Light quality affects light harvesting and carbon sequestration during the diel cycle of crassulacean acid metabolism in *Phalaenopsis*

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Abstract

Crassulacean acid metabolism (CAM) is a specialized photosynthetic pathway present in a variety of genera including many epiphytic orchids. CAM is under circadian control and can be sub-divided into four discrete phases during a diel cycle. Inherent to this specific mode of metabolism carbohydrate availability is a limiting factor for nocturnal CO₂ uptake and biomass production. To evaluate the effects of light quality on the photosynthetic performance and diel changes of carbohydrates during the CAM cycle *Phalaenopsis* plants were grown under four different light qualities (red, blue, red+blue and full spectrum white light) at a fluence of 100 μmol m⁻² s⁻¹ and a photoperiod of 12 h for 8 weeks.

In contrast to monochromatic blue light, plants grown under monochromatic red light showed already a significant decline of the quantum efficiency (Φ_{PSII}) after five days and of the maximum quantum yield (F_v/F_m) after ten days under this treatment. This was also reflected in a compromised chlorophyll and carotenoid content and total diel CO₂ uptake under red light in comparison with monochromatic blue and full spectrum white light. In particular CO₂ uptake during nocturnal phase I was affected under red illumination resulting in a reduced amount of vacuolar malate. In addition red light caused the rate of decarboxylation of malate during the day to be consistently lower and malic acid breakdown persisted until 4 hours after dusk. Because the intrinsic activity of PEPC was not affected the restricted availability of storage carbohydrates such as starch was likely to cause these adverse effects under red light. Addition of blue to the red light spectrum restored the diel fluxes of carbohydrates and malate and resulted in a significant enhancement of the daily CO₂ uptake, pigment concentration and biomass formation.

Keywords: CAM; *Phalaenopsis*; carbohydrates; gas exchange; PEPC; malate.

INTRODUCTION

Crassulacean acid metabolism (CAM) is a specialized photosynthetic pathway that improves atmospheric CO₂ assimilation in water-limited terrestrial and epiphytic habitats and increases water-use efficiency (Yang et al., 2015). CAM species are widely distributed throughout semiarid tropical and subtropical environments, including epiphytes in the humid tropics (Silvera et al., 2010). In CAM plants, a temporal separation of carboxylation (physiological) and decarboxylation (biochemical) events takes place. These temporal events are separated in four discrete phases according to the patterns of gas exchange and stomatal behavior (Osmond, 1978) i.e. nocturnal CO₂ uptake with open stomata in Phase I, early morning CO₂ uptake in Phase II, stomatal closure during the light period in phase III and late afternoon stomatal opening for CO₂ uptake in Phase IV. CAM plants take up atmospheric CO₂ through open stomata mainly via phosphoenolpyruvate carboxylase (PEPC) during the night and store it as malate in cell vacuoles. During the major part of the day (phase III), stomata are closed and malate is remobilized and degraded into CO₂ as source for photosynthetic activity via ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Osmond, 1978). During transition phases II and IV CO₂ can be taken up by PEPC, Rubisco or either a combination of both. Environmental factors such as drought influence the intrinsic CAM activity and thus the employment of the different phases of CAM. For example, phases II and IV are lost under conditions of severe drought stress, and stomata close throughout day and night while respiratory CO₂ is recycled as source for malic acid accumulation (Dodd et al., 2002; Ceusters et al., 2009).

Light, as one of the most important environmental factors, has profound effects on the development and metabolism of plants (Fankhauser and Chory, 1997; Smith, 1982). It is therefore not surprising that light quantity and especially light quality might affect CAM (Ceusters et al., 2010, 2011). Effects of light quality have mainly been studied in facultative CAM plants, which are plants that employ C3 photosynthesis, but under stressful conditions optionally use CAM photosynthesis. In *Kalanchoe blossfeldiana*, CAM is induced by short days through the red-light controlled synthesis of PEPC (Brulfert et al., 1988). In *Clusia minor*, an UV-A/blue light receptor was suggested to mediate the high-light induced C3-photosynthesis/CAM transition (Grams and Thiel, 2002). However, information of light quality on the endogenous circadian rhythm of CAM hardly exists. Effects of light quality on the CAM diel rhythm (diel gas exchange and metabolite turnover) has only been reported previously by Ceusters et al. (2014) on *Aechmea*, an obligate CAM Bromeliaceous plant. They studied effects on CAM by using low fluencies (10 $\mu\text{mol m}^{-2} \text{s}^{-1}$) of red, blue and green light, thus minimizing the direct involvement of photosynthetic processes but sufficient to sustain a typical CAM pattern. This study gave the first clear indication that both red and blue light signaling is inevitable to synchronize the patterns of important carbohydrates such as malate, sucrose and starch in the diel CAM cycle.

As carbohydrate availability and partitioning is considered an important limiting factor for carbon sequestration and as such also biomass formation in CAM plants (Borland et al., 2011; Ceusters et al., 2010), it is essential to comprehend the influences of both light quality and quantity on CAM photosynthetic performance. Moreover novel implications using LED technology become increasingly available for the horticultural industry providing potential benefits for CAM ornamentals such as bromeliads, orchids, *Kalanchoe* ... In the current study 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ fluence rates of blue, red and a combination of both were provided to *Phalaenopsis* orchids in comparison with full spectrum white light. *Phalaenopsis* orchids are epiphytes exhibiting obligate CAM photosynthesis (Guo and Lee, 2006; Mc Williams,

1970; Pollet et al., 2010; Sayed, 2001) and its hybrids became the most important flowering potted plants worldwide. They rank first both in economic turnover and unit sales that are handled through the Dutch auctions (Royal Flora Holland, 2018). To understand the influences of light quality on CAM photosynthetic performance we studied both relative short and long-term adaptations of the photosynthetic performance using chlorophyll fluorescence analysis. Next, we investigated if these light qualities influenced the carbon balance and diel rhythm at both the physiological (CO₂ balance) and biochemical level (malate content, carbohydrate content, PEPC activity).

MATERIALS AND METHODS

Plant material and growth condition

Phalaenopsis hybrid 'Exquisite Edessa' young-plants (Microflor, Belgium) were acquired when the second leaf from the apex had an average length of 6.3 ± 0.8 cm (n=8). They were transplanted in 12-cm plastic pots (600 mL) filled with orchid substrate based on pine bark (*Pinus maritima* Lam.). The acclimation and the light treatments were performed in a growth chamber and the growth conditions were 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD and 28°C day/night temperature with a 12-h day-length (08:00 to 20:00). They acclimated under high-pressure sodium lamps (SON-T, Philips Inc., Eindhoven, The Netherlands). Fertigation with water-soluble fertilizer for orchids (N:P:K = 20:20:20, pH = 6.0, EC = 1.0 dS m⁻¹) was supplied twice a week.

Light treatments

After one week of acclimation, plants were randomly allocated to a light treatment (25 plants per treatment). Light treatment sections were separated with black, plastic curtains in the growth room. Four light treatments were applied namely multispectral white (W, 300-800 nm, light emitting plasma lamp, Gavita BV, The Netherlands), blue (B, peak at 460 nm, Philips, Eindhoven, The Netherlands), red (R, peak at 660 nm, Philips, Eindhoven, The Netherlands) and a combination of red and blue (RB, 60%/40%, CI-800 programmable LED system, CID Bio-Science, WA, USA). As natural daylight is composed of nearly equal amounts of red and blue light, similar quantum fluence rates were applied for the RB combinations. For all treatments, light intensity at the canopy level was 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a photoperiod of 12 h. Light spectral distribution was recorded using a JAZ-ULM-200 spectrometer (Ocean Optics, FL, USA) and converted with Spectrasuite software (Ocean Optics) to $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 1) and the uniformity was verified by measuring the light intensity at five points of each light treatment at the canopy level.

Photosynthesis

Net CO₂ uptake was measured using a LI-6400 portable gas exchange system (Li-Cor Biosciences, NE, USA) on the second expanded leaf counting from the apex, this in four replicates per treatment. Measurements were conducted halfway the leaf avoiding the leaf vein (1 cm away from the main vein) for each treatment. All measurements were made under standard conditions (PPFD at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the day-time and at night the light was switched off in the sensor head, CO₂ concentration at 400 $\mu\text{mol mol}^{-1}$, leaf temperature at 28°C and vapor pressure deficit inside the chamber at 1.4-1.8 kPa.

Measurements were performed every 2 h for a 24 h period to calculate the diel CO₂ uptake respectively one week and eight weeks after the start of the light treatments. For the eight weeks measurement the net absorption/release of CO₂ during the 24-h cycle was divided into the four CAM phases (Griffiths, 1989; Nelson and Sage, 2008). Briefly phase I (night) started at 20:00 and ended at 08:00, phase II started at 08:00 and ended when the CO₂ uptake was negligible, at which Phase III began, Phase IV began when CO₂ uptake was not negligible and malate levels returned to their baseline and continued until 20:00 which is the onset of dark period.

Chlorophyll *a* fluorescence

The leaf chlorophyll *a* fluorescence measurement was conducted on the same leaf and position with a portable PAM 2500 chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The leaf was dark-adapted for 30 min, then 0.6s saturating light (3,450 μmol m⁻² s⁻¹) was given to obtain the maximal and minimal fluorescence yield (F_m and F₀). Next, the leaf was light-adapted with 5 min continuous actinic light at 100 μmol m⁻² s⁻¹ and saturating pulses were given every 25 s, the maximum (F_m') and the steady state fluorescence (F_s) signal were recorded. The actinic light was turned off and a far-red pulse was applied to obtain the minimal fluorescence after the PSI excitation (F₀'). Maximum photochemical efficiency of photosystem II (F_v/F_m) was calculated as (F_m - F₀)/F_m, PSII operating efficiency (Φ_{PSII}) was calculated as (F_m' - F_s)/F_m'; qP was calculated as qP = (F_m'-F_s)/(F_m'-F₀'); NPQ was estimated as NPQ = (F_m-F_m')/F_m' (Baker, 2008; van Kooten and Snel, 1990).

The effects of light quality on the chlorophyll fluorescence parameters were monitored each 5 days for the first 20 days and then at 10 days intervals (total period of 8 weeks), this in five replicates per treatment. The measurements started 2 h after the start of the photoperiod. The diel change of chlorophyll fluorescence parameters were monitored after 8 weeks under the light quality treatments by measuring every 2 h of the day cycle followed by two measurements during the night cycle at 4 h intervals.

Chlorophyll and carotenoids

After 8 weeks of light treatment, pigments were extracted with 80% (v/v) acetone overnight at -20°C. Absorbance at 470 nm (A₄₇₀), 647 nm (A₆₄₇) and 663 nm (A₆₆₃) were quantified spectrophotometrically (Infinite M200, TECAN Group Ltd., Switzerland). Chlorophyll and carotenoid contents were calculated as Chl_a = 12.25 A₆₆₃ - 2.79 A₆₄₇, Chl_b = 21.50 A₆₄₇ - 5.10 A₆₆₃, Carotenoids = (1000 A₄₇₀ - 1.82 Chl_a - 85.02 Chl_b)/198.

Metabolites and PEPC activity

Leaf samples were taken after 8 weeks of treatment each 2 h for a 24 h cycle. The two upper leaves were sampled, immediately frozen in liquid N₂ and stored in -80 °C until further analysis.

Malate was extracted by boiling distilled water for 15 min and quantified by anion-exchange chromatography (Thermo Fisher Scientific, USA) with 17-50 mM NaOH as gradient eluent and electrochemical detection with a Dionex IonPac AS 19 column at 30 °C.

Carbohydrates (200 mg FW) were extracted by 80% ethanol and quantified by means of high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD, Waters, USA) using a CarboPac PA-100

column (Dionex). Starch was extracted from the precipitate by 1M HCl at 95°C for 2 h. Starch content, expressed as glucose equivalents, was determined enzymatically by the reduction of NADP⁺ (measured at 340 nm, UV-VIS, Biotek Uvikon XL) with a hexokinase/glucose-6-phosphate dehydrogenase assay.

PEPC activity was expressed in terms of specific enzyme activity and protein content was determined according to Bradford (1976). The protein extraction buffer contained 100 mM HEPES-KOH (pH 8.0), 5 mM MgCl₂, 10 mM NaHCO₃, 5 mM ethylene glycol tetra-acetic acid (EGTA), 10 mM dithiothreitol (DDT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and polyvinylpyrrolidone (PVPP, 1%, w/v). The PEPC activity was determined according to López-Millán et al. (2000). Briefly, 50 µL of the extraction and 950 µL of enzyme buffer containing 100 mM Bicine [N,N'-bis(2-hydroxyethylglycine)] - HCl (pH 8.5), 5.0 mM MgCl₂, 10 mM NaHCO₃, 2 mM PEP and 0.2 mM NADH were added. PEPC activity (µmol mg⁻¹ protein min⁻¹) was measured by the reduction of the absorbance at 340 nm at 30 °C.

Growth parameters

After 8 weeks under the specific light treatments, four plants per treatment were randomly collected for the biomass determination. Plants were oven-dried at 85 °C for 72 h until a constant mass was reached then the dry mass was determined with an analytical balance (Mettler-Toledo, Greifensee, Switzerland). Leaf area was measured with a leaf area meter (Li-Cor 3000, Li-Cor Inc., USA) and the specific leaf area (SLA) was calculated as SLA= leaf area/leaf dry weight.

Data analysis

Data are presented as means ± SE. Results were analyzed using SPSS Statistics Version 24 (SPSS Inc., Chicago, USA), analyses were carried out using one-way ANOVA and means with significance difference were separated with Tukey's HSD test (P=0.05), figures were made with SigmaPlot 13.0 (Systat Software Inc., USA).

RESULTS

Temporal effects on chlorophyll fluorescence

Effects of an increasing period under monochromatic or dichromatic light in comparison with a control treatment were assessed by the maximum quantum yield (F_v/F_m) and PSII operating efficiency (Φ_{PSII}) (Figure 2). At day one F_v/F_m was 0.74-0.75 for all treatments and Φ_{PSII} ranged between 0.694 and 0.714. After ten days under monochromatic R light a significantly lower F_v/F_m ($P=0.001$) was found while no significant differences among the other light treatments were observed. F_v/F_m continued to decrease for the R grown plants and stabilized at 0.650 after 30 days. No significant time trend was observed for the other treatments. Similarly, Φ_{PSII} decreased under R compared to the other light treatments. R grown *Phalaenopsis* resulted in a significantly lower Φ_{PSII} after five days ($P<0.001$). In the following days, Φ_{PSII} continued to decline and reached a steady lower value ($\Phi_{PSII} = 0.620$) compared with the other treatments after 15 days. No significant differences between B, RB and W were observed and no temporal effect was present.

Diel change of chlorophyll fluorescence parameters

F_v/F_m hardly fluctuated during the 24-h cycle. As already shown in Figure 2, F_v/F_m was significantly lower under R. A slight fluctuation was present ranging from 0.654 (at 08:00) to 0.701 (at 14:00) followed by a slow decrease. For the other light treatments, time course fluctuations were hardly observed. Yet small differences between the treatments were present: at 14:00 F_v/F_m was the greatest under B followed by RB and was significantly lower for W while two hours later no significant differences were found between B, RB and W.

The light-adapted chlorophyll fluorescence parameters were monitored during the day phase (Figure 3). At the start of the day phase (08:00) Φ_{PSII} and qP were high and these values remained rather stable for the next 8 h (16:00), then followed a decrease till the start of the night phase (20:00). This trend was similar for the four light treatments though significant lower values were obtained for R compared to the other light treatments. NPQ fluctuated between 0.085 and 0.221 but no clear differences between the treatments was observed. NPQ is not displayed under R because interpretation of the thermal dissipation process is limited to plants exhibiting similar F_v/F_m values (Baker, 2008).

Effects on leaf gas exchange

To investigate effects of light quality on photosynthesis, both short time (1 week, Table 1) and long-term effects (8 weeks, Figure 4, Table 2) when leaf lengths were already 17.6 ± 0.9 cm ($\pm$ 3-fold increase compared to the start) were investigated. As expected *Phalaenopsis* showed a CAM pattern with nocturnal CO₂ assimilation. CO₂ uptake rates were integrated for 24 h, and after one week the total diel CO₂ uptake under R tended to decline though this was not significant (Table 1).

After eight weeks, the total diel CO₂ uptake was highest under B followed by W and significantly lower under RB and R (Table 2). Taking the data of malate degradation and starch accumulation into account, the four phases of the CAM cycle could clearly be distinguished (Figure 4, Table 2). CO₂ uptake during the night (phase I) was significantly lower for plants under R light (Table 2). During the transition to the light phase, i.e. phase II, integrated CO₂ uptake was significantly greater under B and RB followed by R compared to W (Table 2). The duration of phase II was the longest under B followed by RB and R and significantly shorter under W. In phase III, CO₂ losses tended to be lower under B though no significant effects were present. In phase IV, which was the late afternoon before the night phase started, CO₂ uptake started again in all treatments. This phase started earlier under B followed by RB and R and was the shortest phase under W, however, no significant differences were found.

Diel change of metabolite contents

Malate content: the CAM pattern of nocturnal accumulation and degradation during the light period is clearly shown for all treatments (Figure 4). At the end of phase I (08:00) the malate level of the light treatments was: R 54.3 ± 1.6 , B 60.4 ± 1.6 , RB 66.0 ± 4.5 and W 63.8 ± 2.1 μmol g⁻¹ FW, respectively. Lowest levels were found under R (P=0.01). The dusk-dawn malate accumulation was also significantly lower under R compared to the other light treatments (Figure 5). The kinetics for malate degradation during phase III were clearly different for the treatments. Under B and RB basal malate levels were obtained at 16.00, a slower decrease was observed for W where basal levels were reached at 18.00. The decrease of malate in R grown *Phalaenopsis* was significantly retarded compared to the other treatments

and continued during the first four hours of the night phase. Upon onset of the dark period (20:00), malate levels increased in B, RB and W, whilst this increase was observed only four hours later for R.

Carbohydrate and starch content: Figure 4 shows the diel changes of starch and sucrose contents in *Phalaenopsis*; both displayed an inverse diel pattern compared to malate content. As storage sugars, they accumulated during the day period due to the carboxylation of CO₂ released from malate and degraded at night to provide PEP for nocturnal CO₂ fixation. At the time of the start of the light period (08:00), starch content of the four light treatments were low and averaged around 2.2 ± 0.4 $\mu\text{mol D-glucose equivalents g}^{-1}$ FW. During the light period starch values increased to reach their highest levels at the start of the night period (20:00) with averages of 18.3 ± 1.6 , 17.9 ± 1.4 , 21.5 ± 1.7 and 25.8 ± 0.1 $\mu\text{mol D-glucose equivalents g}^{-1}$ FW for R, B, RB and W respectively. W resulted in significant higher values compared to R (Figure 4). Starch contents decreased during the night from 22:00 for W and from 24:00 for the other light qualities.

The diel pattern was less pronounced for sucrose. At the start of the light period (08:00) it was respectively 2.8 ± 0.3 , 2.2 ± 0.1 , 4.2 ± 0.3 and 4.0 ± 0.6 $\mu\text{mol g}^{-1}$ FW for R, B, RB and W. Fluctuations during the light period were observed but only after two hours night phase a significant decrease was recorded for all light treatments. The difference between the start of the photoperiod (08:00) and the start of the nocturnal period (20:00) (Figure 4) was 2.7, 1.8, 1.4 and 1.7 $\mu\text{mol g}^{-1}$ FW for R, B, RB and W, respectively.

PEPC activity: The total protein content was not significantly affected by the light treatment ($P > 0.05$) though it tended to be lower under R and W. Figure 6 shows the PEPC activity at four time points: 00:00, 08:00, 13:00 and 20:00. It is clearly shown that at midnight (00:00), PEPC activity was the greatest, whilst only negligible activity was present at noon (13:00). At midnight, PEPC activity was greater under RB and W compared to R and B though this difference was not significant ($P > 0.05$). As expected, dawn activity was lower than dusk activity ($P = 0.02$), but no significant differences underpinned by light quality were found concerning PEPC activity.

Growth and pigment contents of *Phalaenopsis*

Table 3 presents chlorophyll and carotenoid contents after 8 weeks of growth under the four different light treatments. The highest content of all pigments was found under B. In detail, Chl *a* was significantly higher under B and RB, while it decreased under W and was lowest under R; Chl *b* was significantly higher under B, declined under RB and W while it was lowest under R. Chl *a/b* ratio was unaffected by light quality. Carotenoid content was significantly higher under B, followed by RB and W and the lowest content was found under R.

The total leaf area, specific leaf area and dry biomass are shown in Table 4. The highest dry mass was obtained for RB and W compared to R, while B had an intermediate dry mass. No significant effects of light quality on total leaf area and specific leaf area were found though SLA tended to be higher under R compared to W.

DISCUSSION

Chlorophyll fluorescence

Chlorophyll fluorescence analysis has widely been used to assess the functioning of photosystem II under abiotic and biotic stresses (Jones and Hoegh-Guldberg, 2001; van Kooten and Snel, 1990). F_v/F_m of unstressed plants varies typically between 0.75 and 0.85 (Quiles, 2005). At the start of the experiment values for *Phalaenopsis* ranged between 0.75-0.80 which is in agreement with values reported by Ouzounis et al. (2015) and Pollet et al. (2009).

When grown under monochromatic red light a significant decline of both F_v/F_m and Φ_{PSII} appeared after respectively 10 and 5 days (Figure 2). A decline in F_v/F_m is correlated with loss of PSII photosynthetic activity of isolated thylakoids (Krause et al., 1990), which is interpreted as PSII down-regulation via sustained components of protective NPQ rather than photodamage (Foyer et al., 2017). This decline of the photosynthetic capacity under R continued and reached a stable significant lower level compared with the other light treatments. These negative effects of R persisted and were also clearly visible in the diel change of F_v/F_m and Φ_{PSII} after 8 weeks of acclimation (Figure 3). Although a short term exposure to high fluence, broad spectrum red light has been reported previously to result in higher photosynthetic performance (McCree, 1971) and in a most efficient deacidification and photosynthesis (measured as photosynthetic oxygen evolution) in isolated leaves of *Kalanchoe daigremontiana* (Barrow and Cockburn, 1982); it is no surprise that long-term red light exposure to whole plants abated photosynthetic performance. Previous studies showed that monochromatic red light reduced F_v/F_m in different C3 species (Trouwborst et al., 2016), *Chrysanthemum*, *Cordyline*, *Ficus*, *Spathiphyllum* (Zheng and Van Labeke, 2017a, 2017b) and rapeseed (Shengxin et al., 2016). If only red light is provided a strong overall phosphorylation of both the PSII core and LHCII takes place and PSI excitation is strongly favored over PSII, leading to imbalances in photosystem excitation (Ferroni, 2012; Tikkanen et al., 2010). Our results show that although the negative effects of red light on F_v/F_m and Φ_{PSII} occur within days after the start of the red light treatment in the *Phalaenopsis* leaves, this is hardly affecting daytime CO_2 uptake in both transition phases II and IV but significantly less CO_2 was sequestered during the night (Table 2). Obligate CAM plants such as *Phalaenopsis* typically engage in main nocturnal carboxylation and only marginal daytime CO_2 uptake. In CAM plants a decline in PSII efficiency might bring about limitations in subsequent nocturnal uptake by limiting the energy-requiring export of malic acid out of the vacuole and/or malic acid decarboxylation in the cytosol during daytime (Holtum et al., 2005; Ceusters et al., 2011). It has already been shown that inhibition of the electron flow in PSII by DCMU (diuron) markedly inhibited deacidification in *Kalanchoe pinnatum* (Nishida and Hayashi, 1981). We also observed a slower deacidification under R during phase III (Figure 4B). If only monochromatic blue is provided no negative effects on F_v/F_m and Φ_{PSII} were observed and addition of blue to red (RB) restored the imbalances as well.

The observed long-term reaction of both F_v/F_m and Φ_{PSII} is also reflected in the pigment concentration. Monochromatic red light significantly reduced Chl a , Chl b , total Chl and carotenoid content compared to the other light treatments (Table 3). As chlorophyll absorbs light both in the red and blue spectrum (Terashima et al., 2009), the reduced chlorophyll content under R will also contribute to a lower photosynthetic efficiency as assessed by F_v/F_m and Φ_{PSII} . These observations clearly indicate that blue light is inevitable to accommodate an efficient photosystem processing. On the other hand, monochromatic blue light seemed able to compensate for the lack of red light as higher concentrations of chlorophyll and carotenoids under monochromatic blue resulted in unaffected F_v/F_m and Φ_{PSII} compared to either white light or a combination of red and blue. Blue light has previously been shown to promote biosynthesis of chlorophyll pigments (Hoffmann et al., 2015; Olle and Viršile, 2013; Sæbø et al., 1995).

Diurnal changes were observed for both photochemical quenching (qP) and the quantum yield (Φ_{PSII}). Under a constant light fluence we observed a steady value for the beginning and middle part of the photoperiod when malic acid decarboxylation provided saturating concentrations of intercellular CO₂ for photosynthesis activity (Winter and Lesch, 1992). Towards the end of the photoperiod, the internal pool of malic acid has been consumed, leading to a decrease of Φ_{PSII} and qP (Adams et al., 1989). The steep decrease of qP and Φ_{PSII} at the transition of day to night corresponds to the termination of photosynthetic electron transport due to Calvin cycle inactivation (Pollet et al., 2009). During the light period, qP, as a measure of the proportion of open PSII reaction centers, is always lower under R while no differences between the other light treatments were observed. Although the values for non-photochemical quenching are low, RB resulted in average in the highest NPQ values during the day, W in the lowest and for B a steady increase during the light period was observed.

Gas exchange and metabolite dynamics

After 8 weeks under the respective monochromatic light treatments (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) the different phases in Osmond's framework could all be distinguished. Upon the onset of the day, a short Phase II was observed in the four light treatments. Ceusters et al. (2014) reported only a phase II under W and low-fluence B in the obligate CAM *Aechmea*, while this phase was absent under low-fluence R. In C3 plants it is generally accepted that blue light triggers stomatal opening through the blue light photoreceptors, phototropin and cryptochrome (Boccalandro et al., 2012; Liscum et al., 2003; Shimazaki et al., 2007). This signaling pathway is supposed to be weaker or absent in CAM species (Lee and Assmann, 1992). In addition, recent transcriptomic analyses of the consecutive CAM plant *Agave americana* did not reveal a prominent role regarding stomatal regulation (Abraham et al., 2016). However, in *Aechmea* low fluence B clearly induced early morning stomatal opening and phase II occurred while this was not the case for low fluence R (Ceusters et al., 2014). The different response to R in phase II between the results of Ceusters et al. (2014) in *Aechmea* and our data might also be due to the applied fluence levels which were 10-fold higher in our treatments (10 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ respectively). It is without doubt that the role of blue light to induce stomatal opening at the transition of the dark to the light phase needs further investigation, but different mechanisms might exist in CAM species. There are several hypotheses about stomatal opening mechanisms of CAM, such as the internal CO₂ concentration, leaf-air vapor pressure deficit and photoperiodic circadian rhythm (Lee, 2010; Males and Griffiths, 2017).

As mentioned earlier, the rate of decarboxylation under R during the photoperiod was consistently lower compared to the other light treatments and malic acid breakdown persisted until 4 hours after dusk. Together with higher basal levels of malic acid, these observations might indicate that red illumination causes a lowered ME activity in the leaf mesophyll cells or bring about an impaired malate efflux out of the vacuole. Red light is energetically less favorable compared with blue and it has generally been demonstrated in CAM plants that day-time integrated photosynthetic photon flux density is positively correlated with the magnitude of nocturnal malic acid accumulation and its subsequent mobilization from the vacuole and decarboxylation during the following day (Lüttge, 2002). This is also consistent with our findings that B and RB brought about an acceleration of malic acid consumption in comparison to control plants under white light. Starch was the main carbohydrate storage to fuel nocturnal carboxylation in *Phalaenopsis*. Less starch was available under R and B compared to white light illuminated plants. The availability of carbohydrate

storage is generally considered to be a major limiting factor for malate synthesis and consequently for the magnitude of dark CO₂ uptake in CAM plants under different environmental conditions (Borland and Dodd, 2002; Ceusters et al., 2011, 2010). However, comparing only R and B no differences were observed between starch dynamics implying other/more limiting factors to restrict nocturnal CO₂ uptake under red illumination. Since our measurements indicate that intrinsic activity of PEPC was not statistically different among the light treatments it is indeed very likely that daytime processing of malic acid out of the vacuole and consequent decarboxylation by malic acid is reduced under R. Phase IV CO₂ assimilation was also observed in all treatments (Table 2). We recorded an earlier CO₂ uptake in phase IV under blue light and RB leading to a longer duration of phase IV (Table 2, Figure 4). This earlier start of phase IV may be explained by the low intercellular CO₂ concentration induced by the end of malate breakdown which was earlier in B and RB, thus resulting in the reopening of stomata to initiate Phase IV (Males and Griffiths, 2017).

Biomass

The significant decrease in photosynthetic efficiency, diel CO₂ uptake and turnover of starch under monochromatic red light was also reflected in biomass accumulation, but to a lesser extent. R grown *Phalaenopsis* produced about 15% less biomass in comparison to white illuminated plants (Table 4). Ouzounis et al. (2015) even found a $\pm 25\%$ decrease of leaf fresh weight for *Phalaenopsis* under R in a daylight background. Leaf area and specific leaf area were not affected by light quality in our treatments. For a similar period of 8 weeks *Phalaenopsis* ‘Vivien’ increased its leaf area with an increasing blue light fraction while ‘Purple Star’ was not affected (Ouzounis et al., 2015) indicating the species dependency for effects of light quality on leaf development.

CONCLUSION

In conclusion, long-term monochromatic red light induced disorders in the photosynthetic response in comparison with treatments including blue light. This was reflected in reduced maximum quantum yield, quantum efficiency, chlorophyll and carotenoid content, starch and malate formation and gas exchange and biomass. Addition of blue light to the red light spectrum restored the diel fluxes of carbohydrates and malate and resulted in a significant enhancement of the daily CO₂ uptake, pigment concentration and biomass formation. The present study stressed the importance of blue light quality in regulating an efficient photosystem II and carbohydrate availability. Blue light influenced the diel CAM rhythm resulting in earlier CO₂ uptake in phase IV and enhanced malate metabolism. Further investigations on the stomatal behavior explaining the effects of light quality on the regulation of CAM phases are needed.

Compliance with ethical standards

Acknowledgements

The first author received a CSC scholarship and a funding from Ghent University (BOF14/CHN/012).

Conflict of Interest

350 The authors declare that they have no conflict of interest.”

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Table 1. Integrated CO₂ uptake of young *Phalaenopsis* leaves (mmol CO₂ m⁻²) over a 24 h period after 1 week under a light quality treatment (red, blue, red + blue, white).

Light quality	Night	Day	Total 24 h
R	44.3 ± 2.5	-7.8 ± 1.1	36.5 ± 3.4
B	49.2 ± 2.5	-7.1 ± 1.9	42.1 ± 1.4
RB	45.1 ± 3.6	-6.7 ± 0.4	38.4 ± 3.3
W	48.2 ± 1.4	-5.3 ± 1.4	42.9 ± 2.8

Data are mean ± SE (n=4). No significant differences at P=0.05 were observed.

Table 2. Integrated CO₂ uptake amount (mmol CO₂ m⁻²) and phase duration by phase over a 24 h period for each light treatment (red, blue, red + blue, white) after 8 weeks of mature *Phalaenopsis* leaves.

Light quality	CO ₂ uptake amount integrated by phase (mmol CO ₂ m ⁻²)				
	Phase I	Phase II	Phase III	Phase IV	Total 24h
R	34.6 ± 1.0 b (92.1%)	0.9 ± 0.1 ab (2.6%)	-4.4 ± 0.6 a	2.1 ± 0.7 a (5.6%)	33.2 ± 1.8 c
B	45.2 ± 1.2 a (91.9%)	1.5 ± 0.2 a (3.0%)	-3.0 ± 1.2 a	3.2 ± 0.5 a (6.3%)	47.7 ± 2.2 a
RB	42.4 ± 1.6 a (94.5%)	1.3 ± 0.2 a (2.9%)	-5.5 ± 0.4 a	1.1 ± 0.5 a (2.5%)	39.4 ± 1.8 bc
W	48.0 ± 1.5 a (96.9%)	0.5 ± 0.1 b (1.1%)	-6.5 ± 1.3 a	1.0 ± 0.4 a (2.0%)	43.1 ± 1.7 ab
Phase duration (h)					
R	12	1.23 ± 0.05 ab	8.83 ± 0.46 ab	1.95 ± 0.23 a	24
B	12	1.76 ± 0.13 a	6.89 ± 0.41 b	3.38 ± 0.23 a	24
RB	12	1.41 ± 0.22 ab	8.32 ± 0.53 ab	2.27 ± 0.67 a	24
W	12	1.05 ± 0.06 b	9.29 ± 0.38 a	1.66 ± 0.41 a	24

Data present in mean ± SE (n=4). Different letters indicate significantly difference between treatments (P<0.05) according to Tukey's HSD test.

Table 3. Pigments contents of *Phalaenopsis* grown for 8 weeks under different light quality (red, blue, red + blue, white).

Light quality	Chl a (mg g ⁻¹ FW)	Chl b (mg g ⁻¹ FW)	Carotenoids (mg g ⁻¹ FW)	Total Chl (mg g ⁻¹ FW)	Chl a/b
R	15.29 ± 1.51 c	7.62 ± 0.51 d	3.94 ± 0.45 c	22.91 ± 1.96 c	2.0 ± 0.1 a
B	34.14 ± 1.05 a	15.68 ± 0.19 a	8.65 ± 0.11 a	49.82 ± 1.24 a	2.2 ± 0.0 a
RB	30.33 ± 2.44 a	13.62 ± 0.62 b	7.28 ± 0.44 b	43.95 ± 3.06 a	2.2 ± 0.1 a
W	23.73 ± 0.55 b	11.61 ± 0.19 c	6.22 ± 0.08 b	35.33 ± 0.20 b	2.1 ± 0.0 a

Data present in mean ± SE (n=4). Different letters indicate significantly differences between treatments (P<0.05) according to Tukey' HSD test.

Table 4. Total dry biomass, leaf area and specific leaf area of the second leaf from the apex (SLA) of *Phalaenopsis* grown for 8 weeks under different light qualities (red, blue, red + blue, white).

Light quality	Biomass (g)	Leaf area (cm ²)	SLA (cm ² g ⁻¹)
R	1.58 ± 0.02 b	198.3 ± 2.4 a	125.54 ± 3.3 a
B	1.77 ± 0.07 ab	200.9 ± 7.8 a	112.95 ± 3.3 a
RB	1.79 ± 0.03 a	211.2 ± 3.5 a	113.75 ± 4.3 a
W	1.86 ± 0.04 a	192.6 ± 4.6 a	107.73 ± 8.3 a

Data present in mean ± SE (n=4). Different letters indicate significantly differences between treatments (P<0.05) according to Tukey's HSD test.

Figure 1. Relative light spectrum distribution of four light treatments. Spectrum was measured at the plant canopy level with a JAZ spectrometer (Ocean Optics, FL, USA). R, red light; B, blue light; RB, red/blue (60%/40%) polychromatic light; W, white light.

Figure 2. Maximum quantum efficiency of photosystem II (F_v/F_m , A) and quantum yield of photosystem II (Φ_{PSII} , B) changes after transfer to treatments differing in light quality (red, blue, red + blue, white). Values are the means with standard errors shown by vertical bars ($n=4$). Asterisk indicates for significant difference ($P<0.05$) among treatments according to Tukey's HSD test. Time course change for both parameters under R are marked with different letters to indicate significant differences according to Tukey's HSD test ($P=0.05$).

Figure 3. Diel change of chlorophyll fluorescence parameters, maximum quantum efficiency of photosystem II (F_v/F_m , light and dark period, A), quantum yield of photosystem II (Φ_{PSII} , light period, B), photochemical quenching (qP , light period, C) of *Phalaenopsis* under different light qualities (red, blue, red + blue, white) and non-photochemical quenching (NPQ, light period, D) under blue, red+blue and white. The horizontal black bar indicates the night period. Data represent the mean of four individual plants with standard errors shown by vertical bars ($n=4$). Asterisk indicates for significant difference ($P=0.05$) among treatments according to Tukey's HSD test.

Figure 4. Diel change of the CO₂ uptake (A), malate content (B) and storage carbohydrates: starch (C) and sucrose (D) in *Phalaenopsis* leaves under different light quality (red, blue, red + blue, white) 8 weeks after the start of the light treatments. The horizontal black bar indicates the night period. Data represent the mean of four replicates with standard errors shown by vertical bars.

Figure 5. Starch content (A) and malate content (B) of *Phalaenopsis* leaves under different light quality (red, blue, red + blue, white) at dawn (08:00) and dusk (20:00), and the dawn and dusk difference of starch content (C) and malate content (D). Data are shown as mean $\pm$ SE (n=4), different letters indicate for significant differences according to Tukey's HSD test (P<0.05).

Figure 6. PEPC activity and total soluble protein content of *Phalaenopsis* leaves at four time points (08:00, 13:00, 20:00 and 24:00) under different light quality treatments (red, blue, red + blue, white). Data shown as means with standard errors (n=4), different letters indicate for significant differences according to Tukey's HSD test ($P < 0.05$), n.s., not significant.