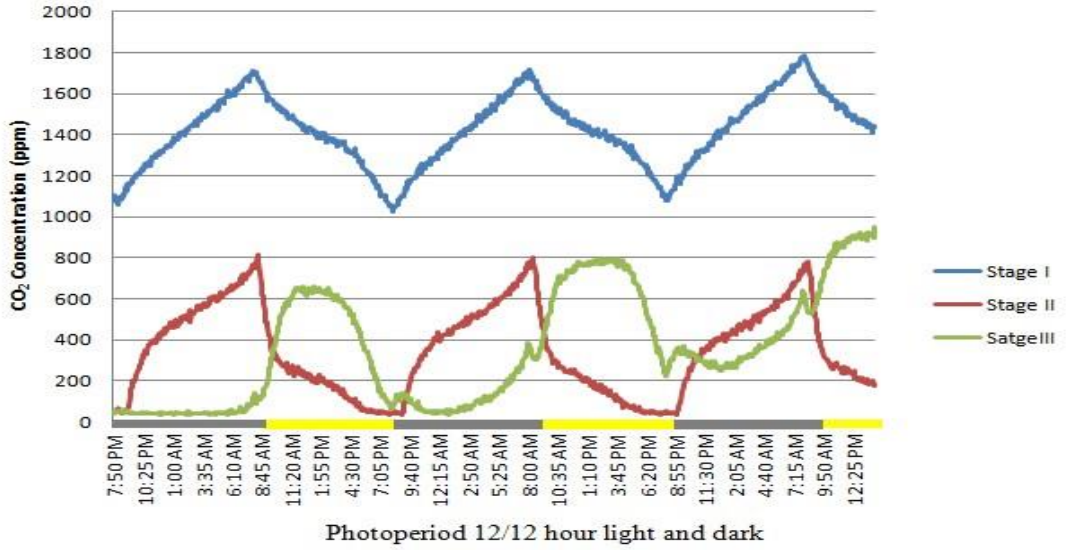


**Fig. 1:** Carbon dioxide rhythm of *Phalaenopsis* 'Fortune Saltzman' seedlings during day time and night time in three different stages. = dark = ght

## Conclusion

After 120 days of culturing, cultured seedlings subjected to 30/25ºC temperature treatment showed significantly longer stem and leaves and higher fresh, dry weight, number of root and root length. The seedlings grown under 25/20ºC and 30/25ºC showed higher levels of Chlorophyll a and total chlorophyll content than those grown under other treatments. Nitrogen, sugar, and starch contents were increased under 30/25ºC temperature treatment. In term of Rubisco and PEPC activity, 30/25ºC temperature treatment also showed significantly higher levels of Rubisco and PEPC activity during the day and night time. This clearly showed that it enhanced *in vitro* seedling growth in *Phalaenopsis* and transform its photosynthesis pattern into CAM, thus 30/25ºC treatments can be recommended for commercial production of *Phalaenopsis*.

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