

Effects of High Temperature Exposure on Chlorophyll Fluorescence of *Phalaenopsis* Leaves

Phunsup Seubma^{1*}, Poonpipope Kasemsap², Olaf van Kooten³ and Jeremy Harbinson³

¹Department of Horticulture, Faculty of Agriculture at KPS, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

²Department of Horticulture, Faculty of Agriculture, Bangkok Campus, Kasetsart University, Bangkok 10900, Thailand

³HPC group, Department of Plant Science, Wageningen University and Research, Wageningen, The Netherlands

*Corresponding author. E-mail: phunsup.s@ku.ac.th

ABSTRACT

The effect of high temperature on photosystem II efficiency was studied in leaves of 16 month-old *Phalaenopsis* 'Sweetheart' plants grown under controlled cabinets. The various temperatures applied to leaves were 28, 35, and 47°C, for 30, 45, and 60 minutes, respectively. The distal half of individual leaves was divided into a shaded zone and illuminated zone and monitored the photosynthetic yield (PSII efficiency) by Walz MINI-PAM system. A PSI Fluorcam 700MF chlorophyll fluorescence imaging system was used to measure the dark-adapted F_v/F_m of the different leaf zones before and at the end of high temperature treatment, and then after 24 hours of recovery at 28°C. The *Phalaenopsis* leaves maintained a high photosynthetic yield (within 83% to 100% of maximum values) over a wide range of leaf temperatures, from 21°C to 45°C. The critical temperature, at which photosynthesis yield began to decrease sharply, was approximately 45°C. Leaf zones exposed to high temperature (47°C leaf temperature) showed an approximately 22% recovery in mean F_v/F_m after being transferred to 28°C growth cabinet for 24 h. After the 47°C leaf treatment, CF imaging system revealed the variation in the F_v/F_m distribution increased significantly and after 24 h of recovery reduced this variation substantially. The main source of variation in the F_v/F_m results was variation between leaves rather than within leaves. 'High temperature stress test' based on CF technology could be developed to sort *Phalaenopsis* plants by temperature tolerance levels to guarantee the certain quality for commercial application.

Key words: Chlorophyll fluorescence imaging system, PSII efficiency, F_v/F_m , Photosynthetic yield, High temperature exposure, *Phalaenopsis*, CAM, Heterogeneity

INTRODUCTION

Global warming affects all living things on Earth. Higher temperatures are likely to have the largest negative impacts on plants productions due to temperature trends exceeding more than twice the historical standard deviation in many locations since 1980 to 2008 (Lobell et al., 2011). The economic yield lost as a result of rising temperature is going to be an important agricultural problem in many areas in the world (Wahid et al., 2007). Extensive damage to thylakoid and inactivation of PSII can be caused by heat shock or heat stress if temperatures rise more than 10-15°C above the ambient level (Wahid et al., 2007; Baker and Rosenqvist, 2004). The earlier to diagnosis the estimated impacts, the faster to solve the problem to decrease yield losses. Advanced techniques which can detect early inside plants should be investigation.

As one of the most valuable potted flowering plants in the world in past years, good quality young *Phalaenopsis* plants, produced at a competitive cost from tissue culture laboratories in Netherlands, Taiwan and Thailand, are exported to other countries (Runkle et al., 2007; Blanchard et al., 2007). *Phalaenopsis* production from tropical countries such as Thailand often takes place under high

temperature conditions. Plant may be subjected to momentary stress from exposure to very high temperature for a short duration due to environmental variability, and as a CAM plant transpirative cooling cannot occur during phase III of the CAM cycle, when air temperatures and radiative loads will be highest.

Chlorophyll fluorescence (CF) is a widely used alternative technique for probing the operation of photosynthesis because of it is non-destructive, fast, relatively non-intrusive, precise, and it can be used to continuously monitor the change of photosynthetic activities over the same leaf of plant throughout the process of experiment (van Kooten and Snel, 1990; Maxwell and Johnson, 2000; Rosenqvist and van Kooten, 2003; DeEll and Toivonen, 2003). It has great potential as a non-invasive tool for monitoring the operation of photosynthesis *in vivo* in various crop plants including *Phalaenopsis* (Ali et al., 2005; Jeon et al., 2006; Hsu, 2007). It possible to manage stress at an early stage, and reduce permanent leaf damage and significant yield losses (Chaerle and van der Straeten, 2000; Nilsson, 1995). In addition, Chlorophyll fluorescence can be used as tool to categorize high temperature tolerance (Baker and Rosenqvist, 2004) as a good indicator for heat-resistance in grape cultivars (Kadir et al., 2007).

Imaging techniques as a whole are expected to have great potential for the detection of stress in leaves or canopies (Nilsson, 1995). While a range of techniques have been used in this role, chlorophyll fluorescence imaging technique seems to be one of the most useful (Chaerle and van der Straeten, 2000). It allows the two dimensional mapping over the surface of the leaf or other object of a wide range of parameters that describe the operation and regulation of photosystem II, such as the dark-adapted F_v/F_m . This has great potential in stress research for several clear reasons. First, several plants or leaves can be imaged simultaneously making screening more rapid, second the ability to spatially resolve the effects of stress can help analyse the physiological processes underlying the effect of the stress, and third it is possible to apply a variety of treatments to one leaf thus eliminating the problems caused by leaf to leaf variation as with conventional (i.e. non-imaging) fluorescence techniques. Imaging can be used to detect the onset of damage before any visual signs are apparent, for example injury due to herbicide treated *Arabidopsis* sp. and *Agrostis tenuis* (Barbagallo et al., 2003). Moreover, chlorophyll fluorescence imaging system showed that the coefficient of variation may be an important tool for assessing low temperature stress in *Calathea makoyana* leaves (Hogewoning and Harbinson, 2007).

The objectives of this research are 1) to investigate high temperature response of photosystem II efficiency and 2) to study effects of high temperatures on F_v/F_m and F_v/F_m heterogeneity of *Phalaenopsis*.

MATERIALS AND METHODS

Plant materials

Phalaenopsis 'Sweetheart' were grown in 1:1 bark:peat media in 12 cm pots in a greenhouse. At least 2 weeks prior to the experiment, 16 month-old plants were transferred to a controlled environment Weiss-Technik growth cabinet. In the cabinets plants were grown under a 12 h light and 12 h dark cycle at 28°C, PPF 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The plants were watered every 2 days and 20-20-20 N-P-K liquid fertilizer was applied every 7 days.

High temperature treatments

A lab-built incubator was a based upon modified freezer fitted with heating cables. Air temperature inside the incubator was measured and controlled Cal3300 controller (Cal Controls, Hitchin, UK) fitted with a chromel/alumel thermocouple. RH was automatically maintained at 70-80% by a nebulizer controlled by a simple on/off regulator. Three fans were used to provide sufficient mixing of the air inside the incubator. A laboratory built red/blue LED light source providing an irradiance (PAR) of 90% red and 10% blue was installed on the top of incubator, and supplied 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at plant level during high temperature exposure. Leaf temperature was monitored using the chromel/alumel thermocouple connected to Yokogawa chart recorder model LR4110 [as described

by Schmitt and Olfenbuttel (1994) and Dziejewski et al. (1998)] which was calibrated using an ice/water mixture: the temperature measurement of the Cal 3300 controller was not calibrated but was used only to set a nominal air temperature (Figure 1).

During high temperature treatments, a strip of black opaque plastic was placed in the distal half near the middle of the leaf to provide a transverse non-illuminated zone, which was flanked by illuminated zones. This allowed the differential effect of light and high temperatures combinations on the loss and recovery of F_v/F_m could be tested. Three high temperature treatments were performed by setting air temperature inside the incubator at 31, 39 or 52°C. Plants were exposed to high air temperatures for 70 minutes under a low light intensity ($100 \mu\text{mol m}^{-2}\text{s}^{-1}$) and temperatures of leaves in different treatments stabilized at approximately 28, 35, and 47°C, after 30, 45, and 60 minutes, respectively.



Figure 1. Image of *Phalaenopsis* plants in an opened incubator during high temperature treatment. The numbers represent (1) heating cables, (2) MiniPAM fiberoptic monitoring photosynthesis yield of an illuminated zone, (3) leaf temperature thermocouple attached to abaxial side, a shaded zone, (4) Vaisala HMP233 air humidity probe, (5) a shaded zone of a leaf, and (6) air temperature thermocouple. LED light source was installed on the top-cover and was not visible in this image.

Chlorophyll fluorescence measurement

A Photon Systems Instruments; FluorCam 700MF chlorophyll fluorescence imaging system (PSI, Brno, Czech Republic) was used to measure the dark-adapted F_v/F_m of the different leaf zones at before and the end of high temperature treatment, and then at 24 hours after treatment to monitor recovery. FluorCam v.5 software was used to control the imaging system, to process the images, and to calculate the average value and frequency distribution histogram. One intact leaf from each plant was put inside a controlled atmosphere cuvette in the imaging area, as described by Nedbal et al. (2000). The atmospheric composition inside the cuvette during fluorescence measurements was controlled as described by Nejad et al. (2006). Air composed of $20 \text{ mmol mol}^{-1} \text{ O}_2$ and $360 \mu\text{mol mol}^{-1} \text{ CO}_2$, with the remainder N_2 , was supplied to the cuvette in order to eliminate photorespiration. The relative humidity of the air flowing through the cuvette was $40 \pm 2\%$. The cuvette temperature was $26 \pm 1^\circ\text{C}$. After a dark-adaptation for 20 mins an F_o image was recorded, after which a saturating white light pulse ($2500 \mu\text{mol m}^{-2}\text{s}^{-1}$, generated with a 250 W halogen lamp) was applied for 1.2 s and during which an F_m image was recorded. These two images (F_o and F_m) were then used to calculate an image of the dark-adapted F_v/F_m (where $F_v = F_m - F_o$). The frequency distribution of F_v/F_m images from the shaded and illuminated zones of each treatment was analyzed using the method described by Hogewoning and Harbinson (2007). Once the measurement of fluorescence in the dark-adapted state were complete, continuous actinic irradiance of $100 \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR was provided by two panels each containing 345 orange light emitting diodes. When

the leaf had reached steady-state an image of the relative fluorescence yield was recorded, after which a saturating white light pulse was applied to the leaf for 1.2 s. During this saturating pulse, an F_m' image was recorded; these images were used to calculate Φ_{PSII} [as $(F_m' - F_s)/F_m'$, Genty et al. (1989)]. The plants were immediately put in the incubator to start high temperature treatment.

All experiments were performed on attached and fully expanded leaves of healthy *Phalaenopsis* plants. Prior to each treatment, a dark-adapted F_v/F_m control image was made to verify that the leaves were undamaged. All leaves used in this experiment had an average F_v/F_m greater than 0.77 and very small within-leaf variation of F_v/F_m .

A Walz MINI-PAM system was used to monitor photosynthetic yield of the illuminated mid-leaf zone during the treatment period. This allowed the immediate effects of temperature on photosynthetic yield to be monitored.

At the end of the 70 min high temperature exposure period, chlorophyll fluorescence of the same leaf was measured again with FluorCam system using the same procedure as described above. An image was taken after 20 min in the dark and another image after 20 min in the light. All plants were transferred back to growth cabinets for 24 h of recovery before a final chlorophyll fluorescence measurement using the FluorCam with the same procedure.

RESULTS

The illuminated *Phalaenopsis* leaves maintain a high photosynthesis yield (within 83 to 100% of maximum values) over a wide range of leaf temperatures from 21 to 45°C. The optimal temperature for photosynthesis yield, defined as the leaf temperature within which photosynthetic yield was within 95 to 100% of maximum values, is 25 to 42°C. The critical temperature, at which photosynthesis yield began to decrease sharply, was approximately 45°C (Figure 2).

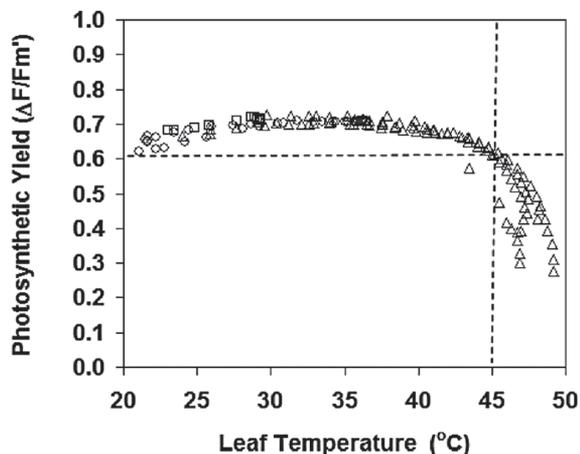


Figure 2. Photosynthetic yield of *Phalaenopsis* leaves during 70 min exposure to high air temperatures. Air temperature treatments were set at 31°C (squares), 39°C (circles) or 52°C (triangles).

Effect of high temperature treatment on PSII photochemical yield

The control F_v/F_m measurement performed approximately 30 min before temperature treatment averaged 0.771, which was 4% less than averaged F_v/F_m (0.804) after the treatment of leaves at 28 and 35°C (Figure 3). This implies that the reduction in F_v/F_m observed following other treatments cannot be attributed to the physical or mechanical stress of placing the leaf in the chlorophyll fluorescence imaging system.

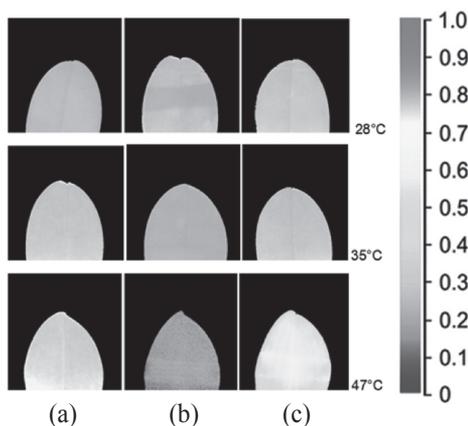


Figure 3. Images of F_v/F_m taken from *Phalaenopsis* leaves before (column a) and after high temperature treatments (column b), and after 24 h of recovery (column c). Images were arranged in rows according to stabilized temperatures of leaves in different treatments: 28, 35, and 47°C. F_v/F_m spectrum bar was shown on the right of the images.

The effect of high temperature treatment on F_v/F_m was similar for leaf zones exposed to high temperatures in the dark and in the light. At moderate temperature treatment (31 and 39°C air, 28 and 35°C leaf temperature, respectively), both shaded and illuminated leaf zones did not show any reduction in mean F_v/F_m measured after the treatment, while at high temperature treatment (52°C air and 47°C leaf temperature), both leaf zones developed an approximately 45% reduction in mean F_v/F_m , regardless of light environment during heat exposure period (Figure 3 and 4).

Both shaded and illuminated leaf zones exposed to 52°C air temperature treatment showed an approximately 22% recovery in mean F_v/F_m after being transferred to 28°C growth cabinet for 24 h (Figure 3 and 4).

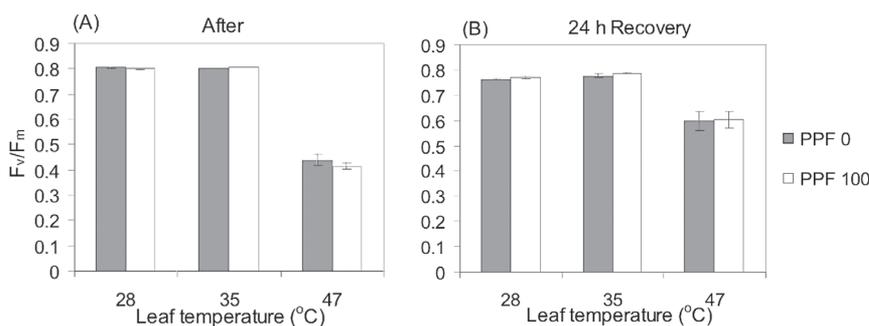


Figure 4. The changes in mean $F_v/F_m \pm S.E.$ of illuminated (PPF=100 $\mu\text{mol m}^{-2}\text{s}^{-1}$) and shaded zones (PPF=0 $\mu\text{mol m}^{-2}\text{s}^{-1}$) after high temperature exposure (A), and after 24 hr of recovery (B) of *Phalaenopsis* leaves exposed to leaf temperatures of 28, 35, or 47°C.

Heterogeneity in PSII efficiency after high temperature treatment

The frequency distributions of F_v/F_m before high temperature treatments (column a, Figure 5) showed very small variations. More than 98% of leaf area had an F_v/F_m within the ± 0.05 ranges.

No systematic difference in F_v/F_m heterogeneity between shaded and illuminated zones of leaves at both 28°C and 35°C temperatures was observed so the data was combined and illustrated in the same frequency distribution graphs. The variation in the F_v/F_m distributions of 28°C leaves

after the treatment and after 24 h of recovery, while that of 35°C leaves after treatment remained almost unchanged but it increased slightly (only 93% of leaf area had F_v/F_m within ± 0.05 ranges) after 24 h of recovery.

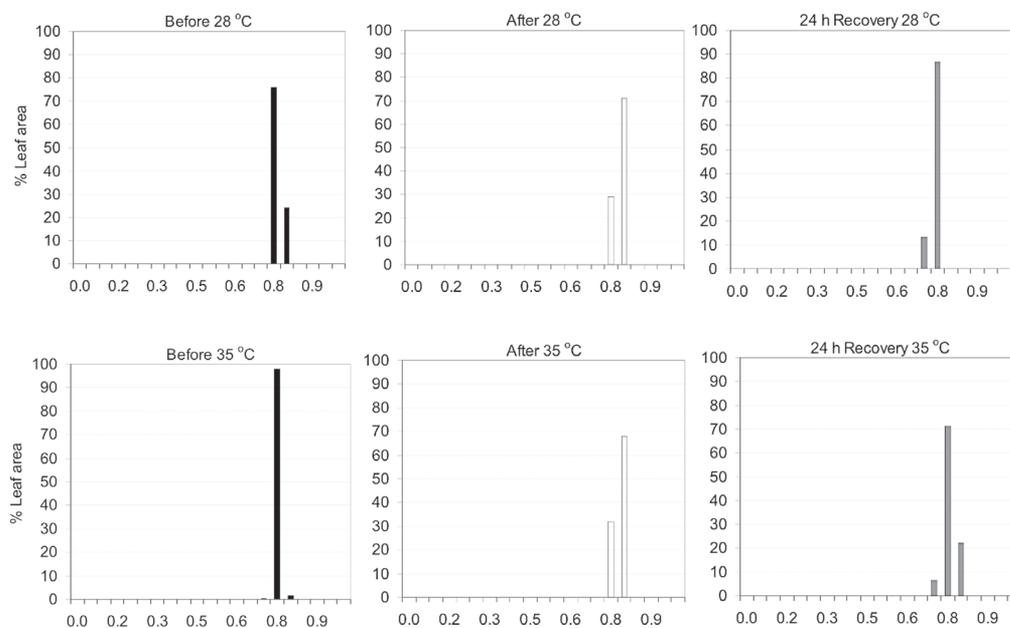
The reduction of F_v/F_m of both shaded and illuminated zones of leaves exposed to 47°C was heterogeneous. After the treatment, the variation in the F_v/F_m distributions increased significantly and a small difference in F_v/F_m heterogeneity between shaded and illuminated leaf zones developed. While all of the shaded area and 97% of illuminated leaf area had an F_v/F_m less than 0.6, the F_v/F_m distribution of the shaded zones had a bimodal distribution with a larger peak at 0.55-0.60 and a smaller peak at 0.20-0.25, while the distribution of illuminated zone had unimodal distribution with a peak at 0.45-0.50.

After 24 h of recovery, the variation in the F_v/F_m distributions was reduced significantly. Almost 70% of shaded leaf area and more than 50% of illuminated leaf area had F_v/F_m greater than 0.60. Comparing the shaded and illuminated leaf zones, there was a substantial difference in the heterogeneity of the F_v/F_m distribution after 24 h of recovery. While F_v/F_m distribution of shaded zones showed a unimodal distribution with one large peak (more than 42% of leaf area) at 0.65-0.70, the distribution of illuminated zone had bimodal distribution with two peaks, one at 0.75-0.75 and the other at 0.55-0.60.

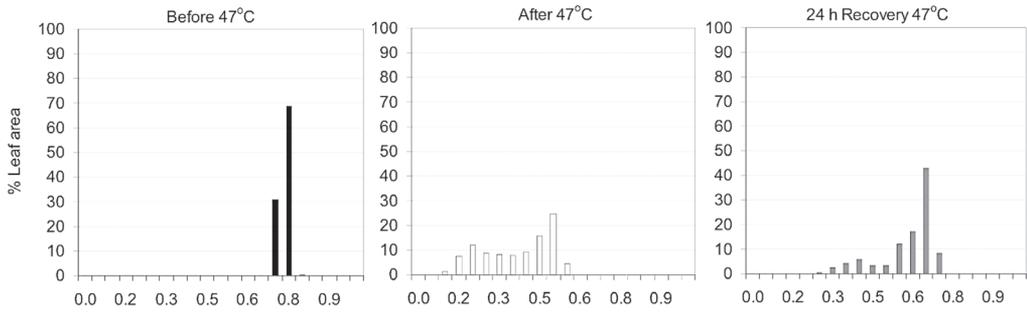
Leaf damage and necrosis after high temperature treatment

After moderate temperature treatments, no visible damage was apparent in any plants. However, one young leaf of one of the high temperature (47°C leaf temperature or 52°C air temperature) treated plants (out of 8 plants) developed small tip burn approximately one week after treatment. Five mature leaves of three high temperature treated plants developed leaf margin necrosis approximately two to three weeks after treatment.

(A) Illuminated and shaded zone



(B) Shaded zone



(C) Illuminated zone

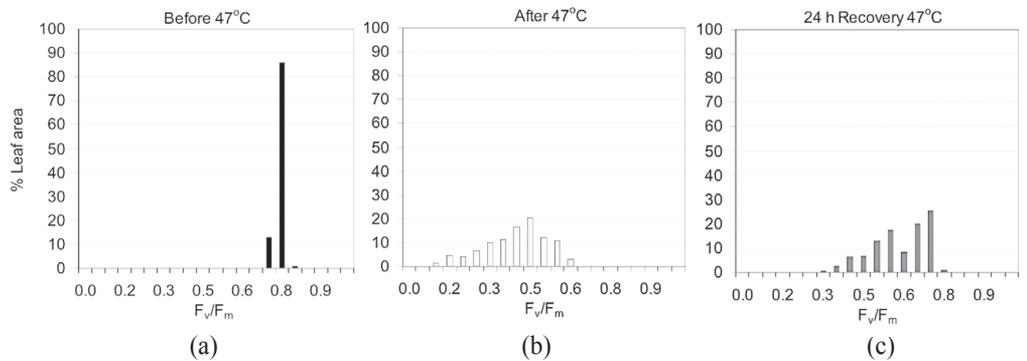
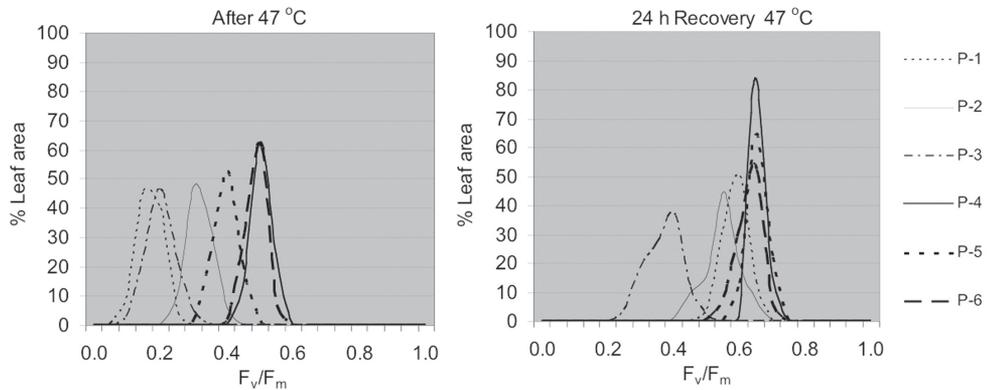


Figure 5. Frequency distribution of F_v/F_m images of *Phalaenopsis* leaves taken before high temperature treatments (column a), after the treatments (column b), and after 24 h of recovery (column c). Images were arranged in rows according to stabilized temperatures of leaves and leaf zones: combined data from illuminated and shaded zones at 28 and 35°C (A), shaded zone of 47°C (B), and illuminated zone of 47°C (C).

(A) Shaded zone



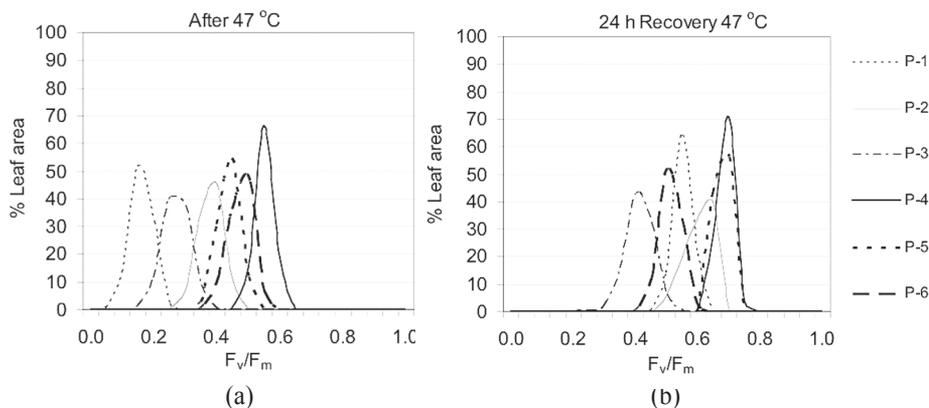
(B) Illuminated zone

Figure 6. Frequency distribution of F_v/F_m images of shaded zones (A) and illuminated zones (B) of *Phalaenopsis* leaves (one leaf per plant) measured immediately after treatment (column a) and 24 h later (column b).

DISCUSSION

Temperature response of photosynthesis yield

The optimal temperatures for photosynthesis are generally broad and match the average daytime growth temperature (Berry and Bjorkman, 1980; Larcher, 1995). *Phalaenopsis* leaves maintain high photosynthesis yield over leaf temperatures ranging from 21 to 45°C. Similarly, values of F_v/F_m of several plant species (the Antarctic hairgrass (*Deschampsia antarctica*), the creosote bush (*Larrea tridentate*), the Andean monocot (*Lysipomia pumila*), the desert shrub jojoba (*Simmondsia chinensis*), spinach (*Spinacea oleracea*), cotton (*Gossypium hirsutum*), and tobacco (*Nicotiana tabacum*)) from contrasting thermal environments were relatively constant over a wide range of temperatures (Salvucci and Crafts-Brandner, 2004). However, the critical temperature, at which F_v/F_m began to decrease sharply, varied greatly among those species. The decrease in F_v/F_m is regarded as an indicator of inactivation of PSII reaction centers, caused by damage to the thylakoid membrane (Rohacek, 2002). Species endemic to colder regions tended to have much lower critical temperatures (35 to 40°C) compared to those endemic to warmer regions (Salvucci and Crafts-Brandner, 2004). Furthermore, regardless of the species, the temperatures that caused F_v/F_m to decrease were higher than those that inhibited net photosynthesis rate (Law and Crafts-Brandner, 1999; Xiong et al., 1999; Georgieva et al., 2000; Hamerlynck et al., 2000). The fact that the critical temperature of *Phalaenopsis* in this experiment was approximately 45°C suggests that *Phalaenopsis* might not be subject to high temperature stress that could cause inhibition to photosynthesis rates in normal production conditions during which temperatures do not exceed 45°C.

Large heterogeneity in PSII efficiency is caused by differential response among leaves

After the high temperature treatment (52°C air and 47°C leaf temperature), averaged F_v/F_m distributions varied from 0.05 to 0.65 in shaded area and from 0.1 to 0.6 in illuminated area, while within individual leaf variation in the F_v/F_m distributions doubled from approximately ± 0.05 to ± 0.1 (Figure 6). The reduction of the average F_v/F_m of individual leaves/plants varied significantly, ranging from as large as 0.6 and 0.55 (leaf 1) to as small as 0.2 and 0.25 (leaf 4) in illuminated and shaded areas, respectively. This indicated that large heterogeneity in PSII efficiency after high temperature treatment was mainly caused by large difference in response to high temperature among leaves/plants and not by small increase in variation of F_v/F_m distribution within individual leaf.

The heterogeneity in PSII efficiency after 24 h of recovery reduced significantly to vary from 0.4 to 0.75 in the shaded area and 0.3 to 0.75 in the illuminated area. After 24 h of recovery, the heterogeneity in PSII efficiency within individual leaves remained large for recovering leaves and tended to reduce for leaves that nearly recovered (with the average F_v/F_m close to original value of 0.7-0.75 before heat treatment). The recovery of the average F_v/F_m of individual leaves/plants varied significantly, ranging from as large as 0.4 in both illuminated and shaded areas of leaf 1, to as small as 0.05 and 0.12 in illuminated and shaded areas, respectively, of leaf 4. Thus, the reduction in heterogeneity in PSII efficiency after 24 h recovery was mainly due to the difference in the recovery related reduction in F_v/F_m between leaves/plants and partly due to reduction in difference within individual leaves (Figure 6).

The magnitude of the recovery (F_v/F_m After – F_v/F_m 24 h) of PSII efficiency depends on both the value of F_v/F_m after the heat treatment and the reduction (F_v/F_m Before – F_v/F_m After) of the average F_v/F_m caused by high temperature treatment. Regression analysis showed statistically significant relations between variations in magnitude of F_v/F_m recovery and either the average F_v/F_m after heat treatment or the reduction of average F_v/F_m caused by heat treatment. While the average F_v/F_m after heat treatment could explained approximately 45% of the variation in the magnitude of the recovery of F_v/F_m , the reduction of the average F_v/F_m explained 53% of variations, and both factors combined to explain approximately 78% of the variation.

The cause of variation in level of F_v/F_m reduction and level of recovery among *Phalaenopsis* leaves/plants after high temperature treatment were not investigated in this research. The study by Lazar et al. (2006) using the frequency distribution of a range of chlorophyll fluorescence imaging derived parameters of Spring barley (*Hordeum vulgare* L. cv. Akcent) showed effects of plant age, with older plants having larger F_v/F_m reduction and greater variations compared to younger control. Further study is needed to determine them if leaf age or other factors are the causes of variation and what are the underlying causes of these changes.

Possibility to develop commercial application in the future

There is a potential to develop a 'high temperature stress test' based on chlorophyll fluorescence technology for commercial application in *Phalaenopsis*. Chlorophyll fluorescence measurements provide a rapid, sensitive and non-destructive method to detect early symptoms of heat stress in chlorophyll-containing fruits and vegetables. (DeEll and Toivonen, 2003). It can be used on whole plants using imaging technology (Baker, 2008) and the result can be analyzed on a whole-plant average measurements, or on target leaves with the same developmental stage (Munns et al., 2010). The imaging technology can be applied to high-throughput monitoring of stress response (Munns et al., 2010) or to automatic sorting systems, to assess quality (Moshou et al., 2005). Furthermore, at harvest chlorophyll fluorescence measurements of an individual, randomly sampled from within a group, has been correlated with postharvest quality of the entire group, as has shown for Iceberg lettuce (*Lactuca sativa* L.) (Schofield et al., 2005). Chlorophyll fluorescence could be combined with other quality measurement techniques to monitor the overall quality of plant or fruits, as has been shown for 'Golden' papaya during fruit ripening (Bron et al., 2004). Finally, the short-term heat treatment used in this research did not cause significant irreversible damage to *Phalaenopsis* plants, and thus could be suitable to use in quality stress test.

Therefore, we believe that in the future it should be possible to develop an automatic system to apply short term high temperature treatment and then to measure chlorophyll fluorescence after the treatment, and then again after a few hours of recovery, to determine the temperature tolerance of *Phalaenopsis* plants for exactly quality classification. Further research is needed to determine the most appropriate measurement procedure, such as temperature and duration of high temperature treatment, the recovery period, leaf age, and recovery environments before this method could be used commercially.

This 'stress test' system could enable growers to sort *Phalaenopsis* plants according to temperature tolerance levels and eventually to predict the period a certain quality level that can be guaranteed to the consumers under certain conditions.

CONCLUSION

Phalaenopsis leaves maintain a high photosynthesis yield over a wide range of leaf temperatures, from 21°C to 45°C. Photosynthesis yield began to decrease sharply at approximately 45°C. A high temperature treatment (47°C leaf temperature) caused substantial reduction in mean F_v/F_m , regardless of light treatment, but there was large variation in the F_v/F_m distributions after the treatment due to differential responses of individual plants. Substantial recovery from high temperature treatment was apparent after 24 h recovery at growth temperature. Further research would allow the development of a 'high temperature stress test' based on chlorophyll fluorescence technology for commercial application to *Phalaenopsis*.

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