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Crassulacean Acid Metabolism in Epiphytic Orchids: Current Knowledge, Future Perspectives

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1. Introduction

1.1 Crassulacean Acid Metabolism (CAM)

Crassulacean Acid Metabolism (CAM) is one of three photosynthetic assimilation pathways of atmospheric CO_2, together with the photosynthetic pathways C_3 and C_4 (Silvera et al., 2010a). The CAM is characterized by the temporal separation between CO_2 fixation and its assimilation into organic compounds. In CAM plants, CO_2 is fixed during the dark period through the action of the enzyme phosphoenolpyruvate carboxylase (PEPC), which uses CO_2 for carboxylation of phosphoenolpyruvate (PEP), giving rise to oxaloacetate (OAA). The OAA formed is converted into malate by the action of malate dehydrogenase (MDH). Then, this organic acid is transported to the vacuole along with H^+ ions, causing the typical nocturnal acidification of CAM plants. During the light period, the decarboxylation of malate and refixation of the CO_2 by the enzyme ribulose bisphosphate carboxylase oxygenase (RUBISCO - C_3 cycle) takes place in the cytosol, causing a decrease of acidity in the tissues (Herrera, 2009; Luttge, 2004; Silvera et al., 2010b) (Figure 1).

The CAM pathway can be separated into four phases (Luttge, 2004; Osmond, 1978; Silvera et al., 2010b). Phase I is characterized by the opening of stomata during the night, the uptake and subsequent fixation of atmospheric CO_2 by PEPC in the cytosol and the formation of organic acids, such as malate. Phase II consists of fixing CO_2 by the enzyme RUBISCO and PEPC concurrently, a phase characterized essentially by the decrease in the activity of PEPC and the start of the activity of RUBISCO. Phase III consists of the reduction of stomatal opening, efflux of organic acids from the vacuole and subsequent decarboxylation of these acids. Finally, phase IV comprises the depletion in the stock of organic acids associated with an increase of stomata conductance.

Due to nighttime fixation of atmospheric CO_2, CAM plants exhibit greater water use efficiency (EUA) when compared with the photosynthetic pathways C_3 and C_4 (Herrera, 2009), given that CAM plants use 50 to 100 g of water per gram of CO_2 fixed, while C_3 plants use 400 to 500 g (Drennam & Nobel, 2000). The ratio of transpiration is 3- to 10-fold lower in CAM plants than in C_3 (Kluge & Ting, 1978). Besides the EUA, another advantage of CAM comprises mechanisms to minimize the damage caused by reactive oxygen species (ROS) (Sunagawa et al., 2010).
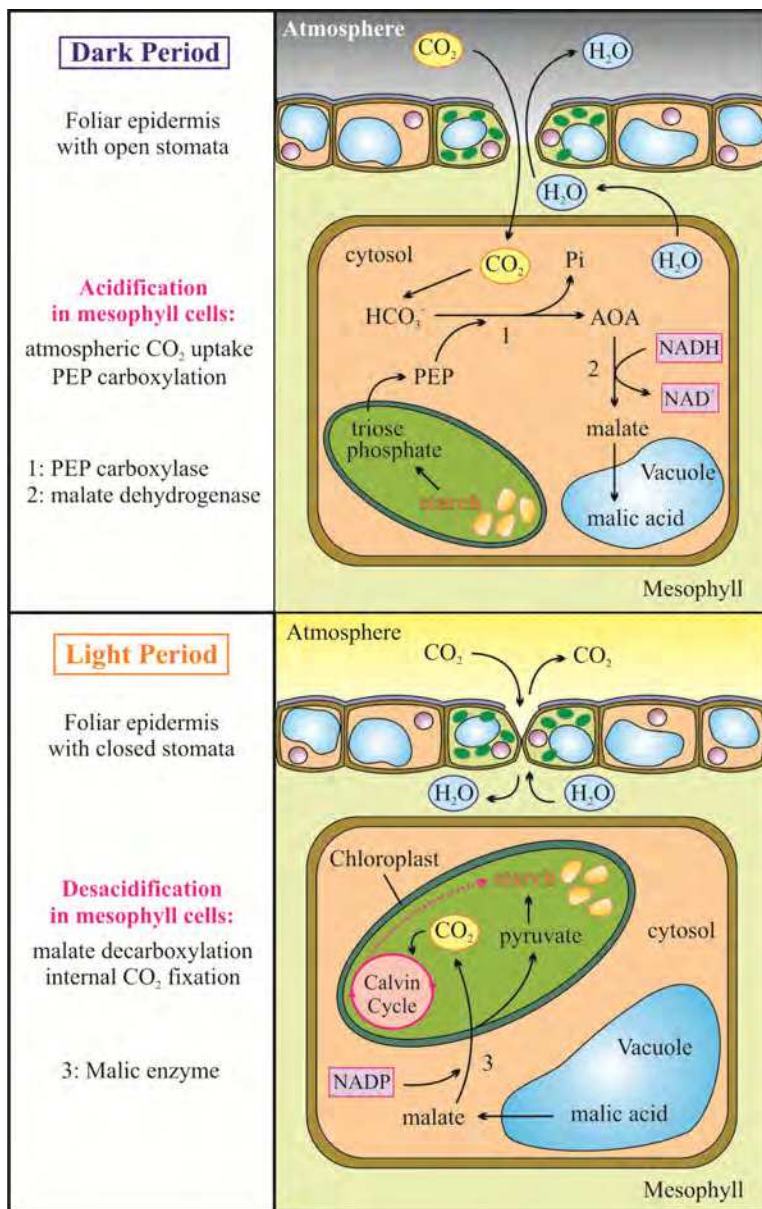
Fig. 1. Temporal separation of CO_2 uptake and fixation typical of the crassulacean acid metabolism (CAM). Atmospheric CO_2 is taken up and fixed during the night (upper panel). Nighttime-accumulated organic acids are decarboxylated during the day, and the internally released CO_2 is refixed (lower panel). The nighttime stomatal opening allows a reduction of water loss by transpiration, which is an important adaptive trait of CAM pathway. PEP: phosphoenolpyruvate, AOA: oxaloacetate.

Regarding its occurrence, CAM species are distributed in semiarid, tropical and subtropical environments, including epiphytic species in the humid tropics (Silvera et al., 2010a, 2010b). The more representative plant families in number of CAM species are Aizoaceae, Asclepiadaceae, Asteraceae, Bromeliaceae, Cactaceae, Crassulaceae, Euphorbiaceae, Portulacaceae and Orchidaceae. In the Orchidaceae, at least half of all species are classified as CAM (Winter & Smith, 1996).

The CAM pathway can operate in different modes: obligate CAM, facultative CAM, CAM-cycling and CAM-idling (Figure 2). Obligate CAM species exhibit high accumulation of organic acids at night and nocturnal CO_2 fixation even under optimal environmental conditions (Kluge & Ting, 1978). On the other hand, facultative CAM species, also known as C_3-CAM, are plants capable of performing C_3 photosynthesis under favorable growth conditions and switching to CAM mode when challenged by environmental constraints, such as water limitation or excessive light incidence. In both constitutive and facultative CAM species, CAM is more strongly expressed in mature tissues (Gehrig et al., 2005), while young plants or young tissues of a mature plant tend to perform exclusively C_3 photosynthesis (Avadhani et al., 1971). CAM-cycling consists of diurnal CO_2 fixation and accumulation of organic acids; however, the stomata remain closed at night, and the CO_2 necessary for the nighttime formation of the organic acids is exclusively obtained from the respiratory activity of the plant tissues. Finally, plants in CAM-idling mode exhibit only a small accumulation of organic acids, and the stomata remain closed both day and night; this nocturnal accumulation of organic acids is also provided by the recycling of respiratory CO_2. Overall, CAM-cycling is considered a weak CAM, while CAM-idling is currently believed to consist of a strong CAM mode (Luttge, 2004). Facultative C_3-CAM species, such as *Guzmania monostachia* (Bromeliaceae) and *Talinum triangulare* (Portulacaceae), can be induced to CAM by various environmental factors, such as drought stress (Freschi et al., 2010; Herrera et al., 1991), photoperiod (Brulfert et al., 1988), salinity (Winter & Von Willert, 1972), nitrogen deficiency (Ota, 1988), phosphorus deficiency (Paul & Cockburn, 1990) and high photosynthetic photons flux (Maxwell, 2002). The CAM induction by environmental factors is usually fast and reversible, a conspicuous example of plasticity. Exemplifying the plasticity of C_3-CAM species, plants of facultative C_3-CAM bromeliad *G. monostachia* have recently been shown to be clearly induced to a CAM-idling mode of photosynthesis when maintained under drought stress during a seven-day period. Interestingly, these same plants returned to a typical C_3 condition after a subsequent period of seven days of rehydration (Freschi et al., 2010).

Despite the above-mentioned favorable adaptive traits, the CAM pathway exhibits some disadvantages related to biomass productivity and photorespiration. The biomass productivity in grams of dry weight/m/day in CAM plants is between 1.5 and 1.8, while in C_3 plants the productivity is 50-200 (Black, 1973). The energy costs for the assimilation of each CO_2 molecule is significantly higher in CAM plants than in C_3 species. The estimated stoichiometry of ATP: NADPH: CO_2 of C_3 plants is 3: 2: 1, while for CAM plants it is 4.8: 3.2: 1 (Winter et al., 1978). Regarding photorespiration, it was verified that the vigorous assimilation of the CO_2 provided by decarboxylation of organic acids after stomata closure can result in increases of up to 40% in the O_2 concentration inside the leaves during phase III (Spalding et al., 1979). Hence, the photorespiration in CAM plants occurs not only in phase IV of CAM, when the stock of malate was exhausted and the stomata are open for CO_2 absorption to occur, but also in phase III, where the CO_2 concentration is counterbalanced by the O_2 concentration (Luttge, 2011).
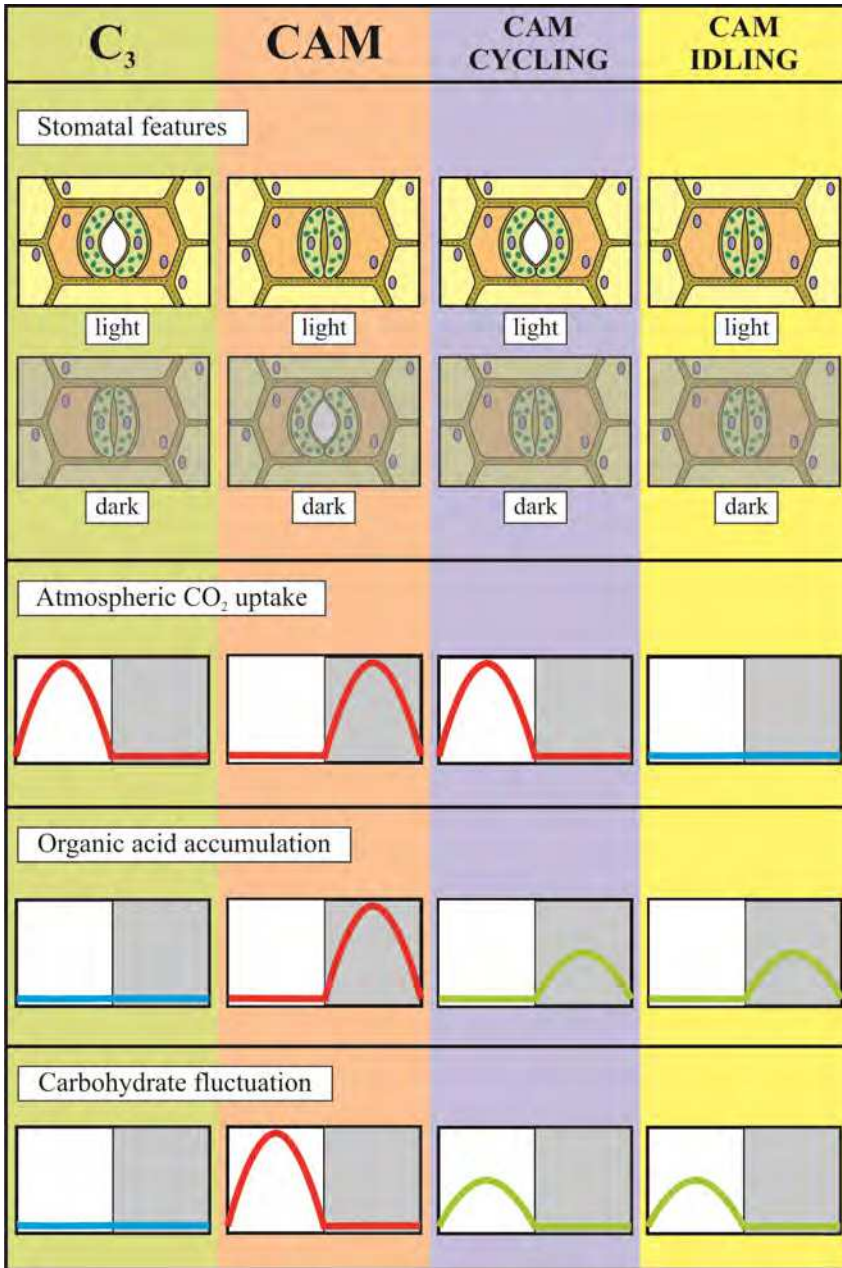
Fig. 2. Different modes of CAM in relation to C_3, constitutive CAM, CAM-cycling and CAM-idling. Differences related to the opening of stomata, atmospheric CO_2 uptake, flux of organic acids and flux of carbohydrates. The gray box represents the dark period, while the white box represents the light period.

Despite the decrease of biomass productivity and photorespiration problems, the fixation of CO_2 via CAM evidently guarantees a maximum carbon gain combined with a minimum loss of water supplies in the plant tissues. In xeric habitats, some plants are only able to survive in low precipitation periods mainly due to the presence of CAM photosynthesis. In this sense, the epiphytic habitat can be considered an excellent example of xeric habitat, where some epiphytic plants, including orchids, often face situations of limited water supply.

2. Crassulacean acid metabolism in the epiphytic habitat

Epiphytes are plants that spend much or all of their lives attached to other plants (Benzing, 1990). They are responsible for much of the biotic diversity that makes humid tropical forests the most complex of all the world's ecosystems (Gentry & Dodson, 1987). Canopy-based species constitute virtually one third of the total vascular flora in some pluvial neotropical forests (Benzing 1990). The majority of epiphytes, including Araceae, Gesneriaceae, Cactaceae and pteridophytes, can be found in the parts of the canopy which have greater quantities of moisture and nutrients than are available to their more xeric counterparts growing in arid zone canopies (Gentry & Dodson, 1987). Their occurrence in drier sites is less common and usually involves fewer taxa but not necessarily lower abundance. Foremost species of these taxa are the few stress-tolerant bromeliads (Bromeliaceae) and orchids (Orchidaceae), which are commonly classified as the extreme epiphytes (Benzing, 1973). The Orchidaceae has been more successful than any other lineage in colonizing tree crowns. About two out of three epiphytes are orchids; at least 70% of the family is canopy-adapted (Benzing, 1990).

Aerial substrata can impose severe environmental stresses on the survival of epiphytic vegetation (Benzing, 1987). The adaptations that facilitate extreme epiphytism are numerous and involve many morphological or anatomical characteristics of the plant body as well all stages of the life cycle. Their success of survival in drier sites of the canopy is associated mainly with refined water-balance mechanisms, singular nutritional modes and efficient reproductive systems (Benzing, 1990). Some of the adaptive traits of epiphytes can be summarized as high water use efficiency, crassulacean acid metabolism, low surface to volume ratio, aerial seed dispersal, mycoheterotrophy (Orchidaceae), holdfast structures, slow growth rate and efficient mineral use (Benzing, 1973; Dodson, 2003; Yoder et al., 2010).

The availability of nutrients and water in the forest canopy is sporadic and totally dependent on the rainy period. Of the natural water supplies available to epiphytes, precipitation intercepted by the canopy and reaching the ground by flowing down the trunks or by falling through the foliage usually contains the most abundant quantities of nutrient solutes (Benzing, 1973). Nevertheless, a liter of stem-flow or fall-through rarely contains more than a few milligrams of any specific mineral nutrient (McColl, 1970; Sollins & Drewry, 1970). Therefore, most extreme epiphytes need to take and accumulate water and all required nutrients during brief intervals (few hours or minutes) when the shoot or root systems are in contact with rainfall and leachates. The extreme epiphytes often have some characteristics that enable plants to absorb and accumulate water and minerals faster and with greater efficiency. In species of the Orchidaceae, the roots of many orchids are covered with a special structure called velamen, which acts as a sponge, allowing the root to immobilize a temporary but highly accessible reservoir of moisture and minerals (Benzing &

Ott, 1981). Moreover, the velamen slows root transpiration, provides mechanical protection and assists in the attachment of orchids to the bark of host trees (Ackerman, 1983; Benzing et al., 1982). Epiphytic orchids have considerable succulence in their shoot organs (leaves and pseudobulbs), which are important reservoir structures for storing water and nutrients (Benzing, 1990).

The vegetative growth is strongly influenced by water supply, the shortage of which can be considered the most severe environmental stress in the epiphytic habitat. The extreme epiphytes need to adjust water-balance mechanisms in all plant tissues rapidly and constantly, via appropriate stomatal and photosynthetic responses, to avoid irreversible drought injuries. The drought endurance observed in the majority of epiphytes is provided by a strong CAM photosynthetic behavior, which promotes a very favorable water economy (Benzing & Ott, 1981).

A high number of epiphytic species perform CAM photosynthesis (Benzing, 1989; Luttge, 2004). Among orchids species it is likely that at least half could perform this type of photosynthetic pathway owing to the high number of epiphytic species, of which the Epidendroideae subfamily is the richest in epiphyte CAM species. This subfamily is believed to have radiated at the beginning of the Tertiary (Ramirez et al., 2007), a period marked by climatic changes such as soil aridification and declining CO_2 concentrations (Pearson & Palmer, 2000), favoring the survival of the epiphyte species displaying CAM photosynthesis. Nevertheless, CAM does not seem to be related to phylogenetic relationships among the taxa. Silvera et al. (2009) suggest that among the Orchidaceae, CAM arose independently at least 10 times from a C_3 ancestor. Indeed, the enzymatic machinery to perform CAM is present in all plants, including those performing C_3 photosynthesis exclusively, and the differences between both photosynthetic pathways are mainly associated with the regulation of such machinery (Silvera, 2010a).

The existence of a significant correlation between photosynthetic pathways and epiphytism has already been observed by Silvera et al. (2009). The phylogenetic analyses showed that C_3 photosynthesis is the ancestral state and that CAM has evolved multiple independent origins, indicating the great evolutionary flexibility of CAM in Orchidaceae (Silvera et al., 2009). Moreover, when using maximum likelihood to trace epiphytism as a character state across the orchid phylogeny, the authors have also verified that the terrestrial habit is the ancestral state within tropical orchids and, similar to CAM, the epiphytic habit is derived. Throughout evolutionary time, the CAM divergence observed by $\delta^{13}C$ analysis is consistently accompanied by divergence in epiphytism, demonstrating a functional relationship between these traits. Correlated divergence between the photosynthetic pathway and epiphytism is likely an important factor contributing to the burst of speciation that occurred in diverse epiphytic orchid clades (Silvera et al., 2009).

Besides epiphytism, it is currently accepted that CAM is also strongly linked with a certain degree of succulence, as commonly observed in members of the Crassulaceae (Kluge et al., 1993) and Orchidaceae (Silvera et al., 2005), in which the leaf thickness provides a higher storage capacity for organic acid accumulation (Ting, 1985). Although succulence and CAM usually coincide, some epiphytes are an exception to this rule (Benzing, 1990) since there are clear demonstrations that some epiphytic orchid species with thin leaves performed CAM, while some species with thick leaves performed C_3 metabolism (Silvera et al., 2005). However, Zotz et al. (1997) have clearly demonstrated the correlation between chlorenchyma thickness and CAM in the leaves of different

orchids. In their study, they have shown that species with strong CAM had thicker chlorenchyma, highlighting that the importance of the relationship between leaf thickness and CAM does not reside in the thickness of the leaf, but rather in the thickness of the chlorenchyma, as in most cases the succulence is due to the presence of a thick hydrenchyma, which does not contribute to CAM (Winter et al., 1983) in terms of organic acid storage capacity.

Most of the knowledge of the distribution of CAM orchids comes from studies in New Guinea, Australia (Earnshaw et al., 1987; Winter et al., 1983), Panama (Silvera et al., 2005, 2009, 2010; Zotz & Ziegler, 1997; Zotz, 2004), Mexico (Mooney et al., 1989) and Costa Rica (Silvera, 2009). These surveys reveal an increase in the number of epiphyte CAM orchid species following the forest precipitation frequency, rising from 25% in wet tropical rainforests and moist tropical forests to 100% in dry forests (Figure 3A). Among the Orchidaceae an evolutionary correlation was found between the photosynthetic pathways and epiphytism, following a direct relation between the epiphytic orchid habit and the presence of CAM, which is more evident in regions ranging from 0 to 500 m when compared to higher regions (Silvera et al., 2009). The same authors attribute this relation to the canopy height of the forests at these altitudes, favoring epiphytism and, consequently, the CAM pathway. Indeed, even in the same location, the presence of CAM epiphytes increases with canopy height, ranging from 7% in the forest understory to 50% in exposed canopy sites (Zotz & Ziegler, 1997). In regions above 2000 m, the tree height is lower and, by consequence, so is the canopy. Therefore, the epiphyte habitat in these regions is reduced (Figure 3B).

It is worth mentioning that the main technique to determine the photosynthetic pathway in the surveys of orchids was based on the quantification of the stable isotope ^{13}C (expressed in $\delta^{13}C$‰) on plants leaves. The typical range of $\delta^{13}C$‰ for C_3 plants is between -33‰ to -22.1‰, while for strong CAM plants it ranges from -22‰ to -12‰ (Elheringer & Osmond, 1989). Several studies done in different taxa have shown a bimodal pattern of the frequency distribution of $\delta^{13}C$‰ values among orchid species, following their photosynthetic pathway (Holtum et al., 2005; Motomura et al., 2008b; Pierce et al., 2002; Silvera et al., 2005, 2009, 2010a, 2010b); this behavior is characterized by a cluster formed by a high number of C_3 species (indicated by values near -28‰) and a smaller cluster formed by CAM species (with values near -16‰); between the clusters are intermediate values that could represent weak CAM or facultative CAM species (usually in response to a stress, such as drought) (Silvera et al., 2009). In fact, it was found that although some orchid species presented $\delta^{13}C$‰ values typical of a C_3 plant, they were capable of nighttime carbon fixation, reflected by an increase in tissue titratable acidity (Silvera et al., 2005). Therefore, the characterization of the photosynthetic pathways by isotopic measurement alone tends to underestimate the number of species capable of performing some degree of CAM (Pierce et al., 2002; Silvera et al., 2005, 2009, 2010a, 2010b; Winter et al., 2008).

Nowadays, it is thought that approximately 40% of the tropical orchid species could exhibit some form of CAM (strong, weak or facultative) (Silvera et al., 2010a), but there is insufficient knowledge about the photosynthetic pathways in other non-leaf organs, such as pseudobulbs or roots. Also, studies about the capacity of a photosynthetic shift between C_3 and CAM triggered by environmental conditions are very rare in the literature.
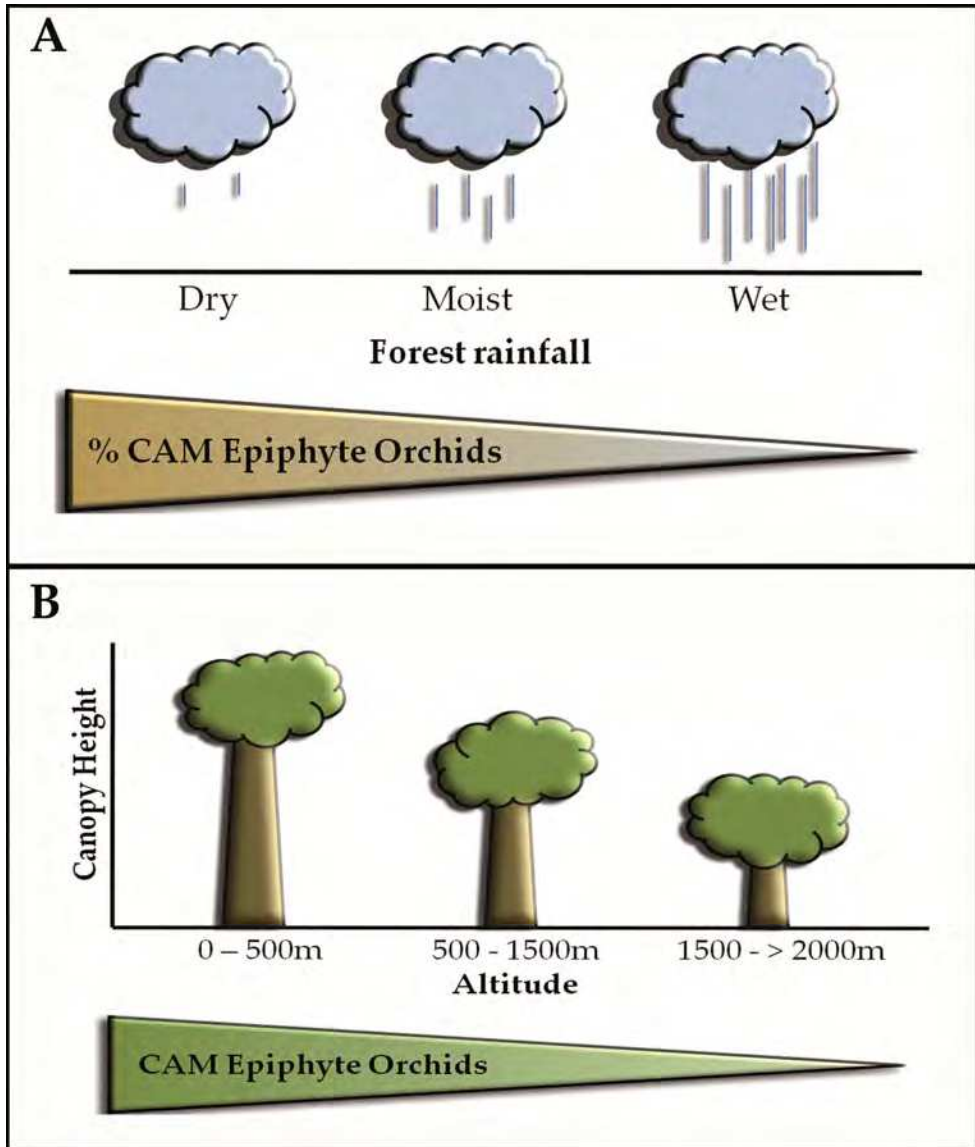
Fig. 3. The occurrence of CAM epiphytic plants according to (A) forest rainfall and (B) variation of altitude and canopy height.

3. Crassulacean acid metabolism in orchid leaves

CO_2 fixation in orchid leaves has been widely studied in the past few years. Most of these studies suggest that thin-leaved orchids present C_3 photosynthesis pathway (Calvin-Benson cycle), while thick-leaved succulent orchids photosynthesize basically via CAM pathway

(Avadhani et al., 1982). Goh et al. (1977) and Winter et al. (1983) observed that the majority of orchid species and hybrids that possess succulent leaves presented nocturnal stomatal opening and acidity rhythms typical of CAM. Orchids like *Cattleya*, *Laelia*, *Brassavola* and *Sophronitis* have thick leaves, and all of those are reported as CAM plants (Avadhani & Arditti, 1981, Avadhani et al., 1982). On the other hand, C_3 orchids like *Arundina graminifolia* and *Oncidium* Goldiana are characterized by thin leaves (Hew & Yong, 2004). When under water stress, daytime CO_2 uptake is greatly reduced by thin or thick orchid leaves. In some CAM orchids submitted to an extended water stress, the nighttime CO_2 uptake can be severely curtailed due to the closure of stomata even at night. These plants under severe drought for a long period of time may convert to a CAM-idling mode, in which organic acids fluctuate without exogenous CO_2 uptake (Fu & Hew, 1982).

In addition to succulence and water storage capacity, there are other leaf features observed by Cushman (2001) and Cushman & Borland (2002) in *Epidedrum secundum*, such as thick cuticles, large and vacuolated cells with capacity to store organic acids and reduced stomata size and frequency. The stomata of some Orchidaceae species occur only on the lower epidermis of the leaves, and occasionally they can be located within hyperstomatic chambers, as seen in *Arachnis* cv. Maggie Oei, *Aranda* cv. Deborah, *Arundina graminifolia*, *Bromheadia finlaysoniana*, *Cattleya bowringiana* X *C. forbesii* and *Spathoglottis plicata* (Orchidaceae) (Goh et al., 1977). Orchid leaf stomata may vary in shape, size and distribution (Goh et al., 1977). In addition, as reported previously by Withner et al. (1974), there are many species in which upper leaf stomata are not seen, contributing to water maintenance. It is commonly accepted that the stomatal rhythm in CAM plants is due to internal CO_2 concentration (Kluge, 1982). In CAM orchids, normally thick-leaved, stomatal opening/closing seems to be regulated by dark fixation of CO_2 in the mesophyll cells, which reduces CO_2 in the internal atmosphere, promoting stomatal opening at night (Goh et al., 1977).

The capacity of the leaves of CAM plants to accumulate acids at night has been shown to increase as they unfold, till the leaves are completely expanded reaching their maturity; however, it decreases in the senescence stage (Ranson & Thomas, 1960). Goh et al. (1984) observed that in *Arachnis* Maggie Oei there was a reduction in acidity fluctuation in young and old leaves by half when compared to mature green leaves and an even smaller fluctuation in yellow senescent leaves, ranging from 20% to 30%. It is worth noting that in some CAM plants the PEPC activity is much higher in mature leaves than in young ones (Amagasa, 1982; Lerman et al., 1974; Nishida, 1978). In full-grown leaves of *Arachnis* Maggie Oei, the stomatal rhythms as well as the CO_2 exchange pattern are consistent with CAM as shown by acidity fluctuations. The stomata open in late afternoon and acidity increases as CO_2 is absorbed. During daytime, de-acidification occurs and stomata are closed (Goh et al., 1977).

In a study of eighteen *Cymbidium* species, Motomura et al. (2008b) verified that there are different CAM intensities, ranging from weak to strong. They found that three strong CAM *Cymbidium* species have thicker leaves than other species: >1.0 mm and <0.7 mm, respectively. In contrast, weak CAM species displayed thin leaves, like C_3 species. Their studies corroborate preview data that emphasize the existence of a tendency for less negative $\delta^{13}C$ values as the leaf thickness increases, while in thinner leaves a wide range of $\delta^{13}C$ values can be found (Earnshaw et al., 1987; Silvera et al., 2005; Zotz & Ziegler 1997; Winter et al., 1983).

Moreira et al. (2009), in a comparative study regarding photosynthetic and structural features in leaves of *Dichaea cogniauxiana* and *Epidendrum secundum*, noted that diurnal titratable acidity fluctuations indicate the presence of CAM in the second species. Moreover, several morphological features in *E. secundum* leaves are typical of plants with this photosynthetic pathway, including the occurrence of a thick mesophyll with few stomata and a wide cuticle. On the other hand, in *D. cogniauxiana*, a C_3 species, there was a negleglible diurnal acidity variation and the leaves were less succulent and covered by a thin cuticle.

In order to investigate whether the major compounds which are produced during the dark $^{14}CO_2$ fixation in orchids are similar to those in other plants, Knauft & Arditti (1969) undertook a study of *Cattleya* leaves. A high dark CO_2 uptake and a prominent diurnal acidity rhythm were reported for *Cattleya* orchids, which have succulent leaves (Borriss, 1967; Nuerenbergk, 1963), while in thin-leaved orchids such as *Cymbidium*, they did not observe organic acid production from CO_2 dark fixation (Hatch, et al., 1967). They also noticed that three organic acids arose during CO_2 dark fixation: malate, citrate and a third unidentified one whose relative amounts varied based on temperature change (from 18°C to 29°C). The major acids at 18°C were malate and citrate, while at 29°C the predominant acid was malate. The unidentified acid increased from 13% to 30% with rising temperature (Knauft & Arditti, 1969).

4. Crassulacean acid metabolism in orchid pseudobulbs

Pseudobulbs are commonly described as enlarged internodes provided with a thick cuticle, epidermis devoid of stomata with gross cell walls resulting in an impervious organ, fundamental parenchyma, vascular bundles and water storage cells that determine a succulent aspect. Although devoid of stomata, the pseudobulbs have chloroplasts in part of their cells indicating, therefore, a certain capacity of photosynthetic activity (Milaneze-Gutierre & da Silva, 2004; Oliveira &, Sajo 2001). The pseudobulb is a characteristic of epiphytic orchids and secondary terrestrial orchids (Hew et al., 1998; Kozhevnikiva & Vingranova, 1999, Stancato et al., 2001; Zimmerman, 1990). This organ has been frequently studied as a storage organ capable of storing minerals (Zimmerman, 1990), water (Stancato et al., 2001; Zimmerman, 1990) and carbohydrates (Hew et al., 1998; Stancato et al., 2001; Zimmerman, 1990). Although there is considerable information about its storage function, there are few studies focusing on pseudobulb photosynthesis, especially regarding the occurrence of the CAM pathway.

Aschan & Pfanz (2003) revised stem photosynthesis, and distinguished it in four categories: two characterized by internal CO_2 refixation (wood photosynthesis and cortical bark photosynthesis) and two characterized by net photosynthesis (stem photosynthesis and CAM in stem succulent plants).

The hermetic feature of most orchid pseudobulbs do not allow them to fix carbon from the air. The only possibility is recycling the respiratory CO_2 generated by the voluminous underlying parenchyma (Ng & Hew, 2000). Hew et al. (1998) detected the presence of chlorophylls and PEPC and Ribulose-1,5-biphosphate carboxilase/oxigenase (Rubisco) activities in pseudobulbs and other non-foliar organs of *Oncidium* Goldiana and suggested that the photosynthesis in non-foliar organs was regenerative. Nevertheless, in the same study they observed that the $\delta^{13}C$ values of pseudobulbs were not characteristic of a CAM plant.

Winter et al. (1983) studied stems and leaves of 82 species from the Orchidaceae and discovered that 53 species exhibited $\delta^{13}C$ values compatible with dark CO_2 fixation. Species , possessing pseudobulbs and having $\delta^{13}C$ values of a CAM plant in leaves presented had similar $\delta^{13}C$ values in pseudobulbs as well. Curiously, the majority of pseudobulbs examined exhibit an enrichment in ^{13}C by up to 3% compared to corresponding leaves, suggesting that there is less discrimination against this isotope in these organs. This could not been explained at the time since, according to the authors, virtually nothing was known about their gas exchange. Another interesting fact was that the leafless species *Bulbophyllum minutissimum*, which have pseudobulbs with thick chlorenchyma and stomata in a depression, had $\delta^{13}C$ values characteristic of CAM. In this case, the pseudobulbs may play a considerable role in performing the CAM pathway and, therefore, contribute to the carbon acquisition in this species.

One of the most interesting studies about the role of pseudobulbs for net CO_2 uptake in light and dark period was done by Ando & Ogawa (1987). They measured CO_2 exchange of shoots of *Laelia anceps* and noted that pseudobulbs alone were apparently not able to assimilate carbon due to their absence of stomata, unlike the leaves. However, when the entire shoot was illuminated during the light period, the leaf assimilated carbon during both the light and dark periods. Notwithstanding, when the pseudobulbs were submitted to dark conditions during the light period, the leaf only assimilated carbon at night revealing a possible influence of the pseudobulbs in the light and dark CO_2 uptake by the leaf. The authors hypothesized that the organic acid, fixed during the night by the leaf, is transported to the pseudobulbs and decarboxylated in this organ during the day. This transport is up-regulated by the exposure of the pseudobulbs to light, causing a decline in the amount of organic acid in the leaf and, therefore, stimulating the net carbon uptake during the day.

The above-mentioned studies clearly raise questions about the occurrence and functionality of the CAM in pseudobulbs of orchids. In fact, current data on this issue remain remarkably scarce and more studies are needed to clarify the mechanisms of gas exchange, discrimination of carbon isotopes, types of organic acids produced or stored in pseudobulbs and translocation of those compounds between leaves and pseudobulbs.

5. Crassulacean acid metabolism in orchid roots

5.1 Structure and function of velamen on aerial roots

A typical orchid root has a cortex with chloroplasts in cortical cells and an exodermis enveloped by the velamen, a multilayered epidermis originating at the root tip. The numbers of cell layers depends on the species and growing conditions (Benzing et al., 1982; Dycus & Knudson, 1957). There is some disagreement about the importance of velamen in aerial roots. Dycus & Knudson (1957), in a study involving the species *Laelia purpurata*, *Cattleya labiata* and *Vanda* hybrid, observed no active phosphorus absorption on aerial roots even after immersion in a solution of this nutrient, except after mechanical injury of the velamen or after roots entered a solid substrate. They proposed that aerial roots which were not able to absorb the phosphorus had lost the ability to retain water and nutrients. However, more recent studies of several species proved that salts in solution can be taken and translocated by aerial roots, an interpretation supported by Benzing et al. (1982). Based on morphological and anatomical observations with the terrestrial orchid *Sobralia macrantha*, the authors proposed that velamen act as a sponge, due to the presence of an internal dead space, which can be a temporary and accessible source of water and minerals. The presence

of many mitochondria and external passage cells with well-developed membranes support the role of roots in nutrient accumulation. Between rainstorms, velamen and exodermis can act as a boundary layer that slows roots transpiration (Benzing, 1990; Benzing et al., 1983).

The velamen contains living cells on the root tip, which remain green, and dead cells, which can mask the green color of cortex when dry, reflecting light. After absorbing water, the velamen becomes transparent or translucent (Aschan & Pfanz, 2003; Dycus & Knudson, 1957; Goh et al., 1983). According to Benzing et al. (1983), velamentous roots are a basic but important adaptation of Orchidaceae to conquer an epiphytic environment.

5.2 Photosynthesis on aerial roots and presence of CAM

The capacity of green orchid aerial roots to photosynthesize is well known; however, in leafy orchids, roots have a secondary role in photosynthesis, unlike in shootless plants (Aschan & Pfanz, 2003; Benzing et al., 1983; Dycus & Knudson, 1957; Goh et al., 1983). Goh et al. (1983) observed that diurnal CO_2 exchange pattern and acidity fluctuations in the aerial roots of the CAM hybrids *Arachnis* Maggie Oei and *Aranda* Deborah were typical of CAM, but based on $^{14}CO_2$ incorporation studies, the C_3 pathway seemed to operate during the day and might represent the major pathway of CO_2 fixation. The authors concluded that these roots were not completely autotrophic and were still dependent on the leaves.

In a more recent study, Motomura et al. (2008a) identified a low degree of CAM expression in aerial roots of two *Phalaenopsis* species, but there were variations into CAM expression between aerial roots of the same plant. Different regions of aerial roots also seem to have different intensities of CAM expression, as observed by Martin et al. (2010) in 12 epiphytic orchids. They separated aerial roots in green root tips and white portions. Although 11 taxa performed CAM in leaves, three taxa performed CAM in the white portion of the roots (one of them from *Phalaenopsis* genus) and only one taxon performed CAM in the green portion. There was no correlation between the presence and intensity of CAM in leaves and roots from the same plant. They concluded that roots of the CAM species analyzed were too shaded to perform CAM, as proven by high chlorophyll amounts and low chlorophyll a/b ratios.

Apparently, the presence of CAM in roots is not related with its presence in leaves. C_3 root photosynthesis can be found in CAM orchids, but CAM in roots was not found in C_3 orchids. Moreira et al. (2009) worked with leaves and roots of the C_3 *Dichaea cogniauxiana* and the CAM *Epidendrum secundum* and observed that roots of both species had ratios of $^{13}CO_2/^{12}CO_2$ and titratable acid fluctuation typical of a C_3 plant. Gehrig et al. (1998) analyzed the photosynthetic behavior and isolated the different forms of PEPC in leaves, stem and aerial roots of the obligate CAM *Vanilla planifolia.* They demonstrated that aerial roots have low malate accumulation at night and expressed only Ppc V2, the "housekeeping" isoform of PEPC, unlike leaves and stem, which had substantial malate accumulation and expressed the Ppc V1, the isoform related to CO_2 fixation in CAM. They found that loss of CO_2 in the light was low for aerial roots, so it was proposed that during the day, respiratory CO_2 was partially refixed by C_3 photosynthesis.

5.3 Photosynthesis in aerial roots of shootless orchids

In leafy orchids, no expression of CAM or expression of weak CAM is expected in the roots, even in plants classified as CAM since the roots do not possess a mechanism to control water loss like stomata in leaves, so carbon uptake at night would not be advantageous in

terms of water conservation. Moreover, photosynthesis in roots plays only a secondary function since leaves are the main source of photoassimilates. However, this is not the case of shootless orchids, where roots assume most or full responsibility for carbon gain for the whole plant, besides mineral uptake and assimilation. Compared to roots of leafy plants, they usually have a thinner velamen layer and larger, chloroplast-containing parenchyma cells in the cortex (Benzing et al., 1983; Benzing & Ott, 1981; Winter et al., 1985).

The presence of CAM in roots appeared more common in this type of orchid compared to leafy ones, as demonstrated by Benzing & Ott (1981). In a study using 12 orchid taxa, although all leafy plants performed CAM, small quantities of CO_2 were assimilated by their roots during day and night. The only species which exhibited CAM rhythms in roots were shootless taxa and one leafy taxon with a small number of well-developed leaves. Similarly, Benzing et al. (1983) observed that, compared to leaves, roots of *Epidendrum radicans* and *Phalaenopsis amabilis* had much weaker diurnal fluctuations in titratable acid, which was not the case for the shootless orchid *Polyradicion lindenii*, which showed more intense dark acidification than roots of the two leafy plants.

In a study of the leafless orchid *Campylocentrum tyrridion*, Winter et al. (1985) observed a nocturnal increase in titratable acidity content and a daily cycle of CO_2 uptake that resembled classical CAM. Cockburn et al. (1985) found similar results in the shootless orchid *Chiloschista usneoides*. The major acid produced at night was malic acid, which indicates CO_2 uptake via PEPC. They observed that despite the absence of stomata, leakage of CO_2 was low, indicating a balance between CO_2 carboxylation and fixation, so the CO_2 concentration in roots and in the atmosphere is the same. Considering that classical CAM plants maintain the balance of CO_2 by controlling stomata aperture, the authors proposed the term astomatal CAM to this variant.

In all these cases, the functional significance of the CAM for aerial roots is not related to a water conserving mechanism but most probably to the role of CAM as a CO_2 concentrating mechanism. CO_2 uptake is limited by velamen and exodermis; therefore, by performing C_3 photosynthesis the low partial pressure of CO_2 can lead to damage of the photosynthetic apparatus in high light intensities. Another advantage of CAM could be the recycling of CO_2 produced by respiration of the plant tissues and of the endocellular fungi since this kind of association is very common in Orchidaceae (Benzing et al., 1983, Winter et al., 1985).

6. Crassulacean acid metabolism in orchid floral organs

One of the first studies on photosynthesis in orchid flowers was in 1968 by Dueker & Arditti, in which they studied the occurrence and contribution of photosynthesis in two different varieties of green *Cymbidium* flowers, the green color of which is due to chlorophyll present in the floral parts.

Even though green leaves are considered the main sources of photosynthate production, Studies support the notion that reproductive organs, such as greenish flowers, can be photosynthetically active (Dueker et al., 1968; Weiss et al., 1988). Among plants, some have green flowers, while others have photosynthetic parts associated with the inflorescence. These structures may contribute positively to total carbon gain and the energy costs of reproduction (Antlfinger & Wendel, 1997; Bazzaz et al., 1979; Marcelis & Hofman-Eijer, 1995; Reekie & Bazzaz, 1987).

The most important advantage of photosynthesizing flowers is their position relative to light. Sepals or bracts are normally shaped in the outermost layer protecting the reproductive parts throughout the floral bud stage. This provision takes full advantage of radiant light energy and may result in a higher carbon fixation, which means that photoassimilates needed for inflorescence growth can be supplemented at least in part by their own photosynthesis (Antlfinger & Wendel, 1997; Khoo et al., 1997). Despite this, it is noteworthy that these organs are one of the main plant sinks, requiring the input of photoassimilates from the leaves and/or pseudobulbs (Yong & Hew, 1995)

Previous work demonstrated that based on chlorophyll content green parts of reproductive structures have up to three times higher photosynthetic assimilatory capacity than green leaves of the same plant species (Heilmeier & Whale 1987; Luthra et al., 1983; Smillie, 1992; Werk & Ehleringer, 1983; Williams et al., 1985). Surveys of floral orchid organs demonstrated some capacity for $^{14}CO_2$ fixation in the light. These studies also indicated that the fixation rates vary among flower parts, plant varieties and stage of floral development (Antlfinger & Wendel, 1997; Dueker et al., 1968; Khoo et al., 1997). Since the amount of CO_2 fixation is highest in the sepals, lower in the petals (Dueker et al., 1968; Khoo et al., 1997) and lowest in the ovary, despite their similarity in appearance between the petals and sepals, these results suggest certain metabolic differences between them (Dueker et al., 1968).

In order to regulate floral gas exchange, stomata like those in leaves can be found in the epidermal layer of petals, but their density is normally lower than that found in leaves (He et al., 1998; Hew et al., 1980). Hew et al. (1980) observed that stomata in orchid flowers are not functional since they could not respond to light intensity, CO_2 and abscisic acid (ABA). Nevertheless, Goh in 1983 indicated the capacity for photosynthesis in orchid flowers, measuring gas exchange, acidity fluctuation and compounds synthesized when providing $^{14}CO_2$ in three succulent-leaf orchids, *Arachnis*, *Aranda*, *Dendrobium*, and one thin-leaf *Oncidium* Goldiana. They verified that orchids with succulent leaves performed night CO_2 fixation and accumulation of acidity, and radioactive malate was formed when $^{14}CO_2$ was provided characterizing CAM, while *Oncidium* Goldiana exhibited C_3 pattern. Thus, it appears that flowers of at least some succulent orchids are capable of both C_3 and CAM photosynthesis. Dueker & Arditti (1968) also observed a capacity for CO_2 fixation at night in *Cymbidium* flowers.

Given that most existing information on the CO_2 exchange and carbon gain of vascular epiphytes is on leaves, data on the carbon gain in flowers are limited (Zotz & Hietz, 2001). Several studies have characterized the responses of flowers from CAM orchids to environmental conditions such as different light irradiances (Khoo et al., 1997; He et al., 1998; He & Teo, 2007) and temperatures (He et al., 1998) through the contents of pigments, acidity and certain photosynthetic parameters, such as PSII and Fv / Fm. Nevertheless, none of these surveys have, in fact, characterized CAM metabolism in flowers. Since this is an understudied field, more attention is clearly needed to determine whether different organs of a given plant can perform CAM.

7. Conclusion

Crassulacean acid metabolism is characterized by nocturnal CO_2 fixation and organic acid accumulation in the vacuole by the decarboxylation of PEP catalyzed by the enzyme PEPC. The decarboxylation of the organic acids occurs during the day, and the CO_2 released is

reattached by the enzyme RUBISCO after the closing of the stomata. By closing stomata during most of the daytime, CAM plants exhibit greater efficiency in water use. Thus, CAM plants can inhabit semiarid and arid environments, including deserts, exposed rock outcrops and epiphytic habitats. Despite these advantages, the CAM pathway is also accompanied, however, by lower biomass productivity, higher energy expenditure and the occurence of photorespiration during phases III and IV.

The main technique used to distinguish C_3, C_4 and CAM plants is based on the quantification of stable isotopes of ^{13}C in plant leaves. However, this technique has a certain degree of variability among individuals of the same species or between plant material derived from the same plant, as in shaded leaves and leaves exposed to light of the same plant. In fact, for several orchid species, values of $\delta^{13}C‰$ typical of C_3 plants were found, despite the fact that they clearly exhibited the ability to fix some of the carbon at night, as reflected in the increased nocturnal acidity in the tissues. This illustrates why the use of only isotopic measurements to determine the type of photosynthetic metabolism tends to underestimate the number of species capable of expressing CAM photosynthesis. Therefore, other parameters, such as day/night fluctuations in titratable acidity, activity of enzymes of CAM and diurnal patterns of gas exchange, are needed to determine the photosynthetic pathway.

Other problems that researchers need to face when determining the photosynthetic pathway of orchid plants is the fact that many experiments are planned using only some parts of the plant, normally leaves, and other organs such as roots, pseudobulbs and flowers are often not included in the study analysis. Moreover, few studies give attention to the fact that some orchids have plasticity in switching between C_3 and CAM photosynthesis in response of changes in environmental conditions.

Despite the existence of technical difficulties in studying the CAM features, there is no doubt that CAM plants have sparked the curiosity of many researchers around the world for decades. Nowadays, it is well known that the majority of CAM plants have been found living in the epiphytic habitat along with many species of Orchidaceae. The proportion of CAM epiphytic orchid flora is completely associated with the degree of water availability in the ecosystems. The occurrence of CAM orchid species increases from wet tropical rainforest and moist tropical forests to dry forests, and steadily declines with increasing altitude, which is entirely related with the increase of mean annual precipitation, and, within a single site, the percentage of CAM epiphytic orchids increases with canopy height. The scarcity of water is arguably the most important and severe abiotic stress in the epiphytic habitat. The epiphytic orchids need to adjust water-balance mechanisms in all plant tissues rapidly and constantly, via appropriate stomatal and photosynthetic responses, to avoid irreversible drought injuries and maintain water storage. The drought endurance observed in the majority of epiphytic orchids is provided by a strong CAM photosynthetic behavior, which promotes a very favorable water economy.

The CAM features also appear to be linked with succulence in orchid plant tissues. The existence of a thicker chlorenchyma tissue, which was detected in some strong CAM orchids, can be important to increase the capacity of organic acid storage. Interestingly, the thickness of the chlorenchyma is not entirely associated with leaf thickness. In the majority of cases the succulence is due to the presence of a thick hydrenchyma, which does not contribute to CAM in terms of night-produced organic acid storage capacity. Therefore, there are epiphytic orchid species with thin leaves performing CAM, while some species with thick leaves display typical C_3 photosynthesis.

Most of studies on water relation and CAM photosynthesis in vascular epiphytes have only looked at the leaf tissues. According to Zotz (1999), focusing on leaves alone may lead to a skewed picture of plant functioning. Orchids have organs other than leaves that also exhibit considerable succulence (e.g. pseudobulbs) which are important reservoir structures for storing water and nutrients. The translocation of water between organs may be an important mechanism to maintain near-constant water content in leaves even during times of drought, while allowing substantial fluctuations in the water content of stems or roots, indicating that non leaf organs may have great importance and influence in the water relations and, therefore, photosynthetic activity in many orchids.

Despite the fact that the leaf is considered the main site where photosynthesis occurs, other organs are able to perform the photosynthetic functions, as long they have all essential biochemical requisites, such as chlorophyll and functional chloroplasts. In most cases, the leaf assumes a secondary function, like respiratory CO_2 refixation, and assimilates are not exported to sink organs. This carbon recycling enables the plant to reduce water loss, which is extremely important to plant survival in the case of epiphytes. The organs of epiphytic orchids also have many other adaptations which enable water economy. Leaves can be succulent and have stomata only in lower epidermis or, in some cases, inside hyperstomatic chambers, which provides a more stable environment around stomata. In roots, the existence of velamen is associated with epiphytic habitats and provides water absorption and conservation, mechanical protection and attachment to substrate. Pseudobulbs can appear in some orchid species as a variation of stem that provides drought tolerance.

The CAM expression can occur in organs like stems, roots and/or flowers. In stems or pseudobulbs, it seems to be associated with the presence of CAM in leaves. In flowers, it was proposed that both C_3 and CAM genetic information can be expressed in CAM orchids. Photosynthesis in this organ is less intense and can contribute to reproductive costs. In roots, however, no correlation was found and C_3 photosynthesis appears to be more common in roots, even in CAM species. However, when leaves are not present on the plant body, stems or pseudobulbs and/or roots might have an important and more relevant role in CAM expression since they are the unique source for photosynthetic assimilates. The lack of some plant organs, like leaves, enables more water economy and investment in reproductive structures.

It is also relevant to mention the importance of uncovering the interaction between different organs of the plant. According to Zotz & Hietz (2001), it is still premature to link the physiology of a single organ with the entire individual, or the physiology of one individual to the entire community. There are rare studies which seek integration between organs in the physiological studies, as exemplified by the work of Ando & Ogawa (1987), who analyzed the influence of light on both leaves and pseudobulbs. It is important to consider that orchid organs other than leaves (e.g. roots, pseudobulbs and flowers) might also have great importance to the survival of these plants in the dry epiphytic habitat since these organs might perform different physiological functions, including, perhaps, distinct modes of CAM photosynthesis. Certainly, a more integrated view is needed in CAM studies of vascular epiphytes to allow a better understanding of the functional importance of each organ to the whole plant.

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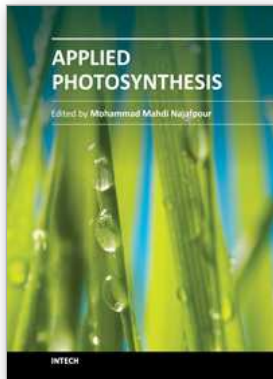
Applied Photosynthesis

Edited by Dr Mohammad Najafpour

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