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BRIEF COMMUNICATION

# Crassulacean acid metabolism in the epiphytic fern *Platycerium bifurcatum*

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## Abstract

The epiphytic fern *Platycerium bifurcatum* grows in different habitats characterized by drought and high irradiance stress. The plant shows diurnal malate oscillations, indicative for CAM expression only in cover leaves, but not in sporotrophophyll. In *P. bifurcatum* cover leaves exposed to high irradiance and desiccation, the decrease in both $CO_2$ assimilation ($P_N$) and stomatal conductance ($g_s$) was accompanied with occurrence of diurnal malate oscillations. Exogenously applied abscisic acid (ABA) induced the decrease in $P_N$ and $g_s$, but no clear change in malate oscillations. The measurements of the maximum quantum efficiency of photosystem 2 ($F_v/F_m$) under high irradiance showed distinct photoinhibition, but no clear changes in $F_v/F_m$ due to desiccation and ABA-treatment were found.

*Additional key words*: abscisic acid; chlorophyll fluorescence; drought stress; malate; net photosynthetic rate; photosystem 2; stomatal conductance.

*Platycerium bifurcatum* (the Elkhorn fern) occurs naturally in New Guinea and Australia, and it is also used as an ornamental plant. This plant belongs to epiphytic ferns (Polypodiaceae) growing in different habitats characterized by limited water availability. Many species of the genus *Platycerium* possess two classes of morphologically different leaves: sporotrophophyll and cover leaves ("niche leaves"). Sporotrophophyll leaves are mainly responsible for photosynthesis and they also produce spores. The epidermis of these leaves is covered by hairs which decrease water loss (Rut *et al.* 2003). The mesophyll cells are equipped with large vacuoles and they can accumulate water. Cover leaves contain up to 95 % of water *per* unit of fresh mass (Rut *et al.* 2001, 2002).

One of the most important adaptations of some epiphytic ferns to water deficit is Crassulacean acid metabolism (CAM) pathway. It enables $CO_2$ assimilation at increased water deficiency and high irradiance stress (Benzing 1986, Kluge *et al.* 1989). CAM plants have evolved a metabolic strategy in which nocturnal $CO_2$ uptake occurs at increased relative air humidity, when stomata open during the night. Thus CAM plants exhibit a highly specific type of $CO_2$-concentrating mechanism, allowing increase in water use efficiency. $CO_2$ enters the mesophyll leaf tissue through open stomata predominantly at night and is fixed in the reaction catalysed by phospho*enol*pyruvate carboxylase (PEPC) in the cytosol. This enzyme combines with phospho*enol*pyruvate (PEP) to form oxaloacetate (OAA). PEPC is a key control point for $CO_2$ fixation (Lüttge 1993, Nimmo 2000), and PEP (the substrate) for this reaction is derived mainly from nocturnal saccharide metabolism; the OAA resulting from PEPC activity is reduced to malic acid, which is accumulated in the vacuole during the night. Nocturnally synthesized malic acid is removed from the cytosol and transported into the vacuole. During the night-day transition the stomata close gradually, malate effluxes from the vacuole (Lüttge 1993, Black and Osmond 2003) and is decarboxylated by NAD(P)-malic enzymes [NAD(P)-ME] or *via* OAA by PEP carboxykinase (PEPCK) giving pyruvate or PEP and $CO_2$. In consequence, pronounced diurnal (day/night) changes in malate contents (Δmalate) usually occur. Variation and plasticity in the expression of this mode of photosynthesis reflects both the diverse,

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*Abbreviations*: ABA – abscisic acid; $F_v/F_m$ – the maximum quantum efficiency of photosystem 2, $g_s$ – stomatal conductance; NAD(P)-ME, NAD(P) – malic enzymes [EC 1.1.1.38(40)]; OAA – oxaloacetic acid; $P_N$ – net photosynthetic rate; PEP – phospho*enol*pyruvate; PEPC – phospho*enol*pyruvate carboxylase (EC 4.1.1.31); PEPCK – phospho*enol*pyruvate carboxykinase (EC 4.1.1.32).

polyphyletic, taxonomic incidence of CAM, and the variety of environments occupied by CAM species. CAM has been described in terrestrial, lithophytic, epiphytic, and aquatic species from 328 genera in 33 families that include 28 angiosperm families (Magnoliophyta), one family in the Gnetopsida, two families in the Filicopsida, and one family in the Lycopodiopsida (Smith and Winter 1996). In the Polypodiaceae CAM has been found in five species of *Pyrrosia*, a relatively derived genus that contains about 100 species, all are epiphytic. The CAM *Pyrrosia* species are *P. confluens* (R.Br.) Ching, *P. dielsii* (C. Chr.) Tindale, *P. lanceolata* (= *P. adnans* Forst.), *P. piloselloides* (L.) Price (= *Drymoglossum piloselloides* Presl), and *P. longifolia* Burm. Morton (Hew and Wong 1974, Wong and Hew 1976, Winter *et al.* 1983, Sinclair 1984, Ravensberg and Hennipman 1986). Nocturnal acidification has been reported also in one species of *Dictymia* J. Sm. (Griffiths 1989), a less derived polypodiaceous genus with three species all of which are epiphytic, and in the epiphytic fern *Vittaria lineata* (L.) J.E. Smith within the family Vittariaceae (Carter and Martin 1994). The expression of CAM can differ in various plant organs, and Libik *et al.* (2004) have shown that in the $C_3$-CAM intermediate plant *Mesembryanthemum crystallinum*, diurnal malate oscillations are different even within one leaf.

It is not clear why the reported incidence of CAM in tropical epiphytic ferns is so low in comparison to the epiphytic vascular plants that inhabit similar tropical habitats. Holtum and Winter (1999) emphasized that the occurrence of CAM in epiphytic ferns is more common. The knowledge concerning $CO_2$ fixation in various organs of epiphytic ferns is rather scant. This is why we tested the hypothesis that the incidence of CAM in *Platycerium bifurcatum* may lead to functional CAM. We checked also its dependency on stress conditions.

Six-week old gametophytes (prothallia) of *P. bifurcatum* were used in the experiments with haploid generation. Spores collected from sporotrophophyll leaves were sown on mesh (0.1 mm) on Petri dishes in culture medium of pH 5.5, containing 1 % agar (m/v) according to Menon and Lal (1974). Petri dishes were placed in the chamber and gametophytes (prothallia) were cultured in 12-h photoperiod at PAR of 100 µmol $m^{-2}$ $s^{-1}$ and 20±2 °C. After 6 weeks the content of malate was determined at the end of light and dark phases.

The experiments with diploid generation were performed on 3-month-old sporotrophophyll and cover leaves of *P. bifurcatum*. The ferns were grown in 12-h photoperiod at 100 µmol $m^{-2}$ $s^{-1}$ (PAR), 25±3 °C, and relative humidity (RH) of 50–60 %. We exposed experimental plants to three kinds of stress: (*1*) PAR of 300 and 400 µmol $m^{-2}$ $s^{-1}$ for 5 d; (*2*) drought stress (desiccation) induced by keeping the plants with anhydrous $CaCl_2$ until the water content was decreased by 20 and 30 % at PAR of 100 µmol $m^{-2}$ $s^{-1}$; (*3*) one set of plants was sprayed with solution of 10 µM abscisic acid (ABA) at the same PAR. The diurnal content of malate and maximal photochemical efficiency of photosystem 2, PS2 ($F_v/F_m$) were measured after 5 d of treatment in every set of plants.

A single leaf was enclosed in a chamber of an infrared gas analysis system (*CIRAS-2*; *PP Systems*, Hitchin, UK) at 25±1 ºC, RH was *ca*. 70–60 %, and airflow through the cuvette was 300 $cm^3$ $min^{-1}$. The net $CO_2$ assimilation rate ($P_N$) and stomatal conductance ($g_s$) for control and all three sets of stressed plants were measured in the middle of the photoperiod in ambient air.

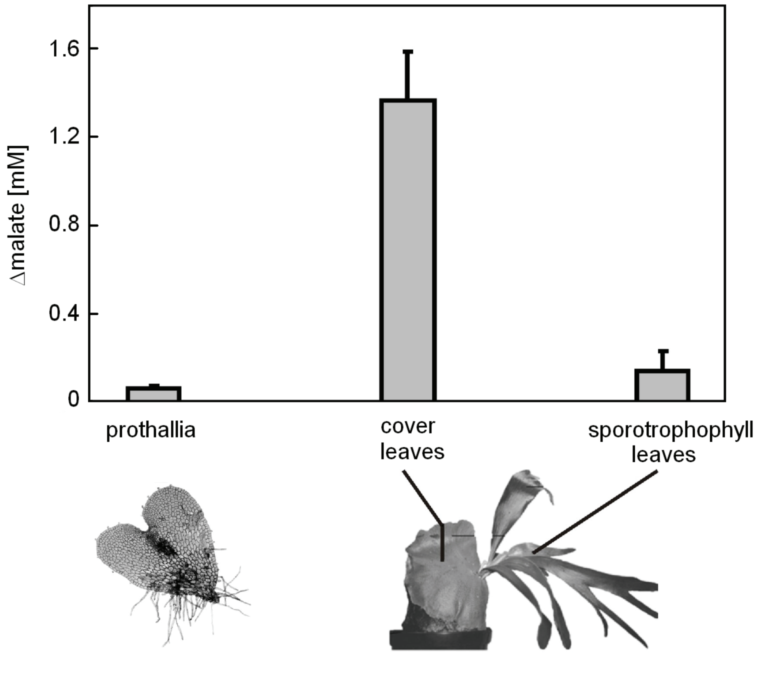

Fig. 1. Diurnal malate fluctuations (Δmalate) in gametophytes (prothallia), and cover- and sporotrophophyll-leaves of *P. bifurcatum* under control growth conditions. Means±SD (*n* = 5).

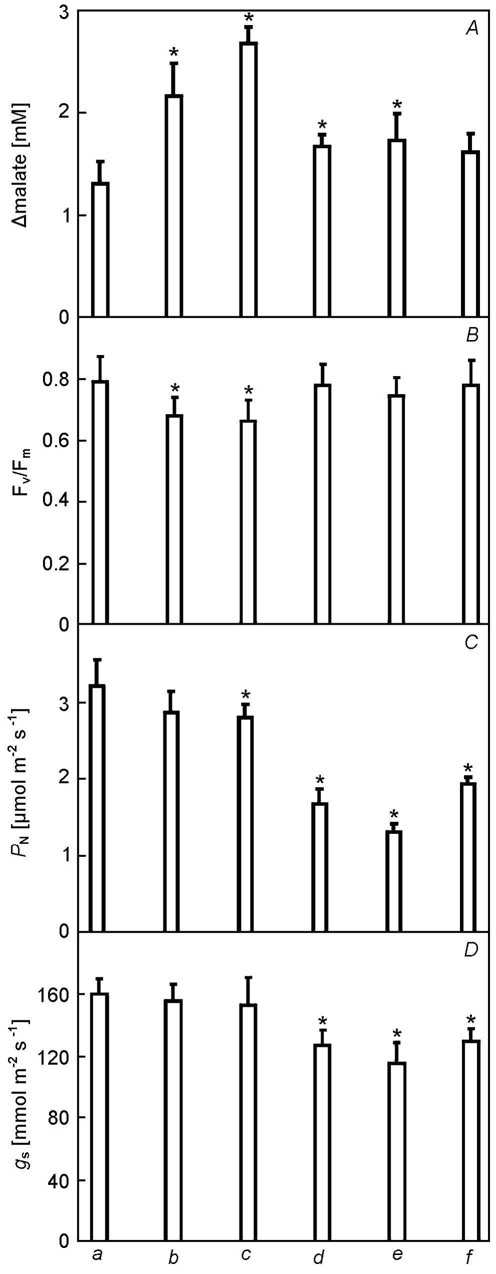

Fig. 2. The changes of (*A*) diurnal malate fluctuations (Δmalate), (*B*) maximum quantum efficiency of photosystem 2 ($F_v/F_m$), (*C*) net photosynthetic rate ($P_N$), and (*D*) stomatal conductance ($g_s$) in cover leaves of *P. bifurcatum* after 5-d growth in different conditions: *a* – control (irradiance of 100 μmol m$^{-2}$ s$^{-1}$), *b* – high irradiance of 300 μmol m$^{-2}$ s$^{-1}$, *c* – high irradiance of 400 μmol m$^{-2}$ s$^{-1}$, *d* – 20 % desiccation, *e* – 30 % desiccation, *f* – ABA-treated leaves (10 μM). Means±SD ($n$ = 5). For experiments with desiccation and ABA, irradiance was as in the control. The asterisks (*) above bars indicate significance of difference from control plants at $p<0.05$.

Chlorophyll *a* fluorescence measurements from photosystem 2 (PS2) were made using a *PSM* (Plant Stress Meter; *BioMonitor AB*, Umeå, Sweden) at 25 °C. Prior to the measurements the leaves were dark adapted for 30 min and the performed measurements allowed for determination of the maximum quantum yield of PS2 ($F_v/F_m$).

The mode of photosynthetic carbon assimilation pathway was checked by determination of the night/day differences of malate concentrations [mM] in the cell sap (Δmalate = $[\text{malate}]_{\text{night}} - [\text{malate}]_{\text{day}}$), as described by Möllering (1985). For isolation of the cell sap, leaf slices of *P. bifurcatum* were frozen at –4 ºC, thawed, and centrifuged for 1 min at 12 000×*g*.

In both gametophytes and sporotrophophyll leaves the diurnal malate oscillations were very small reaching values below 200 μM (Fig. 1). In cover leaves the day/night malate differences were *ca.* 14-fold higher (Fig. 1). Hence in the tested samples of *P. bifurcatum* only in cover leaves the CAM pathway can be expected, induced or enhanced in stress condition (Cushman and Borland 2002). Significantly higher diurnal malate oscillations were observed in cover leaves exposed to both high irradiance and drought, while ABA-treated cover leaves showed no significant increase in diurnal malate oscillations (Fig. 2*A*). No significant differences were observed for drought-stressed and ABA-treated sporotrophophyll leaves (data not shown). In the epiphytic fern *Pyrrosia piloselloides* only sporophytes showed CAM expression in terms of diurnal oscillations in titratable acidity of the tissues (Ong *et al.* 1997).

In the same conditions as described above we determined $P_N$ and $g_s$ for cover leaves. The high irradiance used inhibited both $CO_2$ assimilation and maximal photochemical efficiency of PS2 (Fig. 2*B*,*C*), but it did not affect $g_s$ (Fig. 2*D*). In contrast to that drought stress induced by desiccation and ABA-treatment strongly decreased both $P_N$ and $g_s$ (Fig. 2*C*,*D*), but not $F_v/F_m$ (Fig. 2*B*). Abiotic stress factors such as high irradiance and desiccation may induce the decrease in both $P_N$ and $g_s$ in *P. bifurcatum* cover leaves (Figs. 1 and 2*C*,*D*). Moreover, ABA-induced decrease in $CO_2$ assimilation and stomatal conductance might point out to the involvement of ABA in CAM expression, as it was previously described for transition from $C_3$ to CAM in the $C_3$-CAM intermediate plant *M. crystallinum* (Chu *et al.* 1990). These results taken together support the idea that "CAM-like" pathway might be accelerated in stress situations. In *M. crystallinum* CAM was induced also by salinity and irradiance that exceeded a threshold level (Miszalski *et al.* 2001, Broetto *et al.* 2002, Ślesak *et al.* 2002).

In our experiments the drop of $F_v/F_m$ under high irradiance (Fig. 2*B*) indicates photoinhibition of PS2 (for review see Maxwell and Johnson 2000) and this correlates with CAM indication. In addition, only small trend to decrease of $F_v/F_m$ value at desiccation and ABA-treatment was accompanied by minor tendency to induce CAM symptoms. The decrease in both $P_N$ and $g_s$ during strong irradiation, together with the increase of Δmalate in desiccated plants gave an additional support that some features of CAM might be expressed in *P. bifurcatum* cover leaves when exposed to such stress (Figs. 1 and 2*C,D*). According to our knowledge we present for the first time that *P. bifurcatum* can perform CAM. In previous experiments Holtum and Winter (1999) described small nocturnal fluctuations in titratable acidity, indicating "weak CAM" in litophyte fern *Platycerium veitchii*, but in another fern species *Platycerium grande* CAM mode of photosynthesis was not found (Kluge *et al.* 1989). Also in our experiments CAM was not found in sporotrophophyll leaves. Probably in *P. bifurcatum* in cover leaves we observed so called "weak CAM" or "CAM cycling", where $C_3$ gas exchange pattern was concomitant with diel fluctuations in organic acids, but with little or no net nocturnal carbon assimilation. Plants that perform "weak CAM" grow in habitats with unpredictable daily water supply, and may derive benefits to enter full CAM rapidly when they are in stress environment (Cushman and Bohnert 1999). We also state that desiccation stress despite of causing both significant drops of $g_s$ and $P_N$ did not give a strong signal for CAM development. Similar situation was observed when plants were exposed to exogenously applied ABA (Fig. 2*C,D*). Thus, we suggest that signals for CAM expression operate parallel to photoinhibition. Moreover, our results suggest that in natural habitats the CAM pathway may be realized in *P. bifurcatum* in response to various environmental stresses, and at the same time $C_3$ metabolism occurs in other organs. Such phenomenon might be responsible for stable $CO_2$ fixation, independent of environment fluctuations.

In conclusion, the diurnal malate oscillations reported here for the epiphytic fern *P. bifurcatum* are further evidence for the presence of ancient lineages of CAM that have been reported for higher plants, although the functional CAM is small and is restricted to cover leaves which can accumulate water, as opposed to typically xeromorphic sporotrophophyll leaves. Moreover, the capacity for performing CAM may be important under water shortage, and may be more common among vascular epiphytes than currently considered (Holtum and Winter 1999).

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