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BRIEF COMMUNICATION

# Low irradiance alters carbon metabolism and delays flower stalk development in two orchids

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## Abstract

In *Phalaenopsis*, lowering irradiance has been used to delay flower stalk development but the accompanying biochemical changes remain poorly understood. We cultured two commercial *Phalaenopsis*-type orchids, *Phalaenopsis* cv. Sogo Yukidian V3, and *Doritaneopsis* cv. Walnut Valley Halo ES09 under reduced irradiance by under-bench shading (approximately 15 % of mean control irradiance) for 15 weeks in a greenhouse under the natural photoperiod. Besides delaying flower stalk development as expected, the treatment greatly decreased the activities of ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoenolpyruvate carboxylase, and $NAD^+$-malic enzyme, and reduced the nocturnal malate accumulation and daytime starch deposition, the typical diurnal metabolite fluctuations of crassulacean acid metabolism (CAM) plants. As well, the content of sucrose and starch was reduced at dawn and dusk whereas the content of glucose and fructose only at dawn. The persistent decrease in the sucrose content under shading may be an inhibitory signal of flower stalk induction.

*Additional key words*: CAM metabolism, *Doritaneopsis,* organic acids, PEPC, *Phalaenopsis*, RuBPC/O, shading, sugars.

Lowering irradiance has been recommended to replace warm temperature treatment for delaying flower stalk emergence and flowering date in *Phalaenopsis* (Kubota and Yoneda 1993, Wang 1995, 1997, Hisamatsu *et al*. 2001, Liu *et al*. 2010). Wang (1995) demonstrated that flower stalk induction of *Phalaenopsis* cultured in a growth chamber was inhibited by a treatment with irradiance of 8 μmol $m^{-2}$ $s^{-1}$ under a 12-h photoperiod or darkness for 6 weeks. Wang (1997) also conducted an irradiance-reducing experiment in a greenhouse by covering *Phalaenopsis* with black fabric for different days weekly which resulted in deferred flower stalk development and flowering time. Our previous study showed that a simpler method, namely under-bench shading, which reduces irradiance to 15 % of control on the mobile bench for 14 weeks could effectively delay flower stalk development of mature *Doritaenopsis* in greenhouse (Liu *et al*. 2010). Nevertheless, the biochemical changes in orchids induced by irradiance reduction remain poorly understood.

*Phalaenopsis* is an obligate crassulacean acid metabolism (CAM) plant and exhibits typical diurnal fluctuations of malate and starch content (Endo and Ikusima 1989, Guo and Lee 2006, Chen *et al*. 2008, Pollet *et al*. 2011) which implies a close relationship between sugar and organic acid metabolism.

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*Abbreviations*: CAM - crassulacean acid metabolism; f.m. - fresh mass; $NAD^+$-ME - $NAD^+$-linked malic enzyme; PEPC - phosphoenolpyruvate carboxylase; RuBPC/O - ribulose 1,5-bisphosphate carboxylase/oxygenase.
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In CAM plants, sugar biosynthesis is mainly regulated by activity of ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBPC/O; EC 4.1.1.39) whereas the synthesis of malate is ascribed to activity of phospho-enolpyruvate carboxylase (PEPC; EC 4.1.1.31) and its degradation to activity of $NAD^+$-linked malic enzyme ($NAD^+$-ME; EC 1.1.1.39) (Osmond 1978). Here, we studied changes in the activities of these enzymes to understand how the CAM activity of *Phalaenopsis* is affected by shading treatment.

We used two commercially mass-produced *Phalaenopsis*-type orchids, *Phalaenopsis* Sogo Yukidian V3 (V3) and *Doritaneopsis* Walnut Valley Halo ES09 (ES09). Although ES09 is considered a *Doritis*, it was derived from crossing *Doritaenopsis* Taisuco Firebird and *Phalaenopsis* Hsinging Fair. The genome organization of *Doritaenopsis* Taisuco Firebird is largely derived from *Phalaenopsis* (http://www.wildcattdata.com). In fact, *Doritis* is generally placed within a subgenus of *Phalaenopsis* (Christenson 2001).

The plants with 6 to 7 fully developed leaves were grown in 10.5 cm diameter transparent plastic pots (650 $cm^3$) filled with Chilean sphagnum. They were irrigated once a week, alternating two concentrations (0.2 and 0.5 g $m^{-3}$) of Peters water soluble fertilizer (N:P:K, 20:8.6:16.6, m/m/m) (*Hyponex,* Marysville, OH, USA). The experiment was conducted in a commercial greenhouse at the Taiwan Orchid Plantation of Hobi, Tainan County, southern Taiwan (23° 34' N, 120° 38' E) with day/night temperatures of 25 - 28/18 - 22 °C and the natural photoperiod (Liu *et al*. 2010). The maximum irradiance on the planting bench (controlled by a mobile two-layer nylon net above the roof of the greenhouse) at noon on a fine day was 150 μmol $m^{-2}$ $s^{-1}$, measured by *LI-250* radiometer ( *Li-Cor,* Lincoln, NE, USA) (Liu *et al*. 2010). Plants were randomly divided into 2 groups per cultivar. The control plants were set on a mobile bench positioned 0.7 m above the ground and the shaded plants were placed directly under the bench which was covered with a nylon net that excluded 80 % of the incident radiation. Because of a difference in height of approximately 50 cm between the bench surface and the second leaf of the shaded plants, the actual shade treatment was approxi-mately 15 % of the control level (Liu *et al.* 2010). Each treatment with 60 pots per cultivar lasted 15 weeks (from 14 October 2007 to 30 January 2008).

According to Guo and Lee (2006), the newly and fully expanded leaf (the second leaf from the apex) has the highest net photosynthetic rate in *Phalaenopsis*. Thus, we sampled the second leaf, which was almost two-fold the size of the first leaf, for biochemical analyses. All samples were taken at dusk, from 16:30 to 18:30 on 30 January, and at dawn, from 05:30 to 07:30 on the next day. Leaf disks (approximately 0.28 $cm^2$) were punched out with a borer from the middle and about 0.5 cm away from the main vein of the leaf, weighed, and dropped into liquid $N_2$ until analyses. For organic acid determination, the leaf material was extracted by boiled distilled water and analyzed by anion exchange chromatography (*BioLC, Dionex,* Sunnyvale, CA, USA) with *IonPac AS11* column (Chen *et al.* 2008). For soluble sugar determination, leaf discs were homogenized and extracted with 80 % (v/v) ethanol at 80 °C (Chen *et al*. 2008). The supernatant was evaporated in an $N_2$ gas stream and dissolved in deionized water. Sugars were separated by anion exchange chromatography (*BioLC, Dionex*) with *CarboPac PA10* column (Chen *et al*. 2008). To determine starch content, sediment was digested with a mixture of pullulanase and amyloglucosidase, and the liberated glucose was estimated by the glucose oxidase and peroxidase method (Chen *et al*. 2008). For enzyme extracts, frozen leaf discs collected at dusk were transferred to 2-$cm^3$ Eppendorf tubes containing stainless steel beads and rapidly homogenized in a bullet blender (*CDX24, Next Advance,* New York, USA) with enzyme-specific extraction buffers. The extraction buffer and measurement for RuBPC/O activity were described by Marco and Tricoli (1983), and those for PEPC and $NAD^+$-ME were described by Chen *et al*. (2008).

Data were evaluated by two-way *ANOVA*. Differences in means were determined by the Fisher's protected least significant difference test. Results were considered significant at $P \leq 0.05$.

Many studies have mentioned that low irradiance decreases sugar accumulation in various plant species (Souza *et al*. 2004, Chang *et al*. 2008, Ceusters *et al*. 2011). We found that lowering irradiance decreased the content of glucose, fructose, saccharose (by 80 and 60 % at dawn and dusk, respectively), and starch (by 70 % at dusk) in both cultivars (Table 1). These data are consistent with the lowered activity of RuBPC/O in both cultivars under the shade condition (Fig. 1*A*). Moreover, the lower content of starch under shade treatment at dusk was associated with reduced content of soluble sugars in both cultivars at dawn (Table 1).

Shading significantly reduced the extent of dusk-to-dawn change in the content of starch from 2.8 and 1.4 mg $g^{-1}$(f.m.) to 0.6 and 0.2 mg $g^{-1}$(f.m.) in V3 and ES09, respectively, an almost 4.7- and 7.0-fold decrease (Table 1). Another CAM plant, bromeliad *Aechmea* also shows reduced diurnal fluctuation of starch content under lowered irradiance and after 3 months, the daytime storage sugar is switched from starch to sucrose (Ceusters *et al.* 2011). We did not find this phenomenon in our cultivars (Table 1). Apparently, not all CAM plants exposed to limited irradiance show the same change in sugar pattern.

The synthesis and degradation of malate in *Phalaenopsis* primarily involves PEPC and $NAD^+$-ME, respectively (Chen *et al*. 2008). Malate is an allosteric inhibitor of PEPC (Carter *et al*. 1995, Nimmo 2000). In control plants, PEPC activity was 2 to 3 times that of $NAD^+$-ME in both cultivars at dusk (Fig. 1*B,C*) whereas

Table 1. Effect of under-bench shading on the dawn and dusk content of sugars and organic acids [mg $g^{-1}$(f.m.)] of two *Phalaenopsis*-type cultivars V3 and ES09. Means ± SE, $n$ = 4. Different letters show statistical significance according to LSD test ($P \leq 0.05$) between control and shaded plants of the same cultivar at the same sampling time.

| | | V3 control | V3 shade | ES09 control | ES09 shade |
|---|---|---|---|---|---|
| Glucose | dawn | $0.712 \pm 0.020^{a}$ | $0.324 \pm 0.054^{b}$ | $1.659 \pm 0.238^{a}$ | $0.680 \pm 0.176^{b}$ |
| | dusk | $0.346 \pm 0.108^{a}$ | $0.325 \pm 0.071^{a}$ | $1.727 \pm 0.226^{a}$ | $1.314 \pm 0.502^{a}$ |
| Fructose | dawn | $0.921 \pm 0.047^{a}$ | $0.568 \pm 0.023^{b}$ | $1.855 \pm 0.248^{a}$ | $0.946 \pm 0.141^{b}$ |
| | dusk | $0.479 \pm 0.144^{a}$ | $0.549 \pm 0.061^{a}$ | $2.033 \pm 0.098^{a}$ | $1.499 \pm 0.384^{a}$ |
| Sucrose | dawn | $1.852 \pm 0.393^{a}$ | $0.400 \pm 0.061^{b}$ | $2.204 \pm 0.416^{a}$ | $0.408 \pm 0.087^{b}$ |
| | dusk | $1.434 \pm 0.170^{a}$ | $0.576 \pm 0.018^{b}$ | $2.111 \pm 0.557^{a}$ | $0.773 \pm 0.184^{b}$ |
| Starch | dawn | $1.853 \pm 0.348^{a}$ | $0.888 \pm 0.061^{b}$ | $1.687 \pm 0.053^{a}$ | $0.844 \pm 0.078^{b}$ |
| | dusk | $4.677 \pm 0.538^{a}$ | $1.509 \pm 0.107^{b}$ | $3.060 \pm 0.628^{a}$ | $1.024 \pm 0.188^{b}$ |
| Malate | dawn | $2.217 \pm 0.121^{a}$ | $2.085 \pm 0.190^{a}$ | $2.151 \pm 0.281^{a}$ | $2.234 \pm 0.157^{a}$ |
| | dusk | $0.714 \pm 0.056^{b}$ | $1.350 \pm 0.051^{a}$ | $0.711 \pm 0.140^{a}$ | $1.067 \pm 0.075^{a}$ |
| Citrate | dawn | $2.723 \pm 0.325^{a}$ | $2.057 \pm 0.283^{a}$ | $1.204 \pm 0.308^{a}$ | $1.741 \pm 0.249^{a}$ |
| | dusk | $1.920 \pm 0.178^{a}$ | $1.423 \pm 0.228^{a}$ | $1.265 \pm 0.156^{a}$ | $1.109 \pm 0.170^{a}$ |

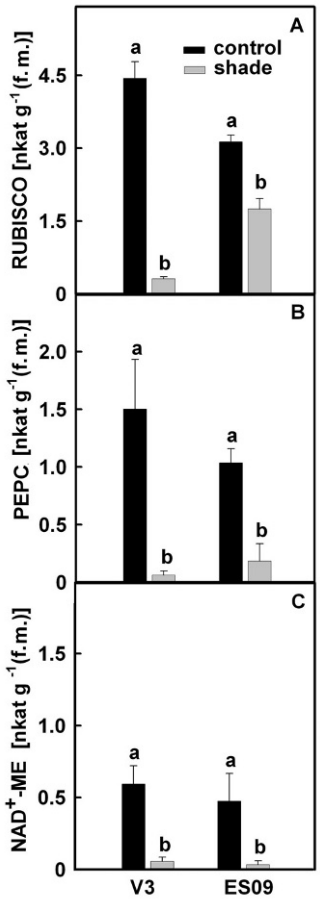


Fig. 1. The activities of RUBPC/O (*A*), phosphoenolpyruvate carboxylase (PEPC) (B), and $NAD^{+}$-linked malic enzyme ($NAD^{+}$-ME) (*C*) in two *Phalaenopsis*-type cultivars under control and shade conditions. Means ± SE, $n$ = 4. Different letters show statistical significance according to LSD test ($P \leq 0.05$) between control and shaded plants of the same cultivar.

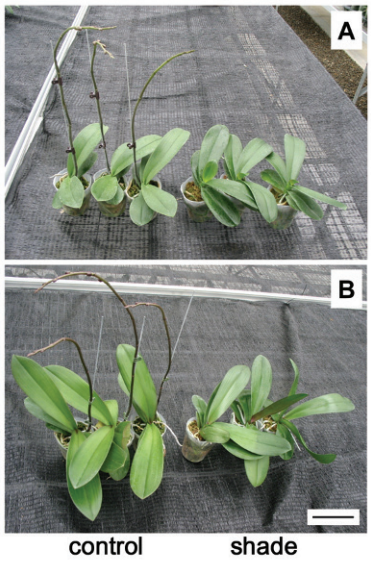


Fig. 2. Flower stalk development of *Phalaenopsis* Sogo Yukidian V3 (*A*) and *Doritaneopsis* Walnut Valley Halo ES09 (*B*) under control and shade conditions for 15 weeks. *Bar* = 20 cm.

the malate content was considerably lower at dusk than at dawn and the dawn-to-dusk change in V3 and ES09 was 1.5 and 1.4 mg $g^{-1}$(f.m.), respectively (Table 1). Malate accumulated during night has been decarboxylated during the daytime by $NAD^{+}$-ME and a low malate content during daytime lead to increased PEPC activity at dusk (Fig. 1*B*). By lowering irradiance, the malate content was slightly lower at dusk than dawn, and the dawn-to-dusk change for V3 and ES09 was 0.7 and 1.2 mg $g^{-1}$(f.m.), respectively (Table 1). The best interpretation of such rather constant content of malate in shaded plants is the simultaneously reduced activities of PEPC (Fig. 1*B*) and $NAD^{+}$-ME (Fig. 1*C*) as compared with the control.

Citric acid is an important organic acid of *Phalaenopsis* (Chen *et al*. 2008, Pollet *et al*. 2011). Despite a similar content of malate in two cultivars, the citrate content was higher in V3 than in ES09, particularly at the control treatment (Table 1). Many reports have suggested that citrate accumulation is favoured in plants to minimize photoinhibition and cope with adverse environmental conditions such as salinity, alkalinity, and excess of iron (Herppich *et al*. 1995, Lüttge 2006, Kornas *et al*. 2009, Sun and Hong 2011, Han *et al*. 2012). In fact, orchid growers consider V3 more tolerant to environmental variations than ES09. Whether the higher citrate content in V3 contributes in part to its greater tolerance than in ES09 needs further clarification.

During our experimental period of 15 weeks, flower stalks completely emerged and were taller than 60 cm for both V3 and ES09 under the control condition, with no flower stalk emergence under the shade condition (Fig. 2). Liu *et al*. (2010) found that 23 % of flower stalks formed in *Doritaenopsis* Nobby's Pink Lady CL200 after 14 weeks of under-bench shading. These data confirm that irradiance is one of the main factors controlling flower stalk development in *Phalaenopsis* and agree with findings by Wang *et al*. (2006) that the efficiency of flower stalk inhibition with shading depends on the cultivar.

Many studies mentioned that sugars act as signals to control the reproductive development of plants (Bernier *et al*. 1993, Gibson 2005, Rolland *et al*. 2006). Kataoka *et al*. (2004) showed that low irradiance for 3-weeks significantly reduced sucrose content but not glucose and fructose content in *Phalaenopsis* leaves and delayed flower stalk emergence as compared with high irradiance. Konow and Wang (2001) also showed that low irradiance did not affect glucose and fructose content but significantly decreased sucrose and starch content and inhibited *Phalaenopsis* flower development. However, Kataoka *et al*. (2004) and Konow and Wang (2001) collected samples at midday and dusk, respectively. We found that lowering irradiance decreased sucrose and starch content at dawn and dusk but the content of glucose and fructose was significantly reduced only at dawn in both cultivars (Table 1). Thus, a persistent decrease in sucrose content may have a key role in delaying flower stalk development in shaded *Phalaenopsis*.

Moreover, ES09 contained more glucose and fructose than V3, particularly at dusk, showing a 3- to 4-fold difference with shading (Table 1). This result was consistent with ES09 showing higher RuBPC/O activity than V3 at the same sampling time (Fig. 1*A*). Sugars promote anthocyanin accumulation and the expression of related genes, such as chalcone synthase and flavanone-3-hydroxylase (Vitrac *et al*. 2000, Hiratsuka *et al*. 2001, Zheng *et al*. 2009). Anthocyanins are responsible for many colours in plants (Taiz and Zeiger 2010) and ES09 is a red-flowered species that inherently possesses a strikingly dark red dorsal leaf surface. The relation between colour appearance and high hexose content in ES09 still needs to be ascertained.

In conclusion, we have demonstrated that shading can effectively delay flower stalk development in the *Phalaenopsis*-type cultivars and this delay is closely related to decreased availability of sucrose.

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