

Light Stress Is Not Effective to Enhanced Crassulacean Acid Metabolism

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Clusia minor L., a C₃-CAM intermediate, and *Clusia multiflora* H. B. K., a C₃ obligate, present two phenotypes of a similar morphotype occurring sympatrically in the field. Both species, exposed 2 days to high light, show similar responses to this kind of stress: (i) the level of xanthophyll pigments in tested plants during the daycourse adapts to stress, (ii) the levels of antheraxanthin and zeaxanthin clearly increase during the afternoon showing increased de-epoxidation, (iii) the changes in the xanthophyll cycle are similar. Exposure to high light increases the malate levels in *C. minor* during the afternoon while decreases the day/night changes of the malate levels, and hence the Crassulacean Acid Metabolism (CAM) expression. It can be concluded that strong light applied as a single stress factor to well-watered plants is not effective in strengthening the CAM metabolism in a C₃-CAM intermediate plant but rather suppresses the CAM activity despite exposure to high light energy. It is suggested that, when water supply is not limiting and other stresses do not prevail, C₃ allows to use up the citrate pool, especially in the afternoon and enables a superior daily photon utilization.

Key words: Oxidative Stress, Xanthophyll Cycle, *Clusia*

Introduction

In plants several reactive oxygen species (ROS) such as: O₂^{•-}, HO[•], H₂O₂, are generated predominantly during the light period in chloroplasts and can cause photooxidative damage (Foyer and Noctor, 2003). ROS affect the redox status of the cells and subsequently have an impact on the regulation of many cellular processes (Inzé and van Montagu, 1995; Bartosz, 1997; Halliwell and Gutteridge, 1999; Mittler, 2002). There are several systems to protect cells from an excess of ROS compounds. It was also shown that due to stress conditions ROS levels increase and plants adapt their levels of low molecular antioxidants (glutathione, ascorbic acid, carotenoids) and the activities of antioxidative enzymes (superoxide dismutase,

catalase, peroxidase, glutathione reductase) (Scandalios, 1993; Adams and Demmig-Adams, 1994; Dat *et al.*, 2000). Carotenoids localized in chloroplasts serve as light-harvesting pigments and also play an important role in protecting the photosynthetic apparatus against photooxidative damage (Siefermann-Harms, 1987). To the most important carotenoids belong: β -carotene and the xanthophylls [neoxanthin, lutein, violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z)]. V, A and Z are pigments participating in the xanthophyll cycle, the reversible cyclic reactions dissipating excess light energy as heat. In plants equipped with the xanthophyll cycle during a change from low light (that does not saturate photosynthesis) to excess light (when more light is absorbed than it can be utilized by photosynthesis), V is reversibly converted to Z. Z constitutes part of the xanthophyll cycle where reversible cyclic reactions dissipate excess light energy as heat. A correlation between the conversion state of the xanthophyll cycle pigments and the level of thermal en-

Abbreviations: A, antheraxanthin; EPS, epoxidation state of the xanthophyll cycle pigments; PPFD, photosynthetic photon flux density; ROS, reactive oxygen species; V, violaxanthin; Z, zeaxanthin.

ergy dissipation was observed in a wide range of different plant species for a given degree of light stress independently of species (Demmig-Adams and Adams, 1996). The degree to which V is de-epoxidized varies widely as a result of variation in the level of V + A + Z as well as the degree of their interconversion (Adams and Demmig-Adams, 1992). However, non-photochemical quenching (NPQ) does not correlate with the amount of Z (Johnson *et al.*, 2008) indicating that control of the xanthophyll cycle pool size is not necessary to optimize the kinetics of NPQ.

In CAM (Crassulacean acid metabolism) plants carbon fixed during the night can be accumulated as malate, citrate or isocitrate and their decarboxylation during the following day may help alleviating photoinhibition (Franco *et al.*, 1992; Borland *et al.*, 1996; Chen *et al.*, 2002). Changes in the mode of photosynthetic metabolism are accompanied by differences in regulation of stomatal opening.

In some plants during C₃-CAM transitions strong changes in the antioxidant system do occur (Castillo, 1996; Miszalski *et al.*, 1998; Niewiadomska *et al.*, 1999; Slesak *et al.*, 2002). However, we do not know the exact mechanism of metabolic changes. Plants performing CAM show very strong diurnal rhythms in the activity of some antioxidants (Niewiadomska *et al.*, 1999, 2004; Slesak *et al.*, 2002; Niewiadomska and Borland, 2008; Kornas *et al.*, 2009). We know that free radicals are involved in CAM induction, but it is not clear whether they are responsible for CAM induction *per se* and/or diurnal CAM regulation. We know that in *Mesembryanthemum crystallinum*, a C₃-CAM intermediate plant, light intensity above some threshold is needed for CAM induction (Misalski *et al.*, 2001), and according to Broetto *et al.* (2002) strong light alone can induce CAM. However, according to other publications light even at the level of 1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ does not result in metabolic transformation (Barker *et al.*, 2004).

In the family of Clusiaceae there are several C₃-CAM intermediate species (Herzog *et al.*, 1999; Lüttge, 2000, 2004). Among the C₃-CAM plants *Clusia minor* is the best studied species which can switch very rapidly (within 1 to 2 days) and reversibly from C₃ photosynthesis to CAM in response to temperature, high light, and drought stress (Borland *et al.*, 1996; Herzog *et al.*, 1999; Mattos *et al.*, 1999; Dodd *et al.*, 2002). As in other

experiments (Herzog *et al.*, 1999; Lüttge, 1999, 2000, 2007) for comparisons aiming to illuminate the functioning of CAM, the putative obligate C₃ plant *C. multiflora* was used. Both species are capable of rapid accumulation of Z for acute photo-protection following high light exposure (Herzog *et al.*, 1999), however, other pigments of the violaxanthin cycle were not analyzed.

Firstly, in the present study it should be found out whether an increased light level can affect CAM – day/night fluctuations. Secondly, does light stress cause similar changes in the xanthophyll cycle activity of different plant species representing different modes of photosynthetic metabolism, *i.e.* CAM and C₃, respectively?

Material and Methods

Plants

Experimental plants of *C. minor* L. and *C. multiflora* H. B. K. originated from cuttings of the *Clusia* plants collection of Botanical Garden of Darmstadt University of Technology (TUD), Germany. After a 2-month rooting period plants were repotted in plastic containers with a defined amount of soil (Fruhsdorfer Einheitserde LD 80, 0.23 l per plant) and were grown under a natural photoperiod at low light intensity [PPFD (photosynthetic photon flux density) = 50–100 $\mu\text{mol m}^{-2}\text{s}^{-1}$; λ = 400–700 nm] in November. Cuttings for propagation were made about 6 months prior to experiments. Thereafter plants were transferred to a semi-controlled environment cabinet in the phytotron of the TUD and exposed to the following conditions: about 70% RH (relative humidity), day/night temperatures at 26 °C/18 °C, and a 12 h/12 h photoperiod (intermediate PPFD of 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at the plane of leaves). Plants were watered and fertilized regularly (Hakaphos mineral nutrients, Compo, Münster, Germany). After 8 d of adaptation all parameters were measured as the control (day 0) and thereafter plants were exposed for 2 d to high light (HL) (PPFD = 650–740 $\mu\text{mol m}^{-2}\text{s}^{-1}$) for 12 h per day. All measurements were performed during the 2nd day of stress application (3rd day of experiments). The irradiance was measured using a LiCor quantum sensor (model Li-190SB, Campbell Scientific, Logan, UT, USA)

Stomatal aperture

The stomatal resistance was measured using a Delta-T Devices Porometer MK III (Burwell, Cambridge, UK), which was calibrated according to the manufacturer's instructions.

Malate and citrate levels

Prior to the cell sap collection leaf discs were heated for 3 min at 100 °C. Malate and citrate levels were determined in cell sap obtained after 3 min of centrifugation (at 12000 x g) of homogenized leaf discs. For determination of the malate level, malate dehydrogenase and glutamate oxaloacetate transaminase were used according to Möllering (1974). For determination of the citrate level, citrate lyase, malate dehydrogenase and lactate dehydrogenase were used according to Möllering (1985).

Composition of carotenoids and EPS value calculations

The carotenoid composition of leaves was determined by HPLC. Two discs (0.5 cm i.d.) were cut out of the leaves and were homogenized in 2 ml of solvent A (acetonitrile/methanol/water, 72:8:1) in a mortar under dim light. The extract was transferred to an Eppendorf tube, centrifuged at 10000 x g for 5 min, and the supernatant was directly injected in a HPLC apparatus [C18 reverse-phase column, 250 x 4 mm, 5 µm (Tracer Analytica, Barcelona, Spain)]. All samples were run isocratically in solvent A at the flow rate of 1 ml/min for 15 min, to separate the xanthophylls. The solvent was changed afterwards to solvent B (methanol/ethyl acetate, 34:16) and a flow rate of 2 ml/min for 35 min to elute chlorophylls and β-carotene. The detection wavelength was 440 nm and the injection volume 100 µl. The relative contents of the carotenoids were calculated from the area of the corresponding peaks. The EPS value (epoxidation state of the xanthophyll cycle pigments), $(V + 0.5 A)/(V + A + Z)$, where V, A and Z are the contents of the corresponding compounds, was calculated according to Thayer and Björkman (1990).

Statistics

Errors are given as standard deviations (SD). Significance of differences between treatments was determined with T-test.

Results

The shape of the daily course of stomatal resistance measured during the 2nd day of exposure to high PPFD for *C. multiflora* (obligate C₃ plant) and *C. minor* (C₃-CAM intermediate plant) was similar from 16:00 to midnight (Fig. 1A). Measurements of V, A and Z contents in both tested species cultivated at low light (day 0) showed no distinct daily fluctuations (before noon and afternoon hours) in both C₃ and C₃-CAM intermediate plants (Fig. 2). In both *Clusia* plants during the 2nd day of exposure to HL also no clear changes in the amount of V were visible (Fig. 2A), while the contents of A and Z increased during the afternoon (16:00–20:00); however, due to the large SD, only for *C. minor* the differences are statisti-

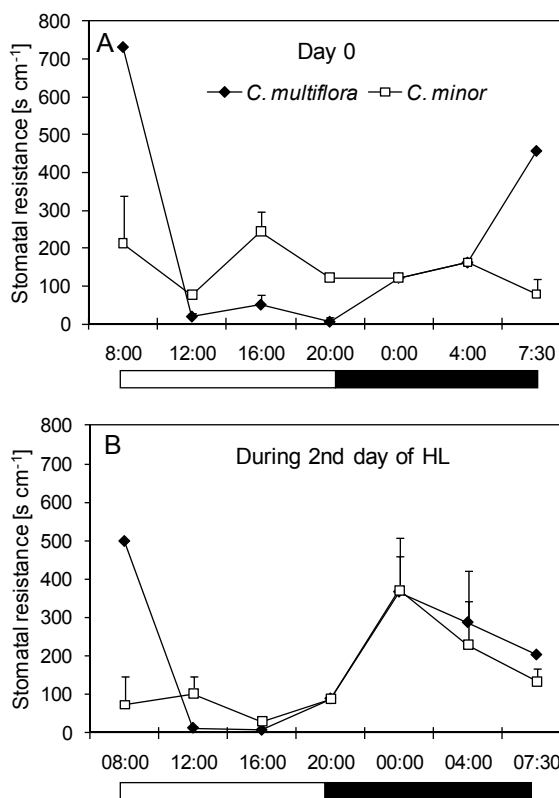


Fig. 1. Daily time course of the stomatal resistance in leaves of *Clusia minor* and *Clusia multiflora* (A) growing at low light (day 0) and (B) during the 2nd day of stress application of high light (HL, 650–740 µmol m⁻² s⁻¹) under well-watered conditions. Data represent mean values ± SD (*n* = 10). Open and closed bars represent light and dark periods, respectively.

cally significant ($p = 0.01$) (Fig. 2B and C). The EPS values were comparable during all periods in both tested plants (Fig. 3). The malate level in *C. minor* was higher than in *C. multiflora*. Its decrease in *C. minor* in the afternoon on day 0 was statistically highly significant ($p = 0.001$) indicating performance of CAM in the absence of light

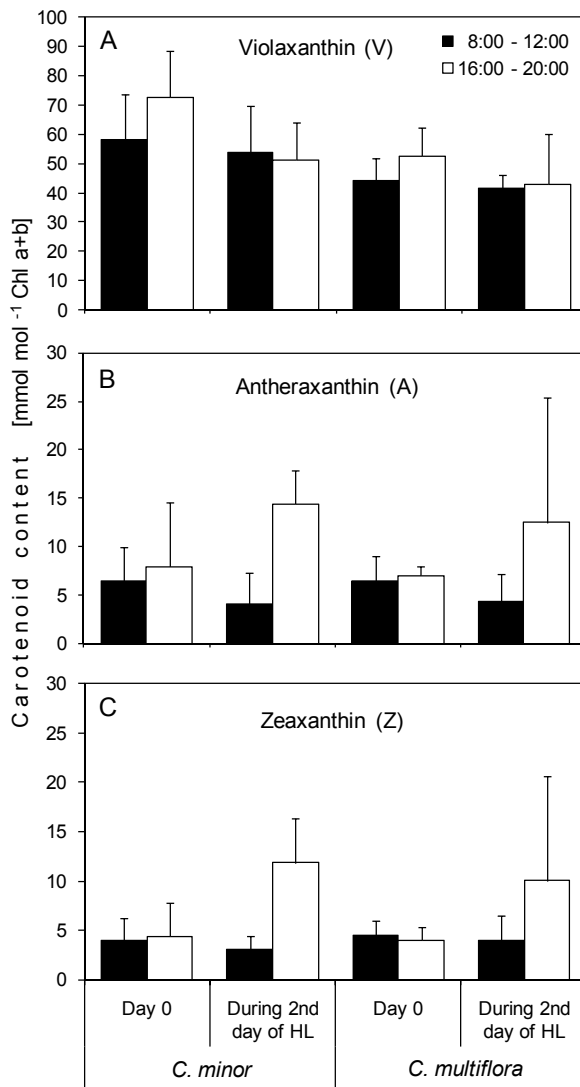


Fig. 2. Daily time course of the levels of the xanthophyll cycle pigments: (A) violaxanthin, (B) antheraxanthin, and (C) zeaxanthin in leaves of *Clusia minor* and *Clusia multiflora* growing at low light (day 0) and during the 2nd day of stress application of high light (HL, 650–740 $\mu\text{mol m}^{-2} \text{s}^{-1}$) under well-watered conditions. Data represent mean values \pm SD ($n = 3$).

stress. On the 2nd day of HL stress the decline of the malate level was not insignificant anymore ($p = 0.02$). In both species only small significant fluctuations in the citrate levels, when growing at low light (day 0) and during the 2nd day of exposure to HL, were observed. In *C. multiflora* on the 2nd day of HL stress the decrease of citrate pool was stated during the morning ($p = 0.02$) and afternoon ($p = 0.006$) hours, when compared to day 0 (Fig. 4).

Discussion

In our experiments we applied HL stress (approx. 650–740 $\mu\text{mol m}^{-2} \text{s}^{-1}$), without causing additional stresses like drought, and kept the plants well watered. During the 2nd day of HL stress in *C. minor* (C_3 -CAM intermediate) a reduction of stomatal resistance, when measured during afternoon hours, was observed as compared to day 0 (Fig. 1). This could be an indication that CAM was not induced (which would lead to an increase in resistance). In both *C. minor* and *C. multiflora* (C_3 obligate) exposed to HL a strong increase in stomatal resistance during the night was observed, thus their sensitivity was similar. This can be regarded as another indication that HL was

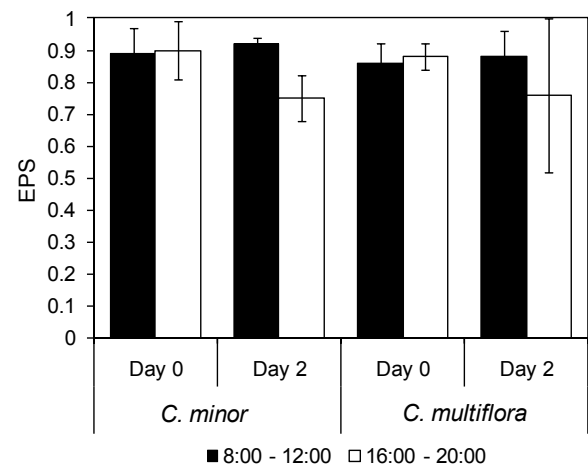


Fig. 3. Daily time course of the level of epoxidation state (EPS) $(V + 0.5 A)/(V + A + Z)$ of the xanthophyll cycle pigments in leaves of *Clusia minor* and *Clusia multiflora* growing at low light (day 0) and during the 2nd day of stress application of high light (HL, 650–740 $\mu\text{mol m}^{-2} \text{s}^{-1}$) under well-watered conditions. Data represent mean values \pm SD ($n = 3$).

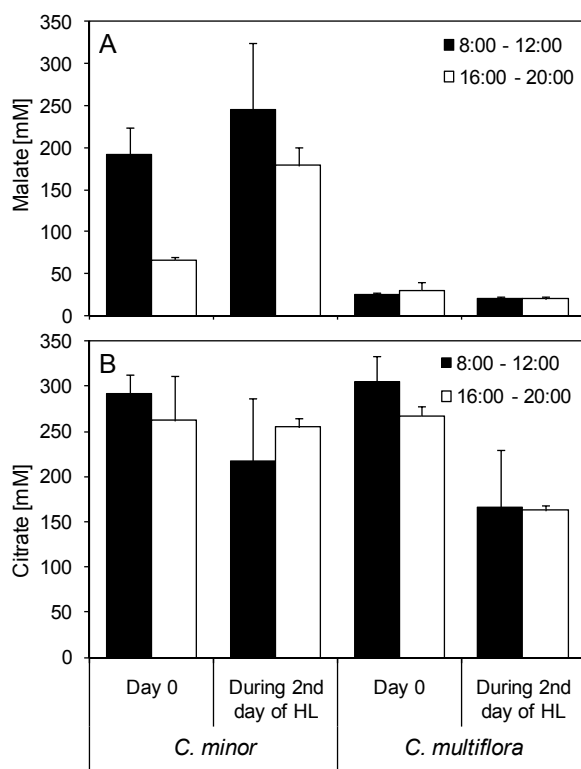


Fig. 4. Daily time course of (A) malate and (B) citrate levels in extracts of leaves of *Clusia minor* and *Clusia multiflora* growing at low light (day 0) and during the 2nd day of stress application of high light (HL, $650\text{--}740\ \mu\text{mol m}^{-2}\text{s}^{-1}$) under well-watered conditions. Data represent mean values \pm SD ($n = 3$).

not effective in CAM induction in *C. minor*, and this paralleled *C. multiflora*.

It is suggested that CAM provides strong protection from photoinhibition during periods of high irradiance (Adams and Osmond, 1988). Some CAM-performing plants are well adapted to strong light (Osmond *et al.*, 1979), and in some CAM plants changes in xanthophyll cycle capacity due to excess light stress have been reported (Lüttge, 2000). In our experiments similar levels of constituents of the xanthophyll cycle were observed in both species (Fig. 2). During the 2nd day of HL treatment independent of the type of carbon metabolism the increases in A and Z levels in the afternoon were 2.5–3.8 times the levels observed before noon (8:00–12:00). Thus, the levels of these pigments in the tested plants during the day course responded to stress. While

the levels of V (double epoxide) did not change significantly during the daily time course the increases of A (single epoxide) and Z (no epoxide), which were significant in *C. minor*, indicated de-epoxidation in the afternoon under HL in both species, *i.e.* increased xanthophyll cycle turnover. The changes of A and Z levels are not seen in V, because the absolute levels of V are larger and the changes of A and Z levels lie within the range of SD of the V level. The small tendency of decrease of the V level on the 2nd day parallel to a significant increase of the A + Z level can be explained by changes in the total pool of pigments. Thus, HL applied in our experiments was effective in xanthophyll cycle regulation, and low light used before (day 0) was not stressful for both plants.

In our earlier experiments on *Clusia* plants we were able to show that HL and drought stress induce a significant lowering of the EPS value (Miszalski *et al.*, 2007). The EPS values measured in the present work in both tested plants were comparable before noon and during the afternoon. A decrease in EPS is a characteristic feature for plants exposed to very strong light (Adams and Demmig-Adams, 1992), but HL applied in our measurements (approx. $650\text{--}740\ \mu\text{mol m}^{-2}\text{s}^{-1}$) did not affect this parameter. The EPS value (equation in Materials and Methods and Fig. 3) is strongly determined by V and thus in the present study does not bear out the more subtle changes in A and Z levels (Fig. 2). In conclusion we can say that very fast changes and reorganization of PSII as shown by Johnson *et al.* (2008) and Horton *et al.* (2005) are not directly connected to CAM-related changes.

CAM plants are able to accumulate high amounts of acids during the night. In CAM-performing *C. minor* plants these fluctuations according to Lüttge (1999) can reach about 200 mM. When comparing malate and citrate fluctuations in our experiments we can state that applied light stress did not increase CAM-characteristic changes (Fig. 4). On the contrary, CAM-characteristic daytime reduction of malate levels observed on day 0 with no HL stress was almost entirely lost (not statistically significant anymore) on day 2 of HL stress. No day/night fluctuations of malate levels, neither at low light nor due to strong light exposure, were observed in *C. multiflora*. The citrate levels in both species were somehow higher in comparison to the malate levels. Two days of HL stress application caused a decrease of the

citrate levels in both *C. multiflora* (statistically significant) and *C. minor* (not statistically significant), however, without daily fluctuations. All of this indicates that HL stress was not effective in enhancing CAM in *C. minor* and by contrast even suppressed the CAM activity in the well-watered plants studied, but we can speculate that the used supply of citrate helps to alleviate HL stress.

In addition to this, it is worth to note that day/night changes in the malate and citrate levels are not parallel. This observation can be compared with results obtained with two obligate CAM species, *C. alata* and *C. hiliariana*. In these plants, exposed to stress conditions, citrate is decarboxylated during the day later than malate (Kornas *et al.*, 2009). As shown by Lüttge (2007) it can be expected that decarboxylation of malate and citrate are controlled separately. There also was a small tendency of absolute levels of malate in *C. minor* to increase under HL stress (Fig. 4). Thus, we could speculate that light stress stimulates higher malate levels, but no CAM behaviour. A similar tendency was observed in experiments on *Mesembryanthemum crystallinum* when exposed to a single oxidative stress factor applied as either ozone, SO₂, or HL (Niewiadomska *et al.*, 2002; Surówka *et al.*, 2007).

In some experiments with *M. crystallinum* light at the level 600–690 $\mu\text{mol m}^{-2}\text{s}^{-1}$ induced C₃-CAM transformation, but in other experiments strong sunlight (1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$) was not effective in CAM induction (Barker *et al.*, 2004). These results are in accordance with our earlier suggestions pointing out that HL stress as a single stress factor can not be responsible for CAM induction, and that, as indicated by Niewiadomska and Borland (2008), several stress factors are necessary for effective C₃-CAM transformation. A similar conclusion can be also drawn from earlier studies on *C. minor* (Haag-Kerwer *et al.*, 1992). Lüttge (1999) also indicated that *C. minor* growing at strong light can be found performing C₃ metabolism. Moreover, the HL-stress-suppressed CAM activity in the well-watered plants of *C. minor* studied here is consistent with earlier observations where nocturnal CO₂ fixation in *C. minor* plants, also well supplied with water, was completely lost when leaves were transferred from 360 to 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPFD (Schmitt *et al.*, 1988, note that in this publication the plant studied is wrongly named *C. rosea* rather than *C. minor*).

Both, C₃ *C. multiflora* and C₃-CAM *C. minor* are fully capable of using the xanthophyll cycle for photoprotection. This is now corroborated by the present analysis of A and V extending the observations of Herzog *et al.* (1999) where only Z could be measured. In addition to the xanthophyll cycle *C. minor* can use CAM for photoprotection but obviously does not necessarily need to use it when it is well watered.

We could speculate that in the field it can happen very often that some leaves even in the same crown are exposed to strong light while others are growing in the shade. From an ecological point of view it is easy to understand that light stress alone is not able to induce the very complicated change in the metabolism from C₃ to CAM, and plants adapt to this stress using violaxanthin cycle pigment enabling fast, efficient and reversible response. In fact, a study of the transition of *C. minor* from CAM to C₃, when water was not limiting and other stresses did not prevail, showed that C₃ photosynthesis implies a superior daily photon utilization (Mattos and Lüttge, 2001). Thus, it can be expected that CAM in comparison to C₃ should not be induced „that readily“. These suggestions also agree with ecophysiological observations reporting that CAM may not be superior to C₃ photosynthesis under stressful conditions of high insolation and reduced water supply, as both species occur sympatrically in a light-exposed savanna (Lüttge, 1999). We can suspect that even in some experiments, where strong light was described as single stress factor, this was accompanied by additional stress factors: heat, stomatal closure, low CO₂ amount. Thus, HL (above some threshold) would be necessary, but as a single factor is not effective in causing metabolic transformation.

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