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# Effects of temperature and relative humidity during *in vitro* acclimatization, on physiological changes and growth characters of *Phalaenopsis* adapted to *in vivo*

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**Abstract**

*Phalaenopsis* plantlets, acclimatized under different air temperatures (15±2, 25±2 and 35±2°C) and relative humidity (RH) (60±5, 80±5 and 95±5%RH), were transferred directly to *in vivo* environments for 14 days. The experiment was done at the Plant Physiology and Biochemistry Lab, National Center for Genetic Engineering and Biotechnology (BIOTEC) in year 2007. Chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$), total chlorophyll (TC) and total carotenoid ($C_{x+c}$) contents in plantlets acclimatized under conditions of low air temperature and low RH were maintained to a higher degree than in those acclimatized under high temperature and high relative humidity by 4.45, 5.79, 4.68 and 4.95 times, respectively. $Chl_a$ and TC contents in acclimatized plantlets were positively related to maximum quantum yield of PSII ($F_v/F_m$) ($r^2$ = 0.61) and photon yield of PSII ($\Phi_{PSII}$) ($r^2$ = 0.82), respectively. $F_v/F_m$, $\Phi_{PSII}$, photochemical quenching (qP) and transpiration rate (E) in plantlets acclimatized under low temperature and low RH were enriched and were greater than those under high temperature and high RH treatment, while stomatal conductance ($g_s$) was lower, leading to enhanced net photosynthetic rate ($P_n$) and growth performances. Low temperature and low RH conditions of *in vitro* acclimatization should be implemented to produce healthy *Phalaenopsis* plantlets, defined by pigment stabilization, chlorophyll *a* fluorescence regulation, $P_n$ and growth characteristics, to enable their rapid adaptation to *in vivo* environments.

**Keywords:** chlorophyll content, chlorophyll *a* fluorescence, growth, net photosynthetic rate, orchid.
**Abbreviations:** $Chl_a$_chlorophyll a, $Chl_b$_chlorophyll b, TC_total chlorophyll, $C_{x+c}$_total carotenoids, E_transpiration rate, $F_v/F_m$_maximum quantum yield of PSII, $g_s$_stomatal conductance, MS_Murashige and Skoog medium, NPQ_non photochemical quenching, $P_n$_net photosynthetic rate, $\Phi_{PSII}$_photon yield of PSII, PPF_photosynthetic photon flux, qP_photochemical quenching, RH_relative humidity.

## Introduction

*Phalaenopsis*, or the moth orchid, is one of the most important genera of ornamental plants in the world. 75% of the market share of orchids produced in the year 2000 was a potted *Phalaenopsis* orchid, representing seventy-five million US dollars. Large scale production of *Phalaenopsis* is carried out in The Netherlands, Germany, China, Taiwan, The United States and Japan (Griesbach 2002). *Phalaenopsis* originates in temperate regions, which have low temperatures (≤25°C). It has been reported as being sensitive to high temperatures and this is especially the case with hybrid types (Kano 2001). In the floral transition stage, low temperatures are necessary for endogenous cytokinin and gibberellin accumulation, as well as photosynthetic enhancement, leading to sucrose gathering for flower bud initiation and stalk elongation (Chou et al. 2000; Su et al. 2001a; Kataoka et al. 2004; Blanchard and Runkle 2006; Lee et al. 2007; Chen et al. 2008; Penfield 2008). High temperature environments strongly affect oxidative stress in *Phalaenopsis* orchids, resulting in inhibition of flower development (Su et al. 2001a; Ali et al. 2005). In the present study, air temperature is mentioned as a key factor in controlling *Phalaenopsis* plantlet growth and development *in vitro*, prior to *in vivo* transplantation. On a commercial scale, *Phalaenopsis* plantlets have been produced by micropropagation through plant tissue culture, which is successfully implemented in many countries, Japan, Taiwan and China (Griesbach 2002). There are many reports into developing an effective protocol of *Phalaenopsis* micropropagation *via* protocorm-like bodies (Islam et al. 1998; Chen et al. 2000; Park et al. 2000; Tokuhara and Mii 2001; Park et al. 2002; Tokuhara and Mii 2003; Liu et al. 2006; Shrestha et al. 2007). Normally, the environments in *in vivo* are quite different when compared to *in vitro* conditions, in terms of relative humidity (RH), constant temperature, air ventilation, nutrient levels, etc (Kozai et al. 1997; Chen 2004; Hazarika 2006). *In vitro* acclimatization, or hardening, is one of the main processes in the production of healthy plantlets before their transplantation to *in vivo* (Pospíšilová et al. 1999a; Hazarika 2003).

Photoautotrophic acclimatization of plantlets using environmental controls has successfully improved the survival percentage rates in *in vivo* conditions (Kozai et al. 1997; Xiao and Kozai 2004). Relative humidity (RH) control of *in vitro* acclimatization is a major factor in enhancing the biochemical, physiological and morphological characters of plantlets when transplanted to *in vivo* (Cha-um et al. 2003; Talbott et al. 2003). There are many techniques for controlling the RH in the culture vessel of plant tissue culture, such as, saturated salt addition to the culture chamber and increasing the air ventilation rate (Cui et al. 2000; Cha-um et al. 2003; Shim et al. 2003). Acclimatized plantlet adaptation is an important mechanism in the transplanting process of plant micropropagation, relating to survival percentage, growth and development (van Huylenbroeck et al. 1998; van Huylenbroeck et al. 2000; Kadleček et al. 2001; Fila et al. 2006). Healthy, acclimatized plantlets have been identified using physiological characteristics including chlorophyll content, chlorophyll *a* fluorescence parameters, $CO_2$ assimilation, net photosynthetic rate ($P_n$), stomatal conductance ($g_s$) and transpiration rate (E), which have been demonstrated in many plants such as orchids (Jeon et al. 2005), *Calathea louisae* (van Huylenbroeck et al. 2000), tobacco (Pospíšilová et al. 1999b; Kadleček et al. 2001), *Spathiphyllum floribundum* (van Huylenbroeck et al. 1998), strawberry (Borkowska 2001), grapevine (Carvalho and Amâncio 2002a; Fila et al. 2006) and chestnut (Carvalho and Amâncio 2002b). Chlorophyll *a* fluorescence parameters, including maximum quantum yield of PSII ($F_v/F_m$), photon yield of PSII ($\Phi_{PSII}$), photochemical quenching (qP) and non photochemical quenching (NPQ), are maintained in orchids grown under *ex vitro* acclimatization (Jeon et al. 2006; Jeon et al. 2006), enriched $CO_2$ (Gouk et al. 1999) and low night temperature (Chen 2008), and also play a role as growth indicators (Hsu 2007). In addition, those parameters have been established as effective indices for directing plant improvements, with many purposes, especially against water deficit stress (Baker and Rosenqvist 2004; O'Neill et al. 2006; Rong-hua et al. 2006; Wu et al. 2008). The aim of this investigation was to acclimatize *Phalaenopsis* plantlets using controlled RH and temperature for rapid *in vivo* adaptation, using pigment content, chlorophyll *a* fluorescence, $P_n$ and growth performances as indicators.

## Materials and methods

### *Plant materials and in vitro acclimatization*

*Phalaenopsis* orchid plantlets (2.5±0.5 cm in height) provided by Prayoon Orchid Lab (Prayoon Orchid Ltd., Pathumthani Thailand) were transferred to sugar-free MS medium (Murashige and Skoog 1962) (one plantlet per glass vessel), using vermiculite as supporting material, for 7 days, at 65±5% relative humidity (RH), 25±2°C ambient temperature and 70±5 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux (PPF) using fluorescent lamps with a 16 h $d^{-1}$ photoperiod. Twenty open capped glass vessels containing orchid plantlets were placed into an aseptic culture chamber box (Carry Box Model P-850, size 26×36×19 cm) in which RH conditions were controlled at 95±5% with 1500 ml distilled water, 80±5% by 1500 ml saturated $CaCO_3$ and 60±5% by 1500 ml saturated NaCl solution. The air exchange rate in the culture chambers was increased to 5.13±0.3 μmol $CO_2$ $h^{-1}$ by punching the sides of the plastic chambers with 32 holes and placing gas permeable microporous polypropylene film (0.22 μm pore size, Nihon Millipore Ltd., Japan) over each hole. The chambers were incubated under conditions of 15±2, 25±2 and 35±2°C, 100±5 μmol $m^{-2}$ $s^{-1}$ PPF, 65±5% RH and $CO_2$-enrichment (1000±100 μmol $mol^{-1}$) in a Plant Growth Incubator (model FLI-2000 EYELA, Japan) for 30 days.

### *In vivo adaptation*

Thirty-day acclimatized plantlets, were transplanted directly to 4.5 cm × 4.5 cm pots containing peat moss and then incubated in a glasshouse at 30±2°C ambient temperature, 75±5% RH and 300-400 μmol $m^{-2}$ $s^{-1}$ PPF light intensity at plant level with 10 h $d^{-1}$ photoperiod, for 14 days. Photosynthetic pigments, chlorophyll *a* fluorescence and net photosynthetic rate ($P_n$) were measured. Fresh weight (FW), dry weight (DW), root length and leaf area measurements were collected as growth characters.

### *Experiment design*

*Phalaenopsis* plantlets were acclimatized under different air temperatures at 15±2, 25±2 and 35±2°C in the Plant Growth Incubator and relative humidity (RH) at 60±5, 80±5 and 95±5%RH using saturated salt solution, subsequently transferred to *in vivo* environments for 14 days. The experiment was arranged as 3×3 factorials in a completely randomized design (CRD) with four replicates and four plantlets per replicate.

### *Measurement of physiological and morphological characteristics*

Chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$) and total chlorophyll (TC) concentrations were analyzed following the methods of Shabala et al. (1998) and total carotenoids ($C_{x+c}$) were assayed according to Lichtenthaler (1987) method. One hundred milligrams of leaf material was collected. The leaf samples were placed in 25 ml glass vials, along with 10 ml 95.5% acetone, and blended using a homogenizer. The glass vials were sealed with parafilm to prevent evaporation and then stored at 4°C for 48 h. The $Chl_a$ and $Chl_b$ concentrations were measured using a UV-visible spectrophotometer (model DR/4000, HACH, USA) at 662 nm and 644 nm wavelengths. The $C_{x+c}$ concentration was also measured by spectrophotometer at 470 nm. A solution of 95.5% acetone was used as a blank. Chlorophyll *a* fluorescence emission from the adaxial surface on the leaf was monitored using a fluorescence monitoring system (model FMS 2; Hansatech Instruments Ltd., UK) in the pulse amplitude modulation mode, as previously described by Loggini et al. (1999). A leaf under dark conditions was initially exposed to a modulated measuring beam of far-red light. Original ($F_0$) and maximum ($F_m$) fluorescence yields were measured under weak modulated red light (<0.5μmol $m^{-2}$ $s^{-1}$) with 1.6 sec pulses of saturating light (>6.8 μmol $m^{-2}$ $s^{-1}$ PAR) and autocalculated using FMS software for Windows®. The variable fluorescence yield ($F_v$) was calculated by the equation of $F_m$–$F_0$. The ratio of variable to maximum fluorescence ($F_v/F_m$) was calculated as maximum quantum yield of PSII photochemistry. The photon yield of PSII ($\Phi_{PSII}$) in the light was calculated by $\Phi_{PSII} = (F_m'-F)/F_m'$ after 45 sec illumination, when steady state was achieved. In addition, photochemical

**Table 1.** Chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$), total chlorophyll (TC) and total carotenoids ($C_{x+c}$) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Errors of mean are represented by ±SD.

| Temp. (ºC) | RH (%) | $Chl_a$ (μg $g^{-1}$ FW) | $Chl_b$ (μg $g^{-1}$ FW) | TC (μg $g^{-1}$ FW) | $C_{x+c}$ (μg $g^{-1}$ FW) |
|---|---|---|---|---|---|
| 15±2 | 60±5 | 829.5±13. 6ab | 223.1±2.8ab | 1052.6±16.3a | 266.2±4.3a |
| | 80±5 | 309.3±18.3cd | 112.1±1.3ab | 421.4±2.0bc | 103.0±1.4bc |
| | 95±5 | 241.0±4.2d | 65.6±2.0ab | 306.6±2.2bc | 74.8±1.4c |
| 25±2 | 60±5 | 861.5±8.4a | 276.2±3.9a | 1137.7±12.2a | 274.2±3.4a |
| | 80±5 | 558.2±12.6bc | 164.9±6.6ab | 723.1±19.2ab | 184.9±4.5ab |
| | 95±5 | 349.5±8.69cd | 105.5±1.3ab | 455.0±9.9bc | 117.8±2.4bc |
| 35±2 | 60±5 | 451.0±4.0cd | 147.5±14.7ab | 598.5±5.5bc | 130.7±1.8bc |
| | 80±5 | 262.7±2.9d | 77.6±1.7ab | 340.3±17.5bc | 64.4±2.5c |
| | 95±5 | 186.4±3.83d | 38.5±5.9b | 224.9±4.2c | 53.8±8.2c |
| Significant level | | | | | |
| Temp | | ** | ** | ** | ** |
| RH | | ** | ** | ** | ** |
| Temp × RH | | ** | ** | ** | ** |

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Turkey's Honestly Significant different test (Turkey's HSD).

**Table 2.** Maximum quantum yield of PSII ($F_v/F_m$), photon yield of PSII ($\Phi_{PSII}$), photochemical quenching (qP), stomatal conductance ($g_s$) and transpiration rate (E) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Errors of mean are represented by ±SD.

| Temp. (ºC) | RH (%) | $F_v/F_m$ | $\Phi_{PSII}$ | qP | $g_s$ (mol $H_2O$ $m^{-2}$ $s^{-1}$) | E (mmol $m^{-2}$ $s^{-1}$) |
|---|---|---|---|---|---|---|
| 15±2 | 60±5 | 0.771±0.016 | 0.441±0.006 | 0.333±0.016 | 15.55±1.79abc | 3.12±0.85b |
| | 80±5 | 0.747±0.018 | 0.423±0.116 | 0.324±0.014 | 18.20±2.08ab | 2.75±0.23bc |
| | 95±5 | 0.731±0.018 | 0.375±0.073 | 0.310±0.011 | 21.60±4.85a | 2.61±0.32bc |
| 25±2 | 60±5 | 0.768±0.018 | 0.465±0.030 | 0.391±0.013 | 11.06±2.93c | 5.25±0.28a |
| | 80±5 | 0.776±0.021 | 0.439±0.066 | 0.359±0.012 | 12.90±0.46bc | 2.01±0.06cd |
| | 95±5 | 0.770±0.017 | 0.390±0.103 | 0.244±0.017 | 13.40±1.85bc | 1.80±0.43cd |
| 35±2 | 60±5 | 0.765±0.006 | 0.404±0.063 | 0.356±0.011 | 12.30±2.31bc | 2.82±0.25bc |
| | 80±5 | 0.741±0.053 | 0.365±0.041 | 0.314±0.093 | 15.76±2.83abc | 1.89±0.08cd |
| | 95±5 | 0.708±0.085 | 0.357±0.033 | 0.249±0.010 | 18.45±3.29ab | 1.32±0.06d |
| Significant level | | | | | | |
| Temp | | NS | NS | NS | ** | ** |
| RH | | NS | NS | NS | ** | ** |
| Temp × RH | | NS | NS | NS | ** | ** |

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Turkey's Honestly Significant different test (Turkey's HSD). $^{NS}$ represents non-significant difference in statistical analysis.

quenching (qP) was calculated as described by Maxwell and Johnson (2000). The net-photosynthetic rate ($P_n$), transpiration rate (E; mmol $m^{-2}$ $s^{-1}$) and stomatal conductance ($g_s$; mol $H_2O$ $m^{-2}$ $s^{-1}$) of *Phalaenopsis* plantlets were measured in dark conditions using an Infra-red Gas Analyzer (IRGA; model LI 6400, LI-COR® Inc, USA). The E and $g_s$ were measured continuously by monitoring the $H_2O$ content of the air entering, and also existing in, the IRGA headspace chamber. The flow-rate of air in the sample line was adjusted to 500 μmol $s^{-1}$. The micro-chamber temperature was set at 25°C (Cha-um et al. 2007). Fresh weight, dry weight, root length, number of roots and leaf area of *Phalaenopsis* plantlets were measured. *Phalaenopsis* plantlets were dried at 110°C in a hot-air oven for 4 days and then incubated in desiccators before measurement of dry weight. The leaf area of plantlets was measured using a leaf area meter DT-scan. The mean values obtained were compared by Turkey's Honestly Significant Difference test (Turkey's HSD) and analyzed using SPSS software.

The correlations between physiological and morphological parameters were evaluated using Pearson's correlation coefficients.

**Results and discussion**

*Phalaenopsis* plantlets were acclimatized under different air temperatures at 15±2 (low Temp), 25±2 (medium Temp) and 35±2°C (high Temp) in the Plant Growth Incubator and relative humidity (RH) at 60±5 (low RH), 80±5 (medium RH) and 95±5%RH (high RH) using saturated salt solution and then directly transferred to *in vivo* environments for 14 days. *In vivo* adaptation, photosynthetic pigments, including chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$), total chlorophyll and total carotenoids ($C_{x+c}$) of plantlets acclimatized under low Temp and low RH were maintained at higher levels when compared to plantlets acclimatized under high Temp and high RH treatments for 4.45, 5.79, 4.68 and 4.95 folds, respectively

**Table 3.** Fresh weight (FW), dry weight (DW), root length (RL) and leaf area (LA) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Errors of mean are represented by ±SD.

| Temp. (ºC) | RH (%) | FW (g) | DW (mg) | RL (cm) | LA ($cm^2$) |
|---|---|---|---|---|---|
| 15±2 | 60±5 | 1.88±0.44ab | 176±4.18a | 4.9±0.4a | 15.61±3.89ab |
| | 80±5 | 1.56±0.40ab | 146±2.21ab | 3.4±0.8abc | 12.36±0.53b |
| | 95±5 | 0.94±0.04b | 128±1.69b | 2.7±0.1c | 12.03±0.76b |
| 25±2 | 60±5 | 2.19±0.27a | 138±1.69b | 3.9±0.9abc | 22.69±1.15a |
| | 80±5 | 1.87±0.36ab | 122±13.16b | 3.0±0.1bc | 16.12±1.88ab |
| | 95±5 | 1.20±0.12ab | 118±1.73b | 2.6±0.9c | 13.99±3.08b |
| 35±2 | 60±5 | 1.99±0.26ab | 186±2.02a | 4.6±0.5ab | 16.94±2.15ab |
| | 80±5 | 1.70±0.07ab | 164±25.5ab | 3.6±0.2abc | 16.50±2.05ab |
| | 95±5 | 1.44±0.19ab | 151±8.31ab | 2.7±0.2c | 14.85±2.63b |
| Significant level | | | | | |
| Temp | | NS | * | NS | NS |
| RH | | ** | ** | ** | ** |
| Temp × RH | | NS | NS | NS | NS |

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Turkey's Honestly Significant different test (Turkey's HSD). $^{NS}$ represents non-significant difference in statistical analysis.

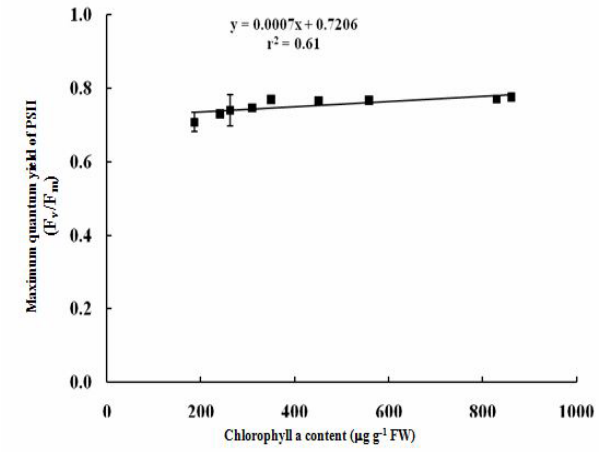


**Fig 1.** Relationship between chlorophyll a ($Chl_a$) content and maximum quantum yield of PSII ($F_v/F_m$) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Error bars represent ±SE.

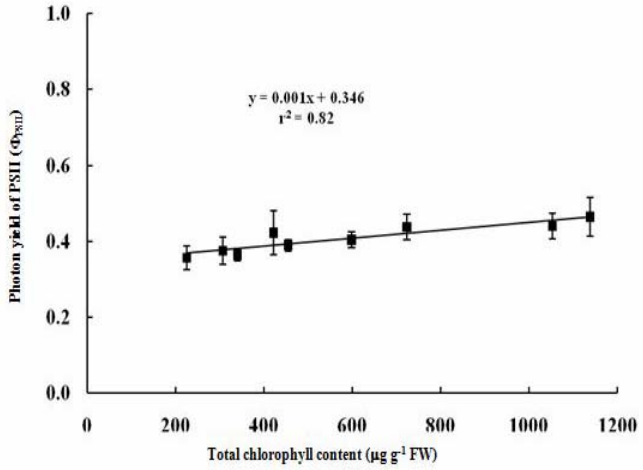


**Fig 2.** Relationship between total chlorophyll content and photon yield of PSII ($\Phi_{PSII}$) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Error bars represent ±SE.

(Table 1). The photosynthetic pigment contents of acclimatized plantlets decreased significantly after transplantation to *in vivo* environments, depending on Temp, RH and their interactions. $Chl_a$ content of plantlets acclimatized under 60±5% RH combined with 15±2, 25±2 and 35±2°C Temp were enriched to a greater degree than those acclimatized under 95±5% RH by 3.44, 2.47 and 2.42 times, respectively. Similar patterns were found in the responses of $Chl_b$, TC and $C_{x+c}$ to *in vivo* conditions (Table 1). The photosynthetic pigments of *in vitro* acclimatized *Phalaenopsis* plantlets grown under low temperature and low RH were maintained after their transfer to *in vivo* for 14 days, leading to high $F_v/F_m$, $\Phi_{PSII}$, qP and $P_n$. A nature of *Phalaenopsis* orchid is a temperate plant species, which is grow well in the low temperature (≤25°C) (Kano 2001). These findings are similar to those of a previous study into *Doritaenopsis* orchids (New Candy), which found that the photosynthetic pigments, $Chl_a$, $Chl_b$, TC and $C_{x+c}$, of *in vivo* acclimatized plantlets were maintained under high RH (90%) and optimum temperature (20-25°C) (Jeon et al. 2006). Concentration of $Chl_a$ and TC in acclimatized plantlets was positively related to maximum quantum yield of PSII ($F_v/F_m$) (Fig. 1; $r^2 = 0.61$) and photon yield of PSII (Fig. 2; $\Phi_{PSII}$) ($r^2 = 0.82$), respectively. Chlorophyll *a* fluorescence parameters i.e. $F_v/F_m$, $\Phi_{PSII}$ and photochemical quenching (qP) in acclimatized plantlets were unchanged (Table 2).

The transpiration rate (E) of acclimatized plantlets was reduced, related to high RH and high Temp, while stomatal conductance ($g_s$) increased (Table 2). Efficacy of $\Phi_{PSII}$ in acclimatized plantlets was positively correlated with net photosynthetic rate ($P_n$) (Fig. 3; $r^2 = 0.42$). $P_n$ in plantlets acclimatized under low RH was higher than that in plantlets

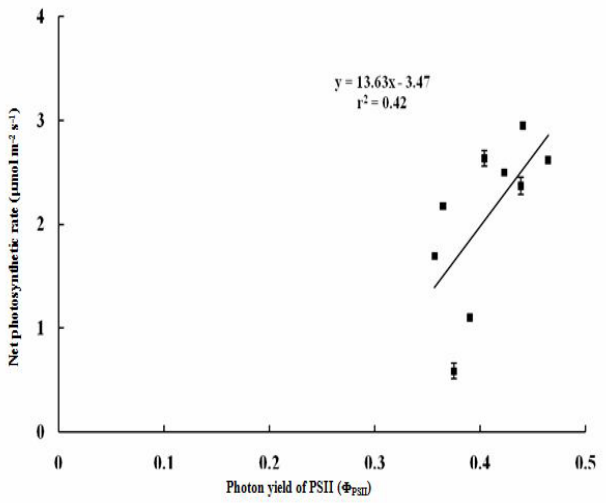


**Fig 3.** Relationship between photon yield of PSII ($\Phi_{PSII}$) and net photosynthetic rate ($P_n$) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Error bars represent ±SE.

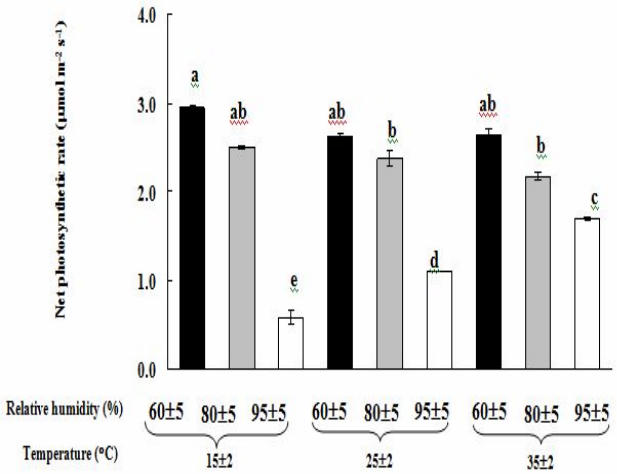


**Fig 4.** Net photosynthetic rate ($P_n$) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Different letters in each bar show significant difference at $p \leq 0.01$ (**) by DMRT. Error bars represent ±SE.

acclimatized under high RH (Fig. 4). Reduction of $P_n$ in acclimatized plantlets was positively related to plant dry weight (Fig. 5; $r^2 = 0.39$). The RH treatment strongly affected fresh weight (FW), root length and leaf area. Those parameters were maintained in plantlets acclimatized under low RH, while the factor of temperature treatment did not have an effect (Table 3). On the other hand, plant dry weight was affected by both factors of Temp and RH. Physiological adaptation, including relative water content, $F_v/F_m$ and $CO_2$ assimilation of *in vivo* acclimatized plantlets has been investigated as an indicator for the rapid acclimatization of *Doritaenopsis* orchids, leading to the improvement of survival percentage rates and overall growth promotion (Jeon et al. 2006). In contrast, orchid plantlets acclimatized under extreme temperature (15 or 35°C) and low RH (50%) conditions showed symptoms of wilting, chlorophyll degradation and growth reduction (Jeon et al. 2006). Chlorophyll *a* fluorescence parameters i.e. water oxidation, quantum efficiency, electron transport and non-photochemical quenching have been widely used as indices for the adaptation of plants to different environments (Su et al. 2001; Lin and Hsu 2004; Hsu 2007). For example, low temperature (25°C) and 70% RH are two environmental factors for storage of bare root *Phalaenopsis* using chlorophyll *a* fluorescence as an indicator (Su et al. 2001). In addition, the $F_v/F_m$ of *Phalaenopsis* seedlings grown under extreme temperatures (11°C or 37°C), decreased significantly when compared to seedlings under incubation at 25°C (Hsu 2007). In the present study, the pigment contents, chlorophyll fluorescence and $P_n$ of plantlets acclimatized *in vitro* in low RH and low air temperature environments, effectively identified healthy plantlets prior to their quick adaptation to *in vivo* environments. The production of healthy plantlets, micropropagated using *in vitro* environmental controls such as increased light intensity, enriched $CO_2$ and reduced sugar in the culture medium has been investigated widely (Lin and Hsu 2004; Ali et al. 2005a; Jeon et al. 2005; Ali et al. 2006; Yoon et al. 2008). In *Phalaenopsis*, 25/20°C day/night temperature has been reported as the optimum temperature for plant growth and development, especially in the flowering stage (Su et al. 2001a; Kataoka et al. 2004; Blanchard and Runkle 2006; Lee et al. 2007; Chen et al. 2008). High temperature incubation of *Phalaenopsis* produces oxidative damage, resulting in biochemical, physiological and morphological changes (Chou et al. 2000; Su et al. 2001b; Wang et al. 2002; Ali et al. 2005b; Ichihashi et al. 2008). In addition, the relative humidity *in vivo* is quite low when compared to *in vitro* environments (Kozai et al. 1997; Chen 2005). Low RH for acclimatizing plantlets is an effective way to harden the plantlets to both physiological and morphological changes before transplantation to *in vivo* (Cha-um et al. 2003), leading to quick adaptation and high survival percentage rates.

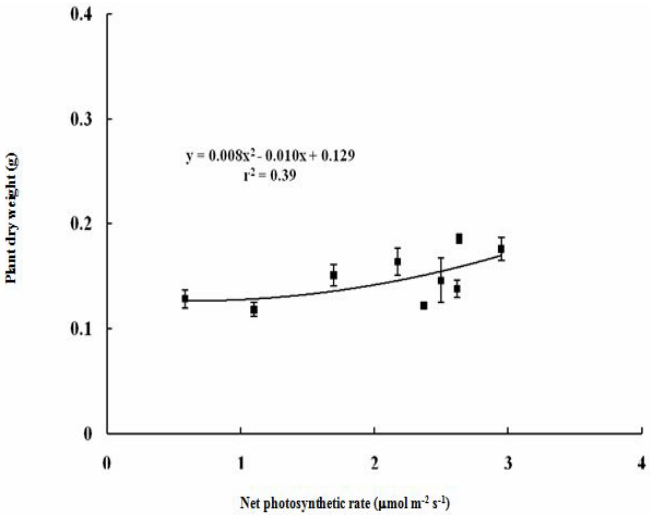


**Fig. 5** Relationship between net photosynthetic rate ($P_n$) and plant dry weight (DW) of *Phalaenopsis* acclimatized *in-vitro* under different temperatures and relative humidity for 30 days and subsequently transferred to *in vivo* for 14 days. Error bars represent ±SE.

## Conclusion

Plantlets of *Phalaenopsis* orchids, acclimatized *in vitro* under low temperature (15-25°C) with low relative humidity (60±5%RH) were well adapted to *in vivo* conditions as identified by their high levels of photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids), net photosynthetic rate, stomatal conductance and low transpiration rate, leading to enhanced growth. The acclimatization stage of *Phalaenopsis* orchid plantlets should be successfully implemented as low air temperature (15-25°C) and low relative humidity (60±5%RH). The basic knowledge of *in vitro* acclimatization of *Phalaenopsis* plantlets, prior to their quick adaption to *in vivo* environments, should be further applied to large scale orchid production.


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## References


Ali MB, Hahn EJ, Paek KY (2005a) Effects of light intensities on antioxidant enzymes and malondialdehyde content during short-term acclimatization micropropagated *Phalaenopsis* plantlet. Environ Exp Bot 54:109-120

Ali MB, Hahn EJ, Paek KY (2005b) Effects of temperature on oxidative stress defense systems, lipid peroxidation and lipoxygenase activity in *Phalaenopsis*. Plant Physiol Biochem 43:213-223

Ali MB, Khatun S, Hahn EJ, Paek KY (2006) Enhancement of phenylpropanoid enzymes and lignin in *Phalaenopsis* orchid and their influence on plant acclimatization at different levels of photosynthetic photon flux. Plant Growth Regul 49:137-146

Baker NR, Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. J Exp Bot 55:1607-1621

Blanchard MG, Runkl ES (2006) Temperature during the day, but not during the night, controls flowering of *Phalaenopsis* orchid. J Exp Bot 57:4043-4049

Borkowska B (2001) Morphological and physiological characteristics of micropropagated strawberry plants rooted *in vitro* or *ex vitro*. Sci Hort 89:195-206

Carvalho LC, Amâncio S (2002a) Antioxidant defence system in plantlets transferred from *in vitro* to *ex vitro*: effects of increasing light intensity and $CO_2$ concentration. Plant Sci 162:33-40

Carvalho LC, Amâncio S (2002b) Effect of *ex vitro* conditions on growth and acquisition of autotrophic behaviour during the acclimatisation of chesnut regenerated *in vitro*. Sci Hort 95:151-164

Cha-um S, Mosaleeyanon K, Supaibulwatana K, Kirdmanee C (2003) A more efficient transplanting system for Thai neem (*Azadirachta siamensis* Val.) by reducing relative humidity. Sci Asia 29:189-196

Cha-um S, Supaibulwatana K, Kirdmanee C (2007) Glycinebetaine accumulation, physiological characterizations, and growth efficiency in salt tolerant and salt sensitive lines of indica rice (*Oryza sativa* L. spp. *indica*) response to salt stress. J Agron Crop Sci 193:157-166

Chen C (2004). Humidity in plant tissue culture vessels. Biosyst Engineer 88:231-241

Chen WH, Tseng YC, Liu YC, Chuo CM, Chen PT, Tseng KM, Yeh YC, Ger MJ, Wang HL (2008) Cool-night temperature induces spike emergence and affects photosynthetic efficiency and metabolizable carbohydrate and organic acid pools in *Phalaenopsis aphrodite*. Plant Cell Rep 27:1667-1675

Chen YC, Chang C, Chang W (2000) A reliable protocol for plant regeneration from callus culture of *Phalaenopsis.* In Vitro Cell Dev Biol-Plant 36:420-423

Chou CC, Chen WS, Huang KL, Yu HC, Liao LJ (2000) Changes in cytokinin levels of *Phalaenopsis* leaves at high temperature. Plant Physiol Biochem 38:309-314

Cui YC, Hanh EJ, Kozai T, Paek KY (2000) Number of air exchange, sucrose concentration, photosynthetic photon flux, and different in photoperiod and dark period temperature affect growth of *Rehmannia glutinosa* plantlets *in vitro*. Plant Cell Tiss Org Cult 62:219-226

Fila G, Badeck FW, Meyer S, Cerovic Z, Ghashghaie J (2006) Relationships between leaf conductance to $CO_2$ diffusion and photosynthesis in micropropagated grapevine plants, before and after *ex vitro* acclimatization. J Exp Bot 57:2687-2695

Griesbach RJ (2002) Development of *Phalaenopsis* orchid for the mass-market. In: Janick J, Whipkey A (eds.), Trends in New Crops and New Uses. ASHS Press, Alexandria, VA, pp 458-465

Gouk SS, He J, Hew CS (1999) Changes in photosynthetic capability and carbohydrate production in an epiphytic CAM orchid plantlet exposed to super-elevated $CO_2$. Environ Exp Bot 41:219-230

Hazarika BN (2003) Acclimatization of tissue-cultured plants. Curr Sci 85:1704-1712

Hazarika BN (2006) Morpho-physiological disorders in *in vitro* culture of plants. Sci Hort 108:105-120

Hsu BD (2007) On the possibility of using a chlorophyll fluorescence parameter as an indirect indicator for the growth of *Phalaenopsis* seedlings. Plant Sci 172:604-608

Ichihashi S, Higuchi T, Shibayama H, Tesima Y, Nishiwaki Y, Ota K (2008) Aspects of $CO_2$ uptake in the crassulacean acid metabolism orchid *Phalaenopsis*. Acta Hort 766:245-256

Islam MO, Ichihashi S, Matsui S (1998) Control of growth and development of protocorm like body derived from callus by carbon sources in *Phalaenopsis*. Plant Biotechnol 15:183-167

Jeon MW, Ali MB, Hahn EJ, Paek KY (2005) Effects of photon flux density on the morphology, photosynthesis and growth of a CAM orchid, *Doritaenopsis* during post-micropropagation acclimatization. Plant Growth Regul 45:139-147

Jeon MW, Ali MB, Hahn EJ, Paek KY (2006) Photosynthetic pigments, morphology and leaf gas exchange during *ex vitro* acclimatization of micropropagated CAM *Doritaenopsis* plantlets under relative humidity and air temperature. Environ Exp Bot 55:183-194

Kadleček P, Tichá I, Haisel D, Čapková V, Schäfer C (2001) Importance of *in vitro* pretreatment for *ex vitro* acclimatization and growth. Plant Sci 161:695-701

Kano A (2001) Analysis of optimal temperature of *Phalaenopsis* based on a parameter derived from photosynthesis and growth. J Japan Soc Hort Sci 70:148.

Kataoka K, Sumitomo K, Fudano T, Kawase K (2004) Changes in sugar content of *Phalaenopsis* leaves before floral transition. Sci Hort 102:121-132

Kozai T, Kubota C, Jeong BR (1997) Environmental control for the large-scale production of plants through *in vitro* techniques. Plant Cell Tiss Organ Cult 51:49-56

Lee HC, Chen YJ, Markhart AH, Lin TY (2007) Temperature effects on systemic endoreduplication in orchid during floral development. Plant Sci 172:588-595

Lichtenthaler HK (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol 148:350-380

Lin MJ, Hsu BD (2004) Photosynthetic plasticity of *Phalaenopsis* in response to different light environments. J Plant Physiol 161:1259-1268

Liu THA, Lin JJ, Wu RJ (2006). The effects of using trehalose as a carbon source on the proliferation of *Phalaenopsis* and *Doritaenopsis* protocorm-like-bodies. Plant Cell Tiss Org Cult 86:125-129

Loggini B, Scartazza A, Brugnoli E, Navari-Izzo F (1999) Antioxidant defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol 119:1091-1099

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence – a practical guide. J Exp Bot 51:659-668

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

O'Neill PM, Shanahan JF, Schepers S (2006) Use of chlorophyll fluorescence assessments to differentiate corn hybrid response to variable water conditions. Crop Sci 46:681-687

Park SY, Murthy HN, Paek KY (2000) Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. Plant Cell Tiss Org Cult 63:67-72

Park SY, Murthy HN, Paek KY (2002) Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. In Vitro Cell Dev Biol-Plant 38:168-172

Penfield S (2008) Temperature perception and signal in plants. New Phytol 179:615-628

Pospíšilová J, Tichá I, Kadleček P, Haisel D, Plzáková Š (1999a) Acclimatization of micropropagated plants to *ex-vitro* conditions. Biol Plant 42:481-497

Pospíšilová J, Synková H, Haisel D, Čatský J, Wilhelmová N (1999b) Effect of elevated $CO_2$ concentration on acclimation of tobacco plantlets to *ex vitro* conditions. J Exp Bot 50:119-126

Rong-hua L, Pei-guo G, Baum M, Grando S, Ceccarelli S (2006) Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. Agric Sci China 5:751-757

Shabala SN, Shabala SI, Martynenko AI, Babourina O, Newman IA (1998) Salinity effect on bioelectric activity, growth, $Na^+$ and chlorophyll fluorescence of maize leaves: a comparative survey and prospects for screening. Aust J Plant Physiol 25:609-616

Shim SW, Hahn EJ, Paek KY (2003) *In vitro* and *ex vitro* growth of grapevine rootstock '5BB' as influenced by number of air exchanges and the presence or absence of sucrose in culture media. Plant Cell Tiss Org Cult 75:57-62

Shrestha BR, Tokuhara K, Mii M (2007) Plant regeneration from cell suspension-derived protoplasts of *Phalaenopsis*. Plant Cell Rep 26:719-725

Su V, Hsu D, Chen WH (2001a) The photosynthetic activities of bare rooted *Phalaenopsis* during storage. Sci Hort 87:311-318

Su WR, Chen WS, Koshioka M, Mander LN, Hung LS, Chen WH, Fu YM, Huang KL (2001b) Changes in gibberellin levels in the flowering shoot of *Phalaenopsis hybrida* under high temperature conditions when flower development is blocked. Plant Physiol Biochem 39:45-50

Talbott LD, Rahveh E, Zeiger E (2003) Relative humidity is a key factor in the acclimation of the stomatal response to $CO_2$. J Exp Bot 54:2141-2147

Tokuhara K, Mii M (2001) Induction of embryogenic callus and cell suspension cultures of from shoot tips excised from flower stalk buds of *Phalaenopsis*. In Vitro Cell Dev Biol-Plant 37:457-461

Tokuhara K, Mii M (2003) Highly-efficient somatic embryogenesis from cell suspension culture of *Phalaenopsis* orchids by adjusting carbohydrate sources. In Vitro Cell Dev Biol-Plant 39:635-639

van Huylenbroeck JM, Piqueras A, Debergh PC (1998) Photosynthesis and carbon metabolism in leaves formed prior and during *ex vitro* acclimatization of micropropagated plants. Plant Sci 134:21-30

van Huylenbroeck JM, Piqueras A, Debergh PC (2000) The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated *Calathea* plants. Plant Sci 155:59-66

Wang WY, Chen WS, Chen WH, Hung LS, Chang PS (2002) Influence of abscisic acid on flowering in *Phalaenopsis hybrida*. Plant Physiol Biochem 40:97-100

Wu FZ, Bao WK, Li FL, Wu N (2008) Effect of water stress and nitrogen supply on leaf gas exchange and fluorescence parameters of *Sophora davidii* seedlings. Photosynthetic 46:40-48

Xiao Y, Kozai T (2004) Commercial application of a photoautotrophic micropropagation system using large vessels with forced ventilation: Plantlet growth and production cost. Hort Sci 39:1387-1391

Yoon YJ, Mobin M, Hahn EJ, Paek KY (2009) Impact of *in vitro* $CO_2$ enrichment and sugar deprivation on acclamatory responses of *Phalaenopsis* plantlets to *ex vitro* conditions. Environ Exp Bot 65:183-188