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Spatial patterns of photosynthesis in thin- and thick-leaved epiphytic orchids: unravelling C_3–CAM plasticity in an organ-compartmented way

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• *Background and Aims* A positive correlation between tissue thickness and crassulacean acid metabolism (CAM) expression has been frequently suggested. Therefore, this study addressed the question of whether water availability modulates photosynthetic plasticity in different organs of two epiphytic orchids with distinct leaf thickness.
• *Methods* Tissue morphology and photosynthetic mode (C_3 and/or CAM) were examined in leaves, pseudobulbs and roots of a thick-leaved (*Cattleya walkeriana*) and a thin-leaved (*Oncidium* 'Aloha') epiphytic orchid. Morphological features were studied comparing the drought-induced physiological responses observed in each organ after 30 d of either drought or well-watered treatments.
• *Key Results Cattleya walkeriana*, which is considered a constitutive CAM orchid, displayed a clear drought-induced up-regulation of CAM in its thick leaves but not in its non-leaf organs (pseudobulbs and roots). The set of morphological traits of *Cattleya* leaves suggested the drought-inducible CAM up-regulation as a possible mechanism of increasing water-use efficiency and carbon economy. Conversely, although belonging to an orchid genus classically considered as performing C_3 photosynthesis, *Oncidium* 'Aloha' under drought seemed to express facultative CAM in its roots and pseudobulbs but not in its leaves, indicating that such photosynthetic responses might compensate for the lack of capacity to perform CAM in its thin leaves. Morphological features of *Oncidium* leaves also indicated lower efficiency in preventing water and CO_2 losses, while aerenchyma ducts connecting pseudobulbs and leaves suggested a compartmentalized mechanism of nighttime carboxylation via phosphoenolpyruvate carboxylase (PEPC) (pseudobulbs) and daytime carboxylation via Rubisco (leaves) in drought-exposed *Oncidium* plants.
• *Conclusions* Water availability modulated CAM expression in an organ-compartmented manner in both orchids studied. As distinct regions of the same orchid could perform different photosynthetic pathways and variable degrees of CAM expression depending on the water availability, more attention should be addressed to this in future studies concerning the abundance of CAM plants.

Key words: *Cattleya walkeriana*, crassulacean acid metabolism, drought, epiphytic orchid, leaf succulence, non-leaf photosynthesis, *Oncidium* 'Aloha', photosynthetic plasticity.

INTRODUCTION

Epiphytic orchids are one of the most species-rich and diverse groups of plants and can inhabit a wide range of niches, varying from almost constantly humid to seasonally dry habitats. Despite the abundance of orchid species occupying the canopy, the epiphytic habitat is the most severe niche in tropical forests because the availability of water and nutrients is sporadic and dependent on atmospheric sources (Kress, 1986; Goh and Kluge, 1989; Benzing, 1990; Gravendeel *et al.*, 2004). Hence, survival and adaptive success of these plants rely largely on their flexible developmental and metabolic responses to environmental conditions (Goh and Kluge, 1989; Benzing, 1990; Sinclair, 1990).

In terms of metabolic strategies, a high number of epiphytic species perform crassulacean acid metabolism (CAM) photosynthesis (Benzing, 1989; Zotz and Hietz, 2001; Lüttge, 2004), which represents an important ecophysiological adaptation that allows plants to reside in habitats with scarce, intermittent and/or seasonal water availability (Cushman, 2001; Silvera *et al.*, 2010*a*; Borland *et al.*, 2011). In fact, the drought endurance observed in the majority of epiphytes is frequently provided by a stronger CAM photosynthetic behaviour that promotes maximum carbon gain combined with minimum water loss (Benzing and Ott, 1981; Dodd *et al.*, 2002; Kerbauy *et al.*, 2012). This is feasible because CAM photosynthesis usually acts as a CO_2-concentrating mechanism through nocturnal CO_2 fixation by phosphoenolpyruvate carboxylase (PEPC) and subsequent vacuolar storage of the fixed CO_2 in the form of organic acids. The following daytime decarboxylation of organic acids releases CO_2, which is refixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and assimilated in the Calvin cycle behind closed stomata (Griffiths, 1989; Lüttge, 2002, 2004).

Concurrently, one essential structural adaptation of most epiphytic orchids against severe drought is a certain degree of tissue succulence, mainly by their leaves and pseudobulbs (Goh and Kluge, 1989; Benzing, 1990). Besides being an efficient way to store water and nutrients during the dry season, succulence is also suggested as an important requirement for CAM expression (Dressler, 1981, 1993; Williams *et al.*, 2001; Griffiths *et al.*, 2008; Silvera *et al.*, 2010*a*; Borland *et al.*, 2011). In fact, a number of reports have indicated a positive correlation between leaf thickness and CAM activity in epiphytic orchids. In addition, it is currently accepted that orchid leaves can show either CAM or C_3 photosynthesis depending on the presence of thick and thin leaves, respectively (Neales and Hew, 1975; Arditti, 1979; Goh *et al.*, 1983). Furthermore, the higher leaf thickness of plants performing CAM is also related to an increased capacity to nocturnally store organic acids inside the vacuoles during the CAM cycle (Nelson *et al.*, 2005; Nelson and Sage, 2008; Silvera *et al.*, 2010*b*). However, this correlation seems to be exclusively valid when leaf succulence is due to increases in chlorenchyma thickness rather than hydrenchyma abundance (Kerbauy *et al.*, 2012).

CO_2 fixation in orchids has been primarily studied in leaves; relatively little attention has been given to the photosynthetic pathways occurring in non-leaf organs, such as pseudobulbs or roots. Although pseudobulbs lack stomata and are impervious to water and gases (Withner, 1974; Hew and Yong, 1994; Ng and Hew, 2000), anatomical and biochemical analyses have revealed the presence of chloroplasts and enzyme activities (Rubisco and PEPC), which, taken together, indicates some photosynthetic activity in these organs (Winter *et al.*, 1983; Stern and Morris, 1992; Hew and Yong, 1994; Sheehan and Sheehan, 1994; Hew *et al.*, 1996). This implies that, in some cases, pseudobulbs might also be capable of expressing some degree of CAM. Moreover, green aerial roots of epiphytic orchids also have the photosynthetic apparatus for CO_2 fixation (Ho *et al.*, 1983; Moreira *et al.*, 2009; Martin *et al.*, 2010) as well as several morphological specializations, such as velamen and exodermis, both designed to trap and absorb water and nutrients (Pridgeon, 1986). However, aerial roots seem to play a minor photosynthetic role for epiphytes (Aschan and Pfanz, 2003), except for some leafless orchids with autotrophic roots. These plants are almost totally dependent on carbon fixation by roots, which can exhibit CAM photosynthesis, even being devoid of true stomata (Benzing *et al.*, 1983; Cockburn *et al.*, 1985). Additionally, roots of some leafless orchids possess well-developed aeration systems with specialized thickened cortical cells that possibly are able to regulate gas exchange (Benzing *et al.*, 1983).

Phylogenetic studies concerning the evolution of photosynthetic pathways in Orchidaceae leaves have shown C_3 as the ancestral state and CAM as having multiple-independent origins with several reversals across the entire family, indicating the great evolutionary flexibility of CAM among orchids. Accordingly, the Epidendroideae subfamily of Orchidaceae is the most species-rich epiphytic clade among all plant groups, which correlates with expressive events of CAM radiation, especially within the Neotropical subtribes Oncidiinae and Laeliinae (Silvera *et al.*, 2009). Moreover, approx. 40 % of the tropical orchid species are considered capable of expressing some form of CAM ('strong' to 'weak') in their leaves (Silvera *et al.*, 2005, 2010*a*).

It is also important to note that several studies have shown that the extent of CAM expression can be highly flexible and dependent not only on plant species but also on tissue characteristics, the phase of organ/plant development and environmental conditions (Nelson and Sage, 2008; Winter *et al.*, 2008; Herrera, 2009; Freschi *et al.*, 2010*a*, *b*; Ping *et al.*, 2010; Borland *et al.*, 2011). Although some valuable investigations have already been performed to verify the expression of CAM in orchid tissues (Goh *et al.*, 1983; Ando and Ogawa, 1987; Cui *et al.*, 2004; Guo and Lee, 2006; Motomura *et al.*, 2008; Moreira *et al.*, 2009; Silvera *et al.*, 2009; Martin *et al.*, 2010), few studies have verified C_3 and/or CAM photosynthetic types in different organs of the same epiphytic orchid or considered that these organs might have plasticity in switching between C_3 and CAM pathways in response to changes in the environmental conditions (Kerbauy *et al.*, 2012), such as drought.

Moreover, CAM plants show evolutionary convergence in terms of particular anatomical and metabolic traits and some of these traits (i.e. succulence and intercellular air space in photosynthesizing tissues) are recognized as putative determinants to constrain the range of photosynthetic plasticity performed by specific plant tissues (Nelson *et al.*, 2005; Nelson and Sage, 2008; Borland *et al.*, 2011). In fact, we have previously reported the existence of distinct degrees of CAM expression in different portions along the leaf blade of the epiphytic bromeliad *Guzmania monostachia* that were modulated by water availability. The drought treatment intensified the CAM expression in this C_3–CAM facultative species, specifically in the apical part of leaves which had a set of physiological and morphological features considered more suitable for the occurrence of CAM photosynthesis (Freschi *et al.*, 2010*b*). This information prompted us to further investigate whether other epiphytes with highly diverse and specialized physiology and morphology, as found among orchids (Kress, 1986; Gravendeel *et al.*, 2004), would also respond to distinct regimes of water availability by exhibiting photosynthetic plasticity and functional compartmentalization of CO_2 fixation in their vegetative tissues.

Based on previous information, the current study investigated the degree to which the photosynthetic modes (C_3 and/or CAM) observed in vegetative organs of two epiphytic orchids from the subfamily Epidendroideae (*Cattleya walkeriana*, subtribe Laeliinae; *Oncidium* 'Aloha', subtribe Oncidiinae) with different degrees of leaf succulence are modulated by water availability. The seasonally dried epiphytic orchid *C. walkeriana*, as with other *Cattleya* species, is characterized by a high degree of leaf succulence and by performing constitutive CAM photosynthesis (Nuerenbergk, 1963; Arditti, 1979; Avadhani and Arditti, 1981; Avadhani *et al.*, 1982; Goh and Kluge, 1989), while most *Oncidium* orchids have thinner leaves and are generally described as C_3 plants (Hew and Yong, 1994, 2004). In fact, the thin-leaved hybrid *Oncidium* 'Aloha' is derived from the varieties *Oncidium* 'Star Wars' and *Oncidium* 'Goldiana' (Wu *et al.*, 2010), the last being largely described as a C_3-shade epiphytic orchid (Hew and Yong, 1994; Yong and Hew, 1995*a*, *b*). Meanwhile, the Brazilian species *C. walkeriana* is usually found in more exposed canopy sites of tropical deciduous forests (Lacerda, 1995). However, both orchids present large heteroblastic pseudobulbs and green roots (Goh and Kluge, 1989; Hew and Yong, 2004), thereby implying some potential for non-leaf photosynthetic activity.

Accordingly, we set out to discover (1) whether distinct regimes of water availability influence the photosynthetic mode (C_3 and/or CAM) in leaves and non-leaf organs of both *C. walkeriana* and *Oncidium* 'Aloha', if so, (2) how drought specifically affects the photosynthetic pathway in leaves of a thick-leaved (*Cattleya*) and a thin-leaved (*Oncidium*) epiphytic orchid, and (3) whether there is a particular degree of compartmentalization in CAM expression among different organs of such epiphytic orchids under either drought or well-watered treatments. Therefore, the present study aimed to study whether a correlation exists between tissue organization and photosynthetic operation (C_3 and/or CAM) in different vegetative organs (leaves, pseudobulbs and roots) of the epiphytic orchids *C. walkeriana* (thick leaved) and *Oncidium* 'Aloha' (thin leaved) subjected to both well-watered and drought conditions.

MATERIALS AND METHODS

Plant material and growth conditions

Plants of *Cattleya walkeriana* and *Oncidium* 'Aloha' (*O.* 'Star Wars' × *O.* 'Goldiana') were obtained by asymbiotic germination and micropropagation techniques, respectively. The plants were cultivated in pots containing moss substrate in greenhouses at São Paulo State until they reached the mature-vegetative age. These orchids were transferred to controlled environment chambers at 25 ± 2 °C with 12-h photoperiod and photosynthetic flux density of about 200 μmol photons m^{-2} s^{-1} supplied by fluorescent lamps (Sylvania, Germany). The plants were watered daily and acclimatized under such conditions for 15 d prior to the treatments. All orchids used in this study were in the rest period of their developmental cycle.

Morphological and histological analyses

The youngest, completely expanded leaf of individuals of *C. walkeriana* and *Oncidium* 'Aloha' and their respective pseudobulb and light-exposed roots were fixed in FAA (formalin, acetic acid and ethanol 50 %) for 24 h (Johansen, 1940) and BNF (buffered neutral formalin) for 48 h (Lillie, 1965). After fixation, leaf samples were dehydrated through a tertiary butyl alcohol series (Johansen, 1940), embedded in paraffin and serial sectioned at 20 μm thickness on a Microm HM340E rotary microtome. Longitudinal and transverse sections were stained with Astra blue 1 % and Safranin O 1 % (Gerlach, 1984) and the slides mounted in Permount. Some material was also freehand sectioned to carry out the histochemical test with Alcian blue (Pearse, 1985) for acidic mucilage and phloroglucinol (Johansen, 1940) for lignin. Observations and photographs were made with an Olympus BX51 light microscope. For scanning electron microscopy (SEM) analysis, samples fixed in FAA were dehydrated in a graded ethanol series and critical-point dried with CO_2, attached to aluminium stubs and coated with gold (30–40 nm). Observations were carried out on a Jeol JSM-5800 LV.

Treatments and tissue sampling for biochemical analyses

Both sets of epiphytic orchids were divided into two experimental groups, each one with five plants submitted to a different watering condition. The well-watered plants were watered daily and maintained under 60–70 % relative humidity, while the drought-treated plants were submitted to 30–40 % relative humidity without watering. After 30 d under these treatments, the youngest leaf and pseudobulb and all light-exposed roots of these orchids were harvested at both dark-to-light and light-to-dark transitions. Roots immersed in the substrate were not used. All harvested samples were immediately fragmented into small pieces of about 2–5 mm and subsequently weighed, frozen in liquid nitrogen and stored at −80 °C until used in the biochemical analyses.

Tissue water content measurements

The tissue water content of the leaves, pseudobulbs and roots was determined according to Barrs and Weatherley (1962). Discs of approx. 1·53 cm^2 of leaves and pseudobulbs and roots of 1 cm length from five different plants were collected 1 h after dawn and immediately weighed to determine the fresh mass (M_f). After determining M_f, the samples were maintained for 24 h in distilled water and in the dark for saturation to obtain the turgid mass (M_t) and then dried to a constant mass at 60 °C and allowed to cool before determining the dry mass (M_d). Tissue water content was calculated using the formula $[(M_f - M_d)/(M_t - M_d)] \times 100$.

PEPC and MDH extraction and assay

Quantification of both PEPC (EC 4.1.1.31) and malate dehydrogenase (MDH, EC 1.1.1.37) activities was performed according to the method described by Freschi *et al.* (2010*a*) with some modifications. For this, 1 g of fresh leaf or pseudobulb or 0·25 g of fresh root was ground in a mortar with liquid nitrogen and polyvinylpolypyrrolidone (PVPP) (0·3 g per fresh mass) until a fine powder was obtained. Frozen samples of leaves, pseudobulbs and roots collected at the dark-to-light transition were extracted in five volumes (v/w) of buffer containing 200 mM Tris-HCl (pH 8·0), 1 mM EDTA, 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 10 % (v/v) glycerol and 0·5 % (w/v) bovine serum albumin. The homogenate was filtered using Millex GV 0·45-μm filters (Millipore), and the supernatant was immediately used for the enzymatic assays. PEPC activity was assayed in a 2-mL standard reaction medium containing 50 mM Tris-HCl (pH 8·0), 1 mM DTT, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 200 mM NADH, 3 mM phosphoenolpyruvate and 0·005 units L-MDH (Sigma-Aldrich). MDH activity was assayed in the oxaloacetate (OAA)-reducing direction in a 2-mL reaction medium containing 50 mM Tris-HCl (pH 8·0), 2 mM OAA, 5 mM $MgCl_2$ and 200 mM NADH. For both enzymatic determinations, the reaction was started by adding an aliquot of enzyme extract, and absorbance was continuously measured at 340 nm. All reported rates were from linear portions of absorbance time curves (usually between 0 and 5 min). The PEPC and MDH enzymes were assayed at 30 °C and their activities were expressed in μmol NADH min^{-1} (mg total chlorophyll)$^{-1}$.

Organic acid quantification

Organic acids were quantified according to the method used by Amorós *et al.* (2003) and Hasegawa *et al.* (2010).

For this, leaf (1 g), pseudobulb (1 g) or root (0·25 g) samples were ground in a mortar with liquid nitrogen and 0·06 g PVPP per gram of fresh mass. When a fine powder was obtained, the samples were transferred to previously cooled micro-tubes, and 600 μL of 0·662 mM formic acid solution was added to each tube. The extracts were mixed vigorously, and an aliquot of cold formic acid solution was added (400 μL). All tubes were centrifuged at 14 000 ***g*** for 6 min at 4 °C. The supernatant was collected and mixed with 200 μL AG3-X4 resin (Bio-Rad) (1 g resin per 4 mL cold distilled water) to extract the acid organics from the mucilage present in large quantities in the orchid tissues. The samples were kept under constant agitation at 4 °C. After 1 h, all tubes were centrifuged at 14 000 ***g*** for 4 min at 4 °C. The supernatant was discarded, and the pellet was re-suspended in 200 μL of the mobile phase (1 % H_3PO_4 solution, filtered in LCR PTFE 0·45-μm filters; Millipore). The tubes were maintained under constant agitation at 4 °C for 1 h, and the samples were then centrifuged at 14 000 ***g*** for 4 min at 4 °C. The supernatant was collected and filtered using Millex GV 0·22- or 0·45-μm filters (Millipore) to remove the resin particles from the extract. Aliquots of 50 μL were injected into a high-performance liquid chromatograph (Waters HPLC, 510 pump, UV/Vis 486 detector, 717 plus autosampler, and Millenium 32 software) equipped with an Aminex HPX-87H ion exclusion column (300 × 7·8 mm; Bio-Rad). The run was carried out isocratically at a flow rate of 0·5 mL min^{-1} and analysed in a UV/Vis detector settled to 215 nm. The column compartment temperature was adjusted to 28 °C. Corrections were made using formic acid as an internal standard.

Leaf gas exchange measurements

As previous photosynthetic experiments with *Oncidium* 'Goldiana' have shown that the leaf tip had the highest photosynthetic capacity along the leaf blade (Hew *et al.*, 1998), gas exchange measurements were made on the apical portion of the youngest, completely expanded leaf of *Oncidium* 'Aloha' plants. This analysis was performed continuously using a portable infra-red gas exchange system (LI-6400, Li-Cor) with all parameters settled as described by Freschi *et al.* (2010*b*). Every analysed leaf was enclosed in a chamber (leaf area within the cuvette was always 4·5 cm^2), which tracked the environmental conditions inside the growth cabinet. Leaf gas exchange parameters were logged automatically every 3 min, and a CO_2 cylinder was used to keep CO_2 concentration constant under 380 p.p.m. Measurement intervals were integrated to show hourly averages.

Statistical analyses

Data are presented as the mean ± s.e. of three replicate samples, with each replicate consisting of plant material collected from five different individuals. Statistically significant differences between means of well-watered and drought-exposed treatments were determined by Student's *t*-test at $P \leq 0{\cdot}05$.

RESULTS

Morpho-histological characteristics of orchid organs

Leaves. Cattleya walkeriana has succulent dorsiventral leaves with the palisade parenchyma formed by elongated cells showing several chloroplasts and large vacuoles (Fig. 1A), whereas *Oncidium* 'Aloha' has non-succulent leaves with intercellular air spaces in the thinner mesophyll and chlorenchyma cells smaller than in *Cattleya* (Fig. 1B). Moreover, *Cattleya* showed the epidermis covered by a conspicuously thicker cuticle (Fig. 1C) than that observed in *Oncidium* (Fig. 1D). While there was no noticeable aerenchyma in *Cattleya* leaves (Fig. 1A, C), this structure was detected in *Oncidium* mesophyll (Fig. 1D). Interestingly, in the leaf base this structure extended towards the pseudobulb at the limit of leaf and stem (Fig. 1E).

Pseudobulbs. The pseudobulbs of both orchids were devoid of stomata (data not shown), with lignified epidermis (Fig. 2A, B), chlorenchyma without intercellular air spaces (Fig. 2C, D), large mucilage idioblasts (Fig. 2G) and vascular bundles scattered throughout the organ (Fig. 2C–F). However, the subepidermal parenchymatous cells of *Cattleya* pseudobulb were also lignified (Fig. 2C), while only *Oncidium* presented aerenchyma together with the vascular bundles adjacent to the phloem (Fig. 2F) throughout the organ length (Fig. 2H).

Roots. The histological features of roots of both orchids were relatively similar (Fig. 3); however, the cortex of *Cattleya* was wider than in *Oncidium*, mainly due to a higher number of chlorenchyma layers formed by smaller cells (Fig. 3A, B). In addition, the exodermal and the velamen cells of *Cattleya* roots seemed to be more strengthened than in *Oncidium* (Fig. 3C, D).

Drought-induced changes in relative water content

After 30 d of drought a significant reduction in the relative water content (RWC) was observed in all organs of *Cattleya* and *Oncidium* (Fig. 4). The water loss was more intense in roots of both orchids with an RWC decrease near 35 and 62 % in *Cattleya* and *Oncidium*, respectively. Besides, pseudobulbs of *Cattleya* and *Oncidium* lost only 10 and 25 % of their RWC, respectively, while leaves of both orchids showed RWC reduction of about 25 %.

Drought-induced modulation of C_3–CAM photosynthesis

Both epiphytic orchids analysed presented distinct spatial responses in terms of CAM expression after 1 month of drought treatment (Figs 5 and 6). In *Cattleya* leaves, for instance, water deprivation induced a marked increase in PEPC and MDH activities (Fig. 5A, B), which was correlated with an expressive increment of nocturnal malate accumulation (Fig. 6B). By contrast, drought-treated *Oncidium* leaves did not show significant changes in PEPC/MDH activities (Fig. 5A, B), nor in night-time organic acid accumulation (Fig. 6A, B). Concurrently, day–night gas exchange analysis (Fig. 7) indicated that under well-watered conditions the *Oncidium* leaves carried out most of the atmospheric CO_2 uptake during daytime, with very modest CO_2 taken up at night. By contrast, when *Oncidium* plants were exposed to water shortage for 30 d, most of the daytime

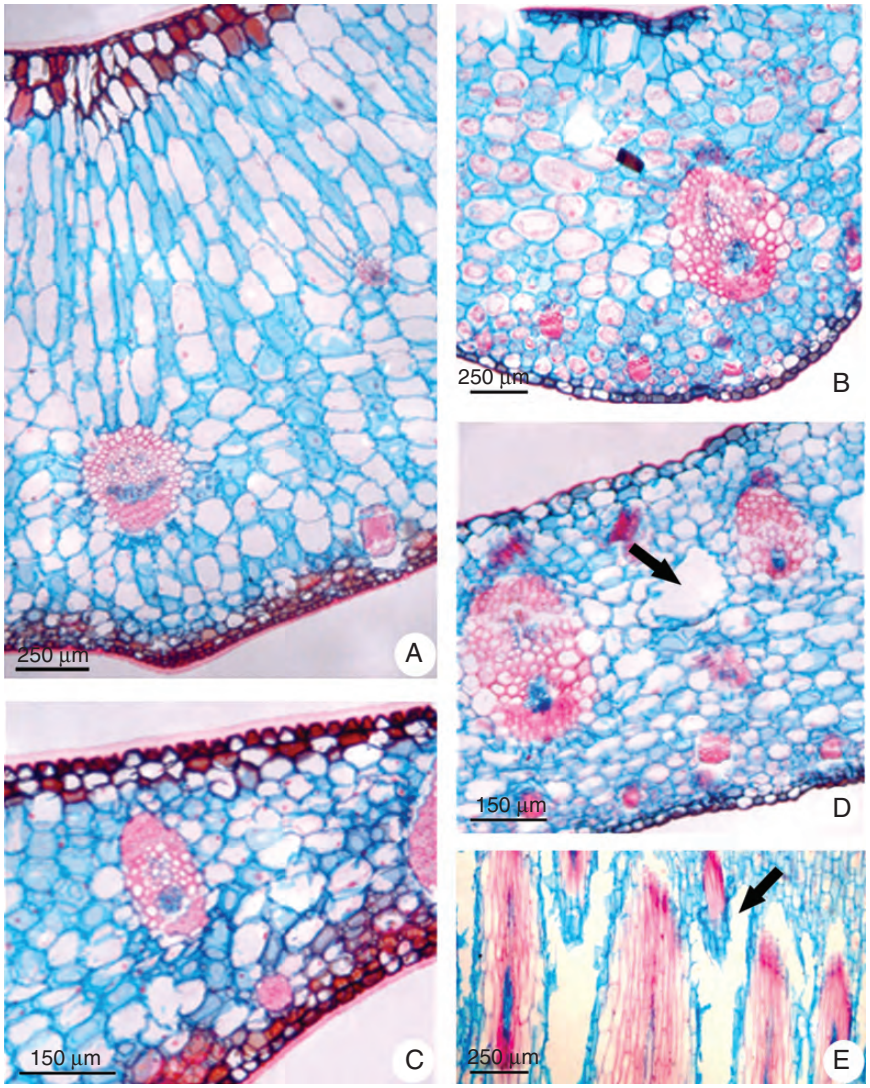

FIG. 1. Comparative view of leaf morphology of (A, C) *Cattleya walkeriana* and (B, D, E) *Oncidium* 'Aloha'. Light micrographs, transverse (A–D) and longitudinal (E) sections. (A, B) General view of transverse sections from the middle region of leaves showing the succulent morphology of *Cattleya* leaf (A) and the non-succulent constitution of *Oncidium* leaf (B). (C, D) Transverse sections from the leaf blade showing the epidermis of *Cattleya* covered by thicker cuticle on both surfaces (C) and the aerenchyma (arrow) between vascular bundles in *Oncidium* leaf (D), with detail of the aerenchyma near the pseudobulb (arrow) in a longitudinal section at the leaf base (E).

CO_2 assimilation disappeared and only minor CO_2 uptake remained in the middle of the afternoon (Fig. 7).

While *Cattleya* pseudobulbs showed no major changes in either CAM enzyme activities (Fig. 5C, D) or organic acid accumulation (Fig. 6C, D) after drought treatment, the *Oncidium* pseudobulbs displayed significant increases in both PEPC and MDH activities (Fig. 5C, D), associated with a remarkable increase in night-time malate and citrate accumulation (Fig. 6C, D). *Oncidium* roots displayed a significant increase in PEPC/MDH activities (Fig. 5E, F), considerable increase in nocturnal malate accumulation (Fig. 6F) and modest citrate loss during the night (Fig. 6E). Conversely, *Cattleya* roots showed fairly stable PEPC/MDH activities (Fig. 5E, F) and no nocturnal malate accumulation in both treatments (Fig. 6F). However, drought-treated *Cattleya* roots presented a slight nocturnal accumulation of citrate (Fig. 6E).

DISCUSSION

Relationship between organ morphology and water status

Thirty days of drought treatment was clearly enough to trigger significant water losses in virtually all organs of both orchids analyzed (Fig. 4). Comparing the overall pattern of water losses in the plant organs, *Cattleya* species (Fig. 4A) appeared to be relatively more resistant to water depletion than *Oncidium* (Fig. 4B). Interestingly, several morphological features were in agreement with the relatively more efficient control against water losses in *Cattleya* than in *Oncidium* plants (Figs 1–3). For instance, *Cattleya* roots showed a higher number of cell layers in the cortex and more lignified cell walls in both velamen and exodermis (Fig. 3A, C). The impermeability of *Cattleya* pseudobulbs was apparently increased due to a considerable deposition of lignin in their subepidermal parenchyma cell walls

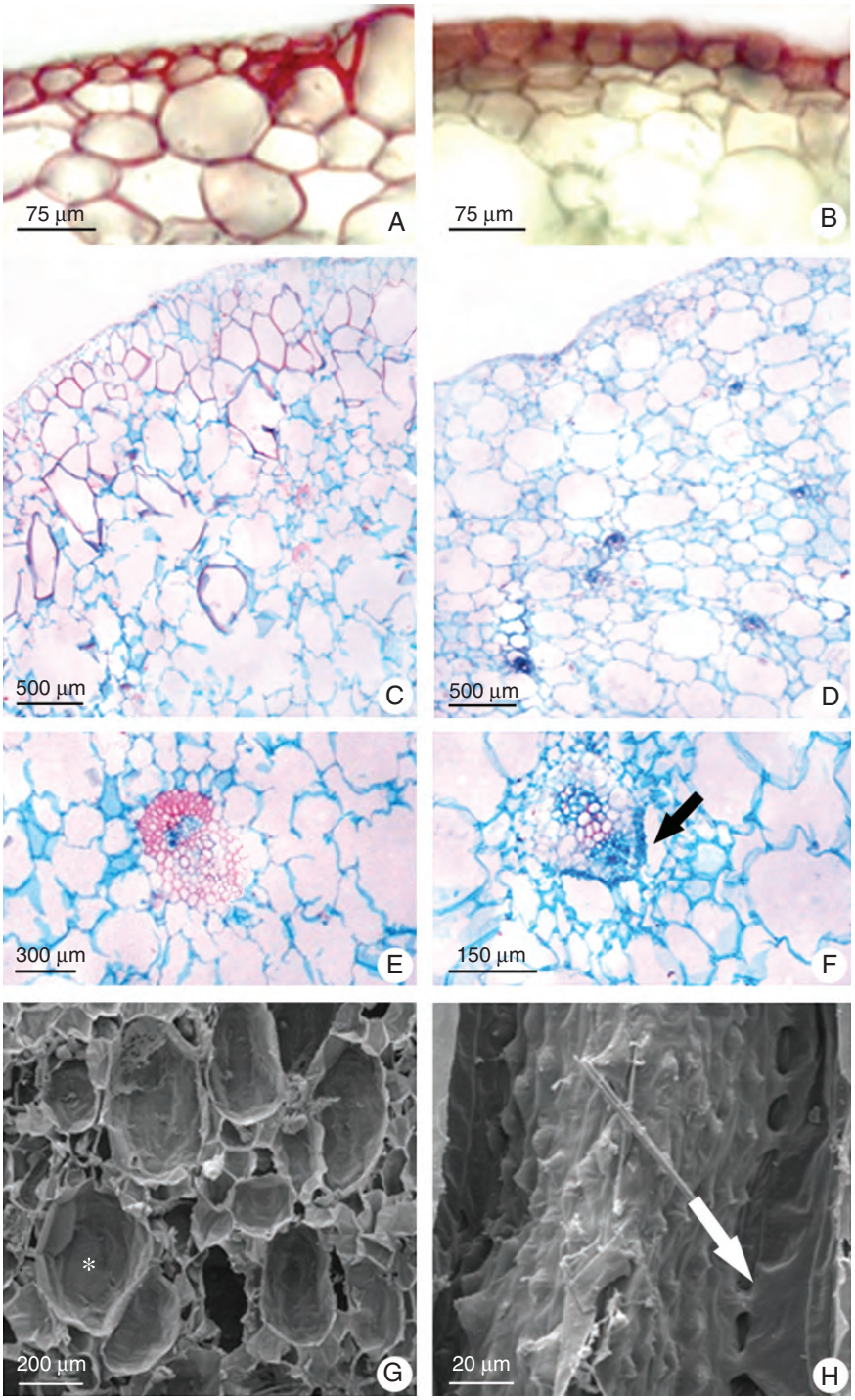

FIG. 2. Comparative view of pseudobulb morphology of (A, C, E, G) *Cattleya walkeriana* and (B, D, F, H) *Oncidium* 'Aloha'. (A, B) Lignin detection by phloroglucinol test (red stained). (C–F) Light microscopy. (C, D) Peripheral portion of the pseudobulb with vascular bundles spread through the organ. (E, F) The vascular bundle surrounded by chlorenchyma and mucilage idioblasts in *Cattleya* pseudobulb (E) and the presence of aerenchyma ducts (arrow) associated with the phloem of the *Oncidium* vascular bundle (F). (G, H) Scanning electron micrographs. Longitudinal sections of pseudobulbs showing mucilage idioblast (asterisk) in *Cattleya* (G) and the aerenchyma (arrow) associated with the *Oncidium* vascular bundle (H).

(Fig. 2A, C). Moreover, leaves of *C. walkeriana* presented lignified epidermis covered by a thicker cuticle and frequently lignified sclereids near stomata (Fig. 1A, C). The elevated density of sclereids in *Cattleya* leaves could be associated with higher hydraulic efficiency as these cellular structures can function as a hydraulic 'shortcut' through the mesophyll apoplast and as collapsible water storage elements that increase leaf capacitance (Brodribb *et al.*, 2010).

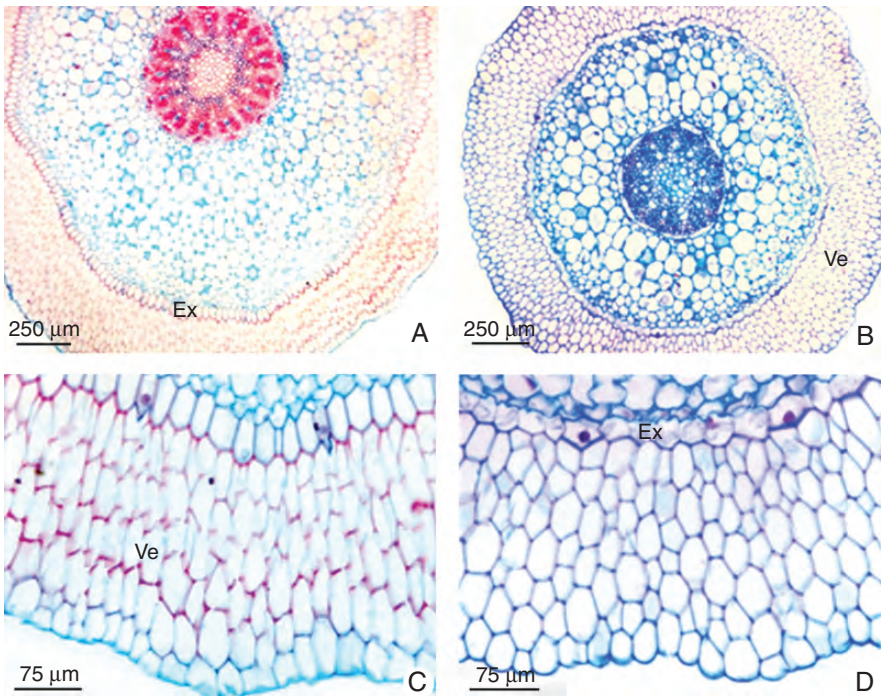

FIG. 3. Transverse sections of the roots of (A, C) *Cattleya walkeriana* and (B, D) *Oncidium* 'Aloha'. (A, B) General view of the roots. (C, D) Detail of the multilayered velamen (*Ve*) and the exodermis (*Ex*).

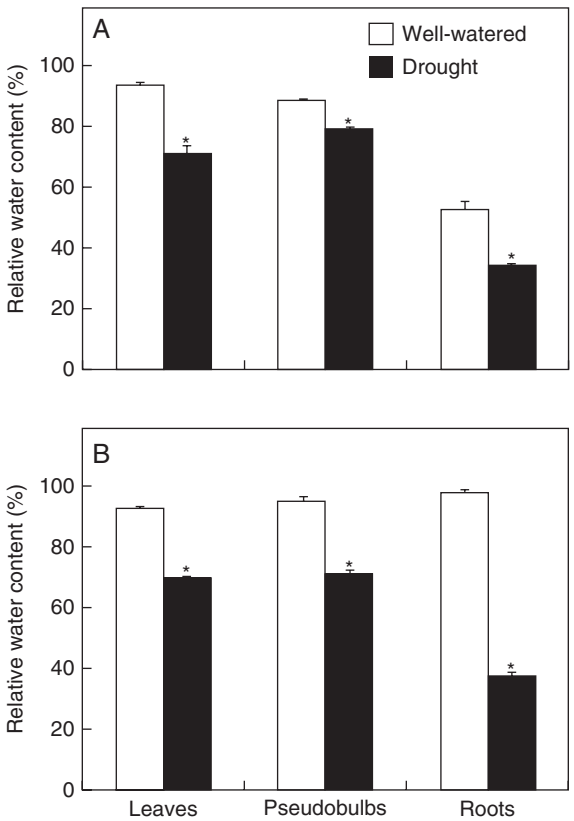

FIG. 4. Relative water content in different organs of (A) *Cattleya walkeriana* and (B) *Oncidium* 'Aloha' after 1 month of well-watered or drought-exposed treatments (as indicated in key). Data are expressed as the mean ± s.e. An asterisk indicates a significant difference between treatments ($P \leq 0{\cdot}05$).

Besides, the higher succulence showed by *Cattleya* leaves was mainly due to the presence of several chlorenchyma layers without noticeable intercellular air spaces (Fig. 1A). Hence, the larger vacuoles observed in the thicker chlorenchyma of this orchid represent a vastly abundant space for nocturnal acid accumulation, as reported in some surveys on *Cattleya* leaf photosynthesis (Nuerenbergk, 1963; Knauft and Arditti, 1969; Goh *et al.*, 1977; Winter *et al.*, 1983). Therefore, *Cattleya* showed a set of morphological characters that suggests ecological adaptations that would aid plants to reduce water loss. These structural features are typically observed in most thick-leaved epiphytic orchids, which, like *C. walkeriana*, are frequently found inhabiting harsher xerophytic environments (Knauft and Arditti, 1969; Avadhani *et al.*, 1982).

Drought-induced modulation of C_3–CAM photosynthesis in leaves

The drought treatment triggered significant increases in PEPC/MDH activities and nocturnal acid accumulation in *Cattleya* leaves (Figs 5A, B and 6B), which are essential metabolic features indicative of CAM expression (Borland *et al.*, 2011). Despite this, no increase in these same parameters was observed in the thin leaves of *Oncidium* (Figs 5A, B and 6A, B). Previous reports have suggested that thick-leaved epiphytic orchids are commonly recognized as performing CAM photosynthesis in their succulent leaves (Avadhani and Arditti, 1981; Avadhani *et al.*, 1982; Fu and Hew, 1982; Hew and Yong, 2004). Furthermore, the pronounced dark CO_2 uptake and diurnal acidity rhythm characteristically found in *Cattleya* orchids are often linked to the succulent morphology of their leaves (Nuerenbergk, 1963; Knauft and Arditti, 1969).

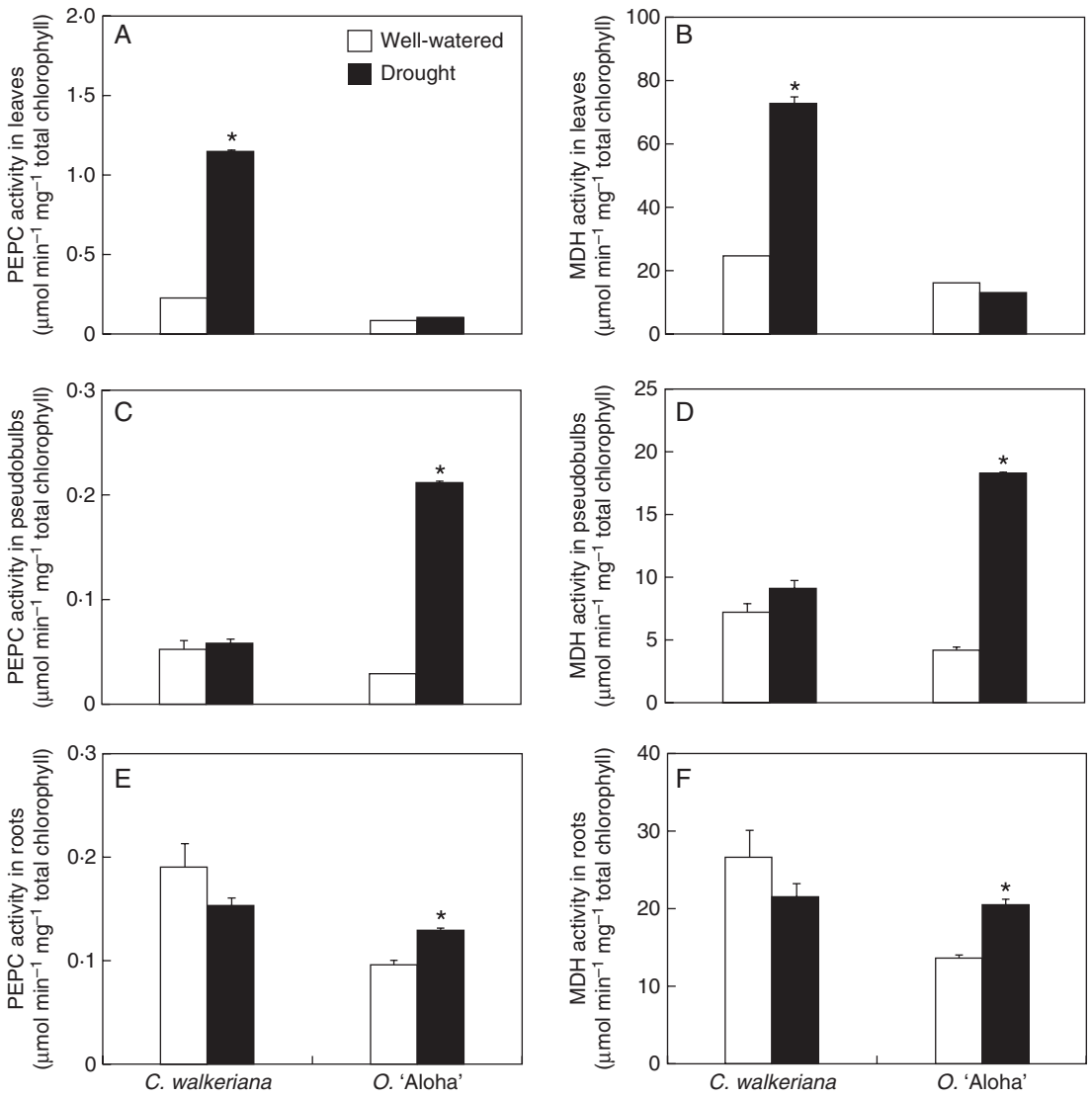

FIG. 5. Activities of (A, C, E) PEPC and (B, D, F) MDH in leaves, pseudobulbs and roots of *Cattleya walkeriana* and *Oncidium* 'Aloha' after 1 month of well-watered or drought-exposed treatments (as indicated in key). Data are expressed as the mean ± s.e. An asterisk indicates a significant difference between treatments ($P \leq 0{\cdot}05$).

However, the present results revealed that mature leaves of *C. walkeriana*, a typical thick-leaved orchid, can display a surprisingly high photosynthetic plasticity under distinct regimes of water availability (Figs 5A, B and 6A, B), which was comparable to the general pattern detected for the epiphytic bromeliad *G. monostachia* (Freschi *et al.*, 2010*b*). Accordingly, well-watered leaves of *C. walkeriana* showed only mild diurnal fluctuations in both organic acid accumulation and PEPC/MDH activities, while the drought treatment induced the up-regulation of these metabolic parameters indicative of CAM expression (Figs 5A, B and 6A, B). These results reveal that even thick-leaved epiphytic orchids included in the genus *Cattleya* present relatively high plasticity in expressing CAM in their succulent leaves. In agreement with the present findings, leaves of the thick-leaved *Phalaenopsis* orchids showed up-regulation of CAM photosynthesis during plant ontogeny and in response to varied thermoperiodic conditions (Guo and Lee, 2006; Ping *et al.*, 2010).

Although some nocturnal organic acid accumulation was observed in both well-watered and drought-treated *Oncidium* leaves (Fig. 6A, B), under well-watered conditions these leaves carried out most of the atmospheric CO_2 uptake during daytime, which almost disappeared when *Oncidium* plants were exposed to water scarcity (Fig. 7). Considering these data, the thin leaves of *O.* 'Aloha' seemed to perform typical C_3 photosynthesis even under drought conditions, which is in accordance with previous reports concerning the photosynthetic mode of leaves in the closely related hybrid *Oncidium* 'Goldiana' (Hew and Yong, 1994; Hew *et al.*, 1996, 1998; Li *et al.*, 2001, 2002).

The results presented here reinforce the idea that a higher degree of chlorenchyma succulence and reduced intercellular air space in leaves might be important for CAM operation (Nelson *et al.*, 2005; Griffiths *et al.*, 2008; Nelson and Sage, 2008; Borland *et al.*, 2011). Hence, the set of morphological traits showed by *Cattleya* leaves might, to a certain extent, favour both water and carbon economy under drought by

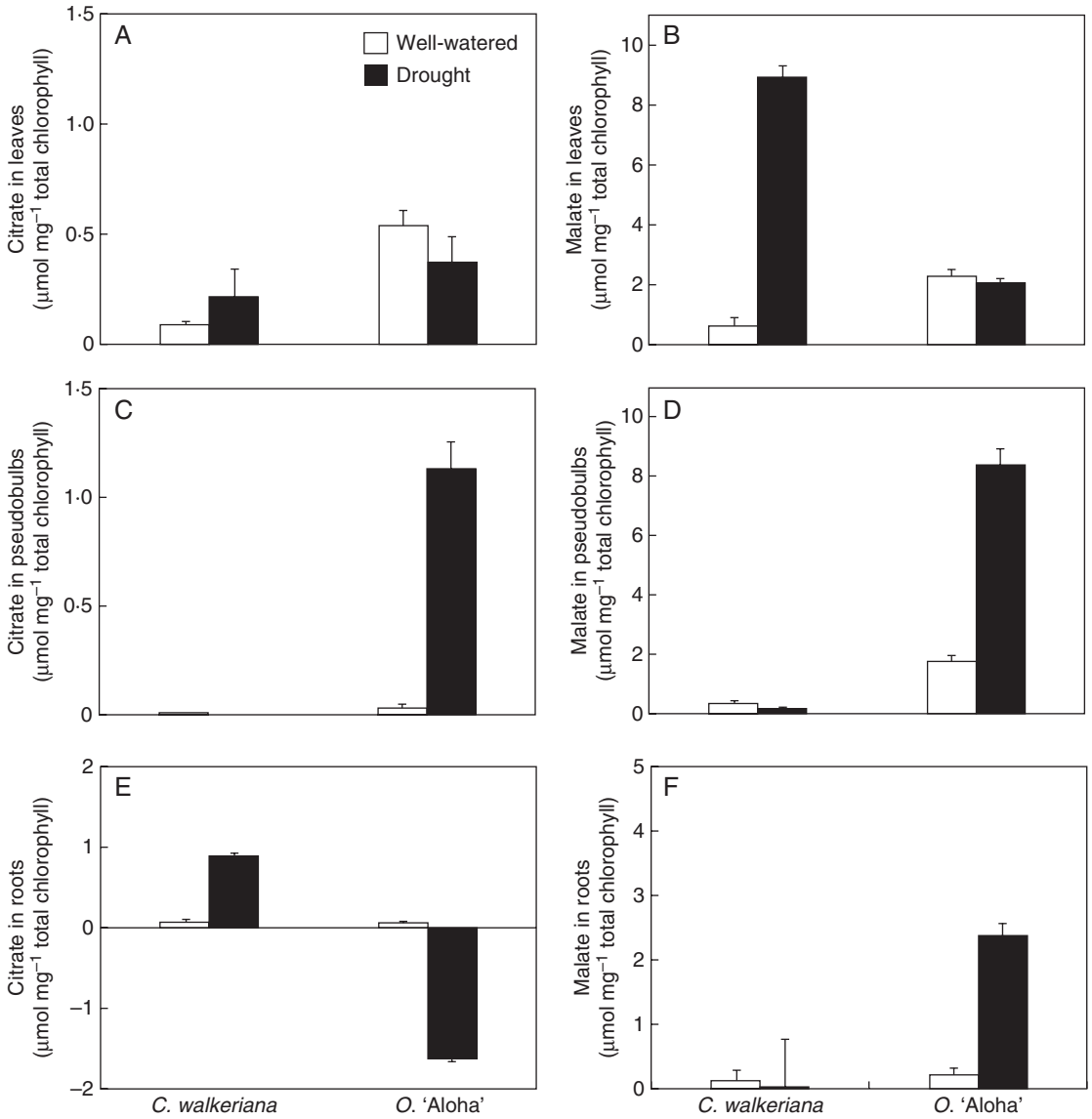

FIG. 6. (A, C, E) Citrate and (B, D, F) malate nocturnal accumulation in leaves, pseudobulbs and roots of *Cattleya walkeriana* and *Oncidium* 'Aloha' after 1 month of well-watered or drought-exposed treatments (as indicated in key). Data are expressed as the differences between dawn and dusk values: organic acid mean = dawn mean − dusk mean. The standard error was calculated by the formula described by Popp *et al.* (2003): s.e. of the dawn − dusk difference = $\sqrt{[(\text{s.e. dawn})^2 + (\text{s.e. dusk})^2]}$. An asterisk indicates a significant difference between treatments ($P \leq 0{\cdot}05$).

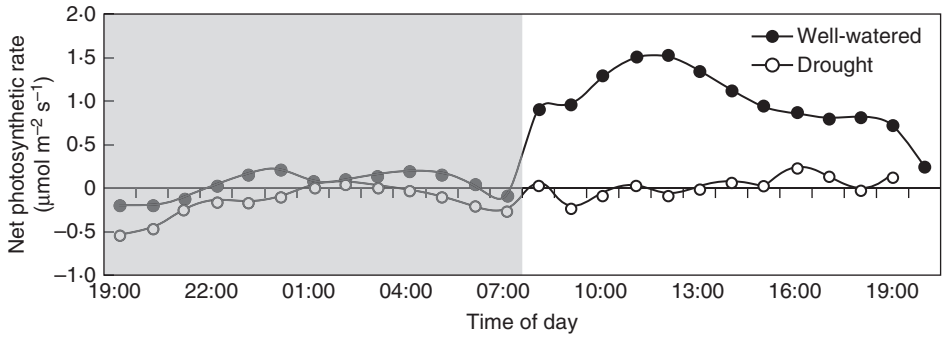

FIG. 7. Diurnal pattern of CO_2 assimilation in the apical leaf portion of *Oncidium* 'Aloha' plants maintained under well-watered conditions or exposed to 30 d of drought (as indicated in key). The shaded area indicates the dark period.

expressing CAM. On the other hand, *Oncidium* leaves might encounter some difficulties in maintaining a positive carbon balance under prolonged drought due to histological features that exhibit an apparently low efficiency in preventing water and CO_2 losses. Therefore, these results suggest that photosynthetic activity in *Oncidium* leaves under water scarcity might be, at least in part, dependent on the non-leaf organs, such as pseudobulbs and roots.

Drought-induced modulation of C_3–CAM photosynthesis in roots

Despite the small amount of citrate accumulation during the night (Fig. 6E), aerial roots of the drought-treated *Cattleya* plants showed an apparent lack of CAM expression (Figs 5E, F and 6F). Therefore, such photosynthetic compartmentalization found among organs of the drought-treated *Cattleya* (CAM in leaves versus C_3 in roots) is in agreement with recent research which has shown that the presence of CAM in leaves of epiphytic orchids did not ensure that their aerial roots would perform the same photosynthetic pathway (Martin *et al.*, 2010). Conversely, the drought-treated roots of *Oncidium* presented a significant increase in nocturnal malate accumulation (Fig. 6F) and also showed a significant increase in PEPC/MDH activities (Fig. 5E, F), indicating that induction, or at least up-regulation, of CAM expression was triggered in aerial roots of this orchid by water limitation. As far as we know, the data obtained with *Oncidium* might be the first demonstration regarding the occurrence of inducible CAM in aerial roots of an orchid performing typical C_3 photosynthesis in its leaves.

The view that aerial roots of leafy orchids possess the photosynthetic apparatus for CO_2 fixation but, in general, are not considered sufficiently autotrophic to maintain themselves (Ho *et al.*, 1983; Hew *et al.*, 1984) suggests a more localized role for the presence of CAM in aerial roots of *Oncidium* under water deficit. However, the larger chlorophyll-containing cells in the *Oncidium* cortex could provide the photosynthetic machinery and the vacuolar space required for nocturnally accumulating organic acids derived from CO_2 fixation through PEPC activity. In support of this hypothesis, autotrophic roots of some leafless epiphytic orchids, although lacking stomata, represent important photosynthetic organs for the plant. The uptake and fixation of CO_2 by these autotrophic roots can occur nocturnally due to the presence of a thinner velamen and larger volume of cortical intracellular space when compared with the same structures in aerial roots of most leafy orchids (Benzing *et al.*, 1983; Winter *et al.*, 1983; Cockburn *et al.*, 1985). Accordingly, studies regarding the relationship between respiration and CO_2 fixation by aerial roots of *Aranda* orchids have suggested that its thick velamen hampers CO_2 uptake from the atmosphere, while the extent of this effect might depend on velamen thickness (Hew *et al.*, 1991).

Drought-induced modulation of C_3–CAM photosynthesis in pseudobulbs

Virtually no nocturnal organic acid accumulation was detected in both well-watered and drought-treated *Cattleya* pseudobulbs (Fig. 6C, D), which was supported by no changes in PEPC/MDH activities (Fig. 5C, D), thus indicating the absence of CAM expression in pseudobulbs of *C. walkeriana*. Alternatively, the drought treatment triggered a remarkable increase in night-time organic acid accumulation in *Oncidium* pseudobulbs, which was followed by a parallel rise in the activities of both PEPC and MDH (Figs 5C, D and 6C, D), thereby implying that *Oncidium* pseudobulbs can be induced to perform CAM photosynthesis depending on environmental conditions.

As most orchid pseudobulbs have a hermetic structure in which the entire organ is covered with thick cuticle and is devoid of stomata, the main explanation for the nocturnal organic acid accumulation in this organ has been based on recycling respiratory CO_2 generated by the highly packed pseudobulb parenchyma (Hew and Yong, 1994; Ng and Hew, 2000). Contrary to the general structural organization observed in ground tissue of *Oncidium*, the outer portion of *Cattleya* pseudobulbs showed a considerable number of dead cells with thickened, lignified walls scattered among assimilatory cells, creating a sclerenchymatous boundary around the periphery of the ground tissue (Fig. 2C). This type of cellular arrangement has been reported for other orchid species (Withner, 1974; Stern and Morris, 1992; Holtzmeier *et al.*, 1998), and, for *C. walkeriana*, it might restrict the amount of living cells with the metabolic requirements for modulating the photosynthetic pathway.

Note that *Oncidium* pseudobulbs, in addition to malate, also nocturnally accumulated citrate, but the amounts were lower than those found for malate (Fig. 6C, D). Although the putative functional advantage of performing CAM with malate and/or citrate is not fully understood, some evidence has indicated that nocturnal accumulation of citrate might be more favourable than malate under certain environmental constraints, such as drought associated with high-irradiance stress (Lüttge, 2002, 2006). Besides, the higher energy demand for citrate cycling in the light period can contribute to energy dissipation and cellular redox balance, thus acting as a protective mechanism against photoinhibition and photodestruction (Kornas *et al.*, 2009; Sun and Hong, 2011).

Hypothetical model for drought-induced C_3–CAM compartmentalization in Oncidium

Previous approaches to the photosynthetic metabolism of *Oncidium* ‘Goldiana’ cast some persistent doubts regarding the actual physiological dynamics behind the complex interdependency observed between leaves and pseudobulbs of this thin-leaved epiphytic orchid. These studies revealed an apparent inconsistency between data obtained by gas exchange and radioactive tracer experiments demonstrating that photosynthates in leaves were somehow transported to the pseudobulb in the first instance and then re-distributed within the shoot organs (Yong and Hew, 1995*a*, *b*). This dilemma, which has remained unclear until now, has been attributed to possible unique patterns of photoassimilate partitioning in tropical orchids which might result from complex vascular connections between source and sink organs (Hew and Yong, 1994; Yong and Hew, 1995*a*; Hew *et al.*, 1996; Ng and Hew, 2000).

Accordingly, the present results showed that in *Oncidium* ‘Aloha’ pseudobulbs all the vascular bundles were associated with an aerenchyma (Fig. 2F), which was present throughout the entire length of the pseudobulb (Fig. 2H) that was able to

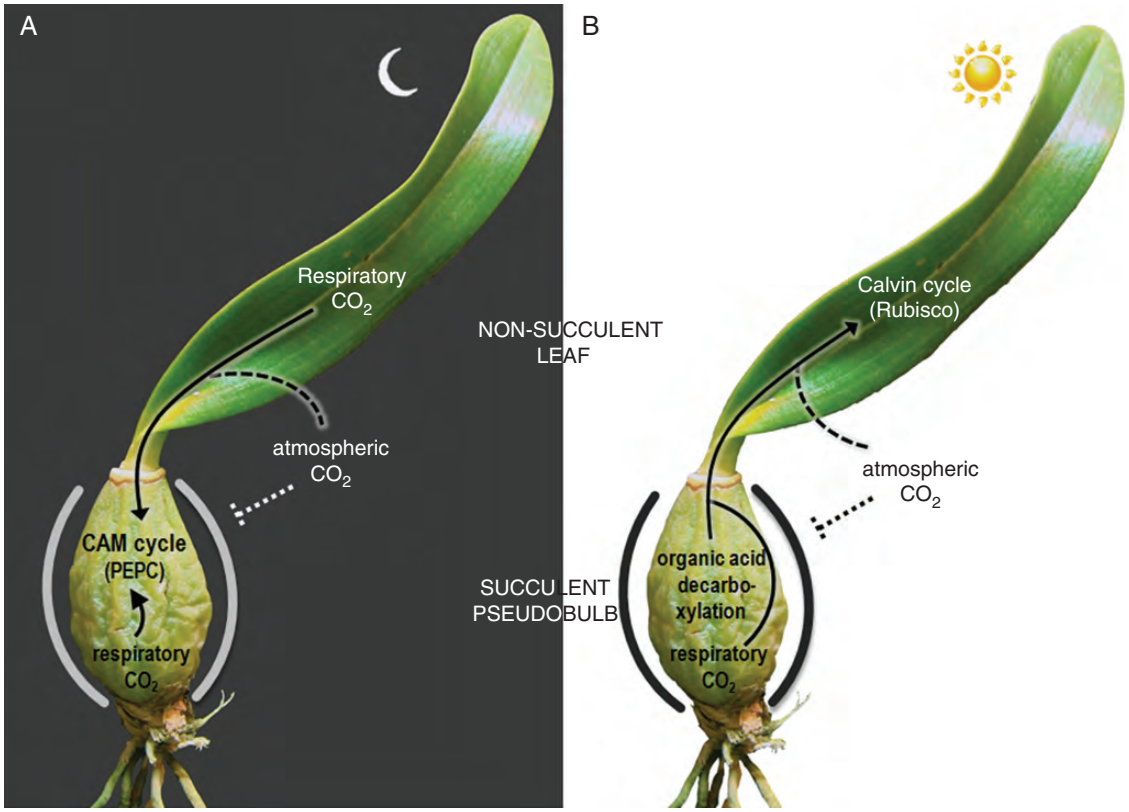

FIG. 8. Schematic representation of the general model suggested for the photosynthetic compartmentalization among organs of the drought-treated *Oncidium* 'Aloha' orchid (C_3 in leaves versus CAM in non-leaf organs) during night (A) and daytime (B). The model is based on the presence of aerenchyma ducts connecting the succulent pseudobulb with the mesophyll of non-succulent leaves (solid arrows) and, consequently, with the atmosphere (dashed lines). The sharper lines surrounding the pseudobulb indicate the hermetic feature of this organ which eliminates direct atmospheric carbon fixation. Details are given in the text.

express CAM under drought conditions (Figs 5C, D and 6C, D). Intriguingly, these aerenchyma ducts along the pseudobulb were connected with the air spaces present at the leaf base of *Oncidium* (Fig. 1E), thus forming free pathways for gas exchange between leaves and pseudobulbs. Equivalent structural organization has been described for some orchids, including species from the subtribe Oncidiinae, where the aerenchyma (termed 'lacunae') is also associated with the pseudobulb phloem (Moreau, 1913; Withner, 1974; Holtzmeier *et al.*, 1998; Stern and Carlsward, 2006; Aybeke *et al.*, 2010).

Therefore, these findings make it possible to suggest a hypothetical model for the photosynthetic compartmentalization found among organs of the drought-treated *O.* 'Aloha' orchid (Fig. 8). Based on this proposition, at least some of the production of the organic acids in this organ might possibly come from the carboxylation of CO_2 originated by the nocturnal respiration of the leaf mesophyll cells, or even from the atmosphere, if some leaf stomata are open at night. By contrast, during the day, if leaf stomata remain closed due to a certain limitation in water supply or other environmental stress, the decarboxylation of organic acids in chlorenchyma cells of the pseudobulbs could possibly provide CO_2 for leaf mesophyll cells through the aerenchyma ducts. If this is the case, the site of organic acid accumulation may be transferred from the non-succulent leaf mesophyll of *Oncidium* to the larger vacuoles of the chlorenchyma cells in the succulent pseudobulb of this orchid. Therefore, in this case, we may have spatial separation between the site of night-time carboxylation via PEPC (pseudobulbs) and the site of daytime carboxylation via Rubisco (leaves). Perhaps, the limited capacity for nocturnal acid storage in the thin leaf mesophyll of *Oncidium* could be surpassed by transferring CAM expression to the pseudobulbs, which besides being succulent are also devoid of stomata, thus limiting any eventual loss of CO_2 to the atmosphere.

This might help to clarify aspects that still persist regarding the complex dynamics that coordinate the photosynthetic interdependency observed between leaves and pseudobulbs of *Oncidium* orchids. However, from the perspective of this evidence, questions remain about whether we should consider *Oncidium* plants as performing C_3 photosynthesis (when taking into consideration just the data obtained from the leaves), or a facultative CAM plant (by considering the drought-induced up-regulation of CAM in the non-leaf organs), or even as an example of a special mode of inducible CAM photosynthesis in which the day–night acid cycle typical of CAM (in the pseudobulbs) is spatially separated from the Calvin cycle (in the leaf mesophyll).

Concluding remarks

Here we demonstrate that water availability is a powerful signal capable of modulating CAM expression in an organ/tissue-compartmented manner in both the thick-leaved (*C. walkeriana*) and thin-leaved (*O.* 'Aloha') epiphytic orchids

studied. Although belonging to an orchid genus classically considered as performing C_3 photosynthesis, *Oncidium* plants under drought seemed to express facultative CAM in its roots and pseudobulbs but not in its leaves; the drought-induced CAM expression in these organs might compensate for the lack of capacity to perform CAM in its thin leaves. On the other hand, *C. walkeriana*, which is considered a constitutive CAM orchid, has shown a clear drought-induced up-regulation of CAM in its thick leaves but not in its non-leaf organs. As distinct regions of the same orchid could perform different photosynthetic pathways and variable degrees of CAM expression depending on water availability, more attention should be given to this during future studies about the abundance of CAM plants in a given plant family, habitat and ecosystem. The data presented highlight the great importance of studying the range of CAM expression/modulation in specific plant tissues while taking into consideration the physiological responses under different environmental conditions and/or developmental phases.

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