BIOLOGIA PLANTARUM 51 (1): 86-92, 2007

Superoxide dismutase activity in C_3 and C_3/CAM intermediate species of *Clusia*

Z. MISZALSKI[1,2]*, A. KORNAS[1], K. GAWRONSKA[1], I. ŚLESAK[2], E. NIEWIADOMSKA[2], J. KRUK[3], A.L. CHRISTIAN[4], E. FISCHER-SCHLIEBS[4], R. KRISCH[4] and U. LÜTTGE[4]

Institute of Biology, Pedagogical University, ul. Podbrzezie 3, PL-31054 Krakow, Poland[1]
Institute of Plant Physiology, Polish Academy of Sciences, PL-30239 Krakow, Poland[2]
Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, PL-30387 Krakow, Poland[3]
Institute of Botany, Darmstadt University of Technology, D-64287 Darmstadt, Germany[4]

Abstract

The C_3-CAM intermediate *Clusia minor* L. and the C_3 obligate *Clusia multiflora* H.B.K. plants were exposed for 7 d to a combination of drought stress and high irradiance of about 1200 μmol m^{-2} s^{-1} for 12 h per day. In both species under these conditions a strong decrease in stomatal conductance was observed at dawn and dusk. Changes in stomatal behaviour of *C. minor* were accompanied by only a low nocturnal accumulation of malate and citrate. Thus, in *C. minor* drought stress applied in combination with high irradiance limited CAM expression, and possibly this is the main reason why *C. minor* prefers semi-shaded sites in the field. The mitochondrial MnSOD, in both well watered and stressed plants of two species showed strong diurnal oscillations with maximum activity at dusk. These oscillations can be explained by the engagement of mitochondria in dissipation of an excess of reducing equivalents. In plants which are able to carry out CAM metabolism tricarboxylic acid cycle is expected to be down regulated in the dark period to prevent breakdown of the entire malate and citrate.

Additional key words: Crassulacean acid metabolism, oxidative stress, stomata.

Introduction

The most important sources of reactive oxygen species (ROS) are mitochondria, chloroplasts, peroxisomes, and the cytosol (*e.g.* Inzé and Van Montagu 1995, Bartosz 1997, Halliwell and Gutteridge 1999, Mittler 2002). Plants exposed to oxidative stress increase the content of low molecular antioxidants (glutathione, ascorbic acid) and the activity of antioxidative enzymes, *i.e.* superoxide dismutases (CuZnSOD, MnSOD, FeSOD), catalase, ascorbate peroxidase, and glutathione reductase (Scandalios 1993, Dat *et al.* 2000, Hernández *et al*. 2006).

Plants performing Crassulacean acid metabolism (CAM) are resistant to many environmental stresses leading to oxidative stress (Nyman *et al.* 1990, Olszyk *et al.* 1987). It was also shown that CAM plants have a strong diurnal rhythm in activity of some antioxidative enzymes such as , catalase, and MnSOD (Niewiadomska *et al.* 1999, 2004, Ślesak *et al.* 2002). Furthermore, in CAM plants much higher diurnal fluctuations in the activity of the photorespiratory pathway have been observed in comparison to C_3 plants (Maxwell *et al.* 1999, Niewiadomska *et al.* 1999). Moreover, in *Mesembryanthemum crystallinum* performing CAM, in comparison to C_3 plants, only a very low activity of fumarase, a mitochondrial marker enzyme (Miszalski *et al*. 2001), and a very high ATP/ADP ratio were detected during the night (Niewiadomska *et al.* 2004). The diurnal regulation of respiration in CAM plants is not known in details. Signals involved in controlling the activity

Received 31 March 2005, *accepted* 6 June 2006.
Abbreviations: A - antheraxanthin; BSA - bovine serum albumin; COX - cytochrome *c* oxidase; CuZnSOD - CuZn-superoxide dismutase; DTT - dithiothreitol; EPS - epoxidation state of the xanthophyll cycle pigments; g_s - stomatal conductance; H_2EDTA - ethylenediaminetetraacetic acid; HEPES - *N*-(2-hydroxyethyl)piperazine-*N*'-(ethanesulphonic acid); MnSOD - Mn-superoxide dismutase; PAGE - polyacrylamide gel electrophoresis; PEG - polyethylene glycol; PEPC - phospho*enol*pyruvate carboxylase; PFD - photon flux density; PVPP - polyvinylpolypyrrolidone; ROS - reactive oxygen species; TCA cycle - tricarboxylic acid cycle; V - violaxanthin; Z - zeaxanthin.
Acknowledgements: This work was supported by the Deutsche Forschungsanstalt für Luft- und Raumfahrt (DLR), the Volkswagenstiftung and the Polish Comittee for Scientific Research (grant number 3P04C 064 23). We thank Silvia Haase for expert technical assistance in immunological experiments.
* Corresponding author; fax: (+48) 12 4251844, e-mail: miszalski@ifr-pan.krakow.pl

of the mitochondrial MnSOD, an enzyme heavily involved in removing ROS produced in the electron transport chain in mitochondria, have not been identified up to now.

Among C_3-CAM intermediate plants, *Clusia minor* is a species that has been studied deeply in the past. *C. minor* can shift rapidly and reversibly from C_3 photosynthesis to CAM in response to low temperature, high radiation, and drought stress (Borland *et al*. 1993, 1996, De Mattos *et al.* 1999, Herzog *et al.* 1999, Dodd *et al.* 2002). The high metabolic flexibility of *C. minor* supports rapid responses to changing radiation. In the present work, a comparison of SOD activities was undertaken, in different diurnal phases of photosynthesis in *C. minor* growing under high irradiance in well watered conditions or exposed to drought. Assessment of SODs focused on mitochondrial MnSOD activity. In addition, we determined the epoxidation state (EPS) of the xanthophyll cycle pigments as a measure of photon efficiency of photosynthesis (Adams and Demmig-Adams 1992). As a control we used *Clusia multiflora,* an obligate C_3 plant. *C. minor* and *C. multiflora* frequently occurring sympatrically in the field, and they have been subjected to comparative studies in the past (Herzog *et al.* 1999, Lüttge 1999, 2000, 2003). No information is available on the activity of their antioxidative system. The present study deals with a role of the antioxidative system in these plants in response to changing levels of high radiation stress.

Materials and methods

Plants of C*lusia multiflora* H.B.K. and *Clusia minor* L. originated from cuttings of the *Clusia* plants collection of the Botanical Garden of the Darmstadt University of Technology, Germany. After a 2-months rooting period plants were repotted in plastic containers with a defined amount of soil (*Fruhsdorfer Einheitserde LD 80*, 0.23 dm^3 per plant) and grown at a natural photoperiod (photon flux density, PFD 50 - 100 μmol $m^{-2}s^{-1}$; λ = 400 - 700 nm). Cuttings for propagation were made about 6 months prior to experiments. Thereafter plants were transferred in December - January to a semi-controlled environment cabinet for 2 weeks: in the day/night average temperatures 28/22 °C and relative humidities 58/76 %. A 12-h photoperiod with PFD of 200 to 400 μmol $m^{-2}s^{-1}$ at the leaf level was used. Plants were watered and fertilized regularly (*Hakaphos* mineral nutrients, *Compo*, Münster, Germany). For high PFD treatment, plants were transferred to approximately 1200 μmol m^{-2} s^{-1} for 12 h per day (~52.8 mol m^{-2} d^{-1}) for 7 d. Plants exposed to drought stress were not watered for 7 d during exposure to high PFD. The PFD was measured using a *LI-COR quantum sensor 190SB* (Lincoln, USA).

Malate and citrate contents were determined in cell sap obtained from previously frozen leaf discs after 3 min centrifugation at 12 000 *g*. For malate detrmination, malate dehydrogenase and glutamate oxaloacetate transaminase were used according to Möllering (1974). For citrate determination, citrate lyase, malate dehydrogenase and lactate dehydrogenase were used according to Möllering (1985). Prior to the cell sap collection, leaf discs were heated at 100 °C for 3 min.

Stomatal conductance: The stomatal conductance was measured using a *DELTA-T Devices* porometer *MK III* (Cambridge, UK), which was calibrated according to the manual instructions.

Pigment analysis: Two discs (φ = 0.5 cm) were cut out of the leaves and homogenised in 2 cm^3 of solvent *a* (acetonitrile:methanol:water; 72:8:1) in a mortar under dim irradiation. The extract was transferred to an Eppendorf tube and centrifuged at 10 000 *g* for 5 min and the supernatant was directly injected on the HPLC C18 reverse-phase column, 250 × 4 mm, 5 μm, (*Tracer Analytica*, Barcelona, Spain). It was run isocratically in the solvent *a* at the flow rate of 1 cm^3 min^{-1} for 15 min to separate the xantophylls and changed afterwards to solvent *b* (methanol:ethyl acetate; 34:16) under the flow rate of 2 cm^3 min^{-1} for 35 min to elute chlorophylls and β-carotene. Detection wavelength was 440 nm and the injection volume 0.1 cm^3. The relative contents of the carotenoids was calculated from the area of the corresponding peaks. EPS value [V+0.5A]/[V+A+Z] was calculated according to Thayer and Björkman (1990).

PAGE analysis of SOD forms: Fresh leaves (1 g) were homogenised in 8 cm^3 100 mM Tricine buffer, pH 8.0, containing 3 mM $MgSO_4$, 1 mM DTT, 3 mM H_2EDTA and 50 g dm^{-3} PVPP, and centrifuged for 3 min at 12 000 *g* and 4 °C. Protein amount was estimated according to Bradford (1976). PAGE was performed using the Laemmli (1970) buffer system with the exception that SDS was absent from all the buffers. For each lane, 8 μg of the protein extract was applied. SOD activity was visualized on 12 % polyacrylamide gels, as previously described (Beauchamp and Fridovich 1971, Miszalski *et al.* 1998). Representative gels are shown in Figs. 2,3. For identification of SOD forms the gels were stained in a buffer containing 5 mM H_2O_2 and 3 mM KCN (data not shown).

Isolation of mitochondria: Fresh leaves (1 g) were homogenised in 30 cm^3 200 mM HEPES-KOH buffer, pH 7.6, containing 200 mM sorbitol, 0.5 mM $MgCl_2$, 5 mM H_2EDTA, 20 mM ascorbic acid, 2 mM DTT, 5 g dm^{-3} BSA, 20 g dm^{-3} PEG and 20 g dm^{-3} PVPP, filtered through 200 μm mesh nylon net and centrifuged for 10 min at 3 000 *g* and 4 °C. The supernatant was centrifuged at 17 000 *g* for 15 min and 4 °C, and the

resulting pellet after its washing with 2 cm^3 200 mM HEPES-KOH buffer pH 7.6 containing 400 mM sorbitol, was suspended in 50 mM HEPES buffer, pH 7.6, containing 350 mM sorbitol, 5 mM H_2EDTA, and used for PAGE and cytochrome *c* oxidase (COX) activity measurements. On the gel, the amount of 8 μg of protein was applied, both mitochondrial and crude extracts.

COX activity: The activity of COX was assayed spectrophotometrically according to Kato *et al*. (1997) by following the decrease in absorption at 550 nm.

Results and discussion

CAM induction in the C_3/CAM intermediate plant *C. minor* among other factors is governed by PFD. Borland *et al.* (1998) and De Mattos *et al.* (1999) induced CAM in *C. minor* growing plants at relatively low PFD of 530 or 400 μmol m^{-2} s^{-1}, respectively. In experiments performed by Herzog *et al.* (1999), CAM induction in *C. minor* was obtained by exposure to a light program simulating field conditions reaching a maximum of 1000 or 1200 μmol m^{-2} s^{-1} at midday and giving an integrated daily PFD of 24.5 or 33.5 mol m^{-2} d^{-1}. PFD above 1200 μmol m^{-2} s^{-1} are described by Roberts *et al.* (1998) as excessive, which *C. minor* is not able to use for photochemical work or to dissipate. Interestingly, Lüttge (1990) reported that in the obligate CAM species *Kalanchoe pinnata,* exposure to high PFD (15.85 - 22.92 mol m^{-2} d^{-1}) resulted in a lower CAM expression compared to low PFD (0.77 - 2.13 mol m^{-2} d^{-1}). In experiments presented in this paper, *C. minor* and *C. multiflora* plants were exposed for 7 d to a combination of drought stress and a high PFD of about 1200 μmol m^{-2} s^{-1} for 12 h per day (~52.8 mol m^{-2} d^{-1}) to impose severe stress. Under these conditions both *Clusia* species developed leaf necrosis. For experiments described below, however, only leaves without any visible damage were used.

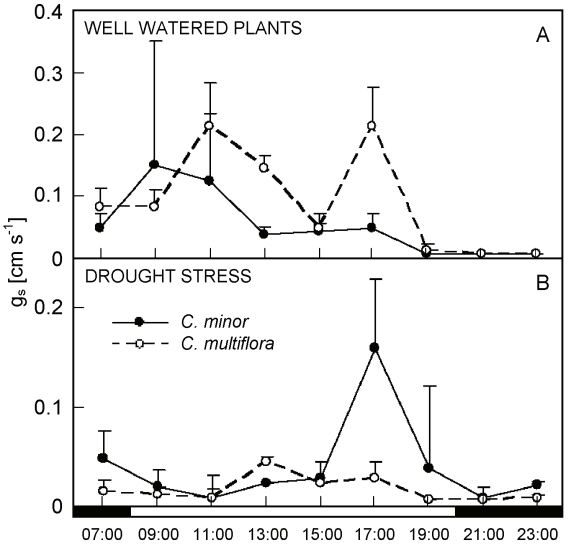

Fig. 1. Daily time-course of stomatal conductance in *Clusia minor* and *Clusia multiflora* growing at 1200 μmol m^{-2} s^{-1} under well watered conditions (*A*), and exposed to drought stress (*B*). *Open* and *closed bars* represent light and dark period, respectively. All analysis were performed using at least 3 plants (n = 15).

The stomatal conductance (g_s) which reflects the ability to take up and fix atmospheric CO_2, was measured during the day-night cycle. In well watered plants of *C. minor* and *C. multiflora* exposed to high PFD, a small tendency to decrease in g_s was observed during the early afternoon (13:00 - 15:00) followed by a clear decrease at dusk (Fig. 1*A*). Decrease of g_s in plants with C_3-photosynthesis with a minimum at about midday is a well known phenomenon (midday depression) and has been reported also for *Clusia* before (Borland *et al.* 1998, De Mattos and Lüttge 2001, Franco and Lüttge 2002). It was shown that *C. minor* in the CAM state is able to fix atmospheric CO_2 in phases II and IV of CAM *sensu* Osmond (1978), in the early morning and late afternoon, respectively (De Mattos *et al.* 1999). This is also in accordance with measurements made in field experiments by Roberts *et al.* (1998). Furthermore, Borland and Griffiths (1997) have shown that phospho*enol*pyruvate carboxylase (PEPC) in *C. minor* is active for 4 h after the onset (phase II) and 3 h before the end of the photoperiod (phase IV) illustrating the possibility of high CO_2 uptake during the light period. Thus, *C. minor* even in the CAM state is able to effectively fix atmospheric CO_2 in the light. Therefore, plant performance and survival is not only due to the magnitude of organic acid oscillations of CAM, but also light utilisation for fixation of atmospheric CO_2 during the day. In our experiments, a strong decrease in g_s was observed at dawn and dusk when *C. minor* and *C. multiflora* were exposed to combination of drought and high PFD (Fig. 1*B*) and this decrease was delayed in *C. minor* compared to *C. multiflora*. De Mattos *et al.* (1999) pointed out that in *C. minor* performing CAM, drought stress strongly limits CO_2 uptake during the day, whereas CO_2 uptake during the night remains unchanged. This is supported by Herzog *et al.* (1999) showing that in *C. minor* plants in the CAM state, exposure to high PFD (33.5 mol m^{-2} d^{-1}) inhibits photosynthesis in phase II at dawn. As expected also the exposure of *C. minor* to an extremely high PFD (~52.8 mol m^{-2} d^{-1}) caused a strong decrease in g_s at the time of phase II (Fig. 1*B*). The changes in stomatal behaviour of *C. minor* under drought stress in addition to high PFD were accompanied by only a relatively low nocturnal accumulation of malate (Δmalate 18 mmol m^{-2}) and citrate (Δcitrate 6 mmol m^{-2}). In well watered plants these values were even lower and reached about 1 mmol m^{-2} of citrate and 5 mmol m^{-2} of malate, while in

C. multiflora no accumulation was observed. In experiments of De Mattos *et al.* (1999) with *C. minor* exposed to drought stress for 16 d at a PFD of only 400 μmol m^{-2} s^{-1}, Δmalate and Δcitrate were as high as 99.4 mmol m^{-2} and 45.0 mmol m^{-2}, respectively. Thus, in *C. minor* drought stress applied in combination with high PFD appears to be a factor limiting CAM expression (low Δmalate and Δcitrate). It is also worth to mention, that most experiments published up to now described high CAM induction (high Δmalate and Δcitrate) in *C. minor* grown in the greenhouse at low PFD. In ecophysiolgical studies based on $\delta^{13}C$-analyses, which indicate the relative contribution of C_4-carboxylation *via* PEPC over the life time of leaves sampled, it is observed that in the field C_3/CAM intermediate *Clusia* species generally make only infrequent use of their CAM option (Lüttge 1999, Holtum *et al*. 2004).

High internal CO_2 concentrations generated in CAM plants during the day by malic-acid remobilization and decarboxylation (phase III) have been widely taken to support a protective mechanism feeding photochemical energy dissipation, and thus, avoiding photoinhibition. However, this is also accompanied by generation of high internal oxygen concentrations (Spalding *et al.* 1979, Lüttge 2002) causing oxidative stress. Stimulation of the alternative respiratory pathway is also discussed as a mechanism participating in avoiding oxidative stress (Hong *et al*. 2005). When, on the other hand, only a low amount of CO_2 is available by decarboxylation of low amounts of malate and citrate, as in the present study, photoinhibition at a high PFD is also expected. Photoprotective strategy due to increased non-photochemical quenching of photosystem excitation involves the xanthophyll cycle for dissipation of excitation energy as heat (Demmig-Adams and Adams III 1996). In some CAM plants including *C. minor*, changes in amount of xanthophyll cycle pigments have been found (Herzog *et al.* 1999). However, the view of CAM as being superior to C_3 photosynthesis in avoiding photoinhibition under high PFD and reduced water supply is not supported by all earlier studies. A comparison of *C. multiflora* and *C. minor* showed that the CAM option is not so much an advantage at strong continuous PFD but allows rapid adaptation to fast changes in PFD (Lüttge 1999, 2000). Our estimation of the epoxidation state of the xanthophyll cycle pigments suggests that in *C. minor* under drought and high PFD the efficiency of photon utilisation measured as epoxidation state (EPS; Table 1) in the morning (phases II+III) was lower compared to well watered plants. Independently of water availability, EPS of *C. minor* in the afternoon (phase IV) was low. In *C. multiflora* EPS remained unchanged during the course of the day and was as high as in *C. minor* in the morning under well watered conditions. In comparison to *C. minor, C. multiflora* showed a considerably lower g_s at the beginning and at the end of the photoperiod when exposed to severe drought and high PFD (Fig. 1). Thus it appears that *C. multiflora* could be more efficient in light and water use at high PFD than *C. minor*. All these arguments could explain why *C. minor* prefer semi-shaded sites in the field (Lüttge 2000).

Table 1. Epoxidation state (EPS; V+0.5 A)/(V+A+Z) of the xanthophyll cycle pigments of *Clusia minor* and *Clusia multiflora* exposed to high irradiance (1200 μmol m^{-2} s^{-1}) alone or to drought stress and high irradiance. Data are means ± SD, n = 3 for EPS values between 07:00 and 13:00. For the time period of 15:00 to 19:00 values represent the mean of two measurements.

	Clusia minor high PFD	drought and high PFD	*Clusia multiflora* high PFD
07:00-13:00	0.64 ± 0.03	0.45 ± 0.06	0.68 ± 0.07
15:00-19:00	0.23	0.28	0.59

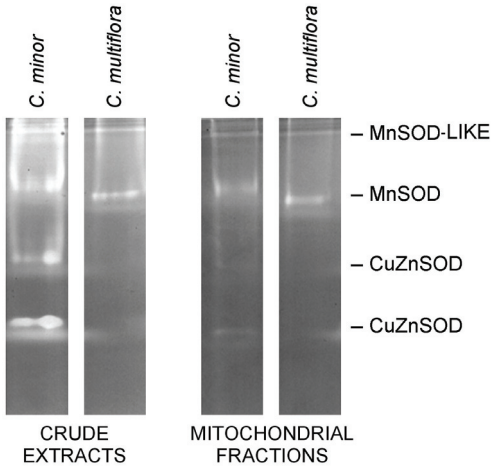

Fig. 2. Enzyme activities of MnSOD, CuZnSOD and MnSOD-like protein on PAGE activity gels of crude extracts and in mitochondrial fractions isolated from *Clusia minor* and *Clusia multiflora* leaves. 8 μg protein was loaded on the gel.

With both, their internal concentrating CO_2 in the light period (phase III) and a well expressed antioxidative response system (Miszalski *et al.* 1998), CAM plants appear to be particularly able to withstand oxidative stress (Miszalski *et al.* 1997). In the C_3-CAM intermediate plant *Mesembryanthemum crystallinum*, the activities of the mitochondrial MnSOD and COX, as well as the ATP/ADP ratio showed distinct diurnal oscillations and these fluctuations were especially pronounced in plants performing CAM (Niewiadomska *et al.* 2004). However, the mechanism of these oscillations is not understood, and it is unclear if they are due to the day/night rhythm or to performance of CAM. In the present work, we evaluated the activity of SOD in *Clusia* plants exposed to drought and high PFD. In leaf mesophyll cells of *C. minor* and *C. multiflora* at least four different forms of SOD were found (Figs. 2, 3). According to their positions on native polyacrylamide

gels, the comparison with the C_3-CAM intermediate plant *M. crystallinum* (Miszalski *et al.* 1998), and inhibitor tests (KCN and H_2O_2, data not shown) these antioxidative enzymes were identified as two forms of CuZnSOD, one form of MnSOD and one MnSOD-like protein. The MnSOD-like protein has also been found recently in *M. crystallinum* (Ślesak and Miszalski 2003). The molecular masses of the MnSOD-like protein subunit and the native form of this enzyme in *M. crystallinum* were estimated to be 57 kDa and 186 - 201 kDa, respectively. In Western blot experiments with leaf extracts of *C. minor* and *C. multiflora* using polyclonal antibodies directed against the human liver MnSOD and the human erythrocyte catalase, only cross reactions with polypetides with a molecular mass of *ca*. 60 kDa were found (data not shown). This indicates that the complex of the MnSOD-like protein (Figs. 2, 3) has either an immunologically different structure than the MnSOD or that the MnSOD-like protein is a complex of several monomers of the MnSOD, and possibly of catalase that is separated under SDS-PAGE denaturating conditions.

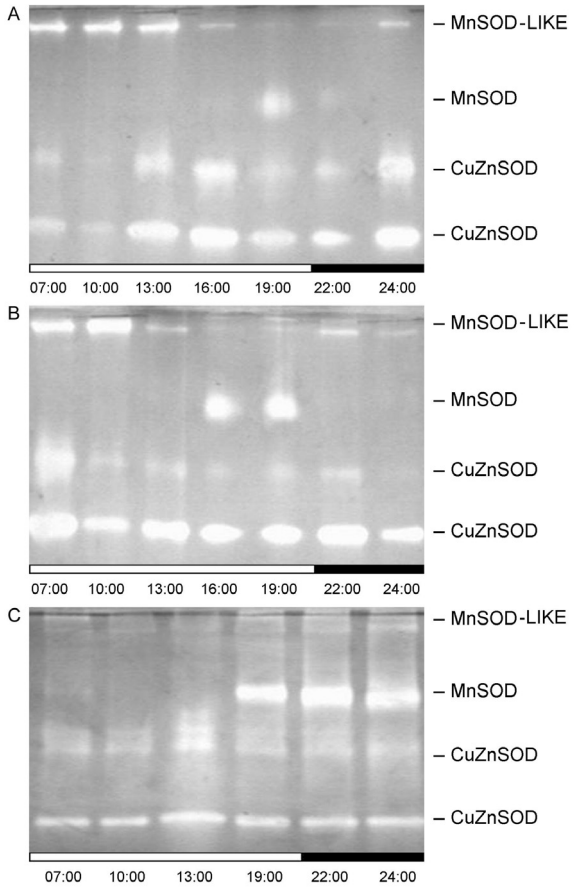

Fig. 3. Daily time-course of SOD activities on PAGE activity gels of leaf extracts: *Clusia minor* exposed for 7 d to drought stress at 1200 μmol m^{-2} s^{-1} (*A*), *Clusia minor* grown under well watered conditions at about 1200 μmol m^{-2} s^{-1} (*B*), and *Clusia multiflora* grown under well watered conditions at about 1200 μmol m^{-2} s^{-1} (*C*). Open and closed bars represent light and dark period, respectively.

In *C. minor* and *C. multiflora*, two CuZnSOD isoforms (Fig. 3) were found assumed to be located in chloroplasts and cytoplasm (Inzé and van Montagu 1995). Both isoforms changed their activity during the day-night cycle, as was shown before for *M. crystallinum* in the CAM state (Broetto *et al.* 2002). In our experiments, no clear correlation of CuZnSODs with MnSOD activity and photoperiod was observed in *C. minor*. In *C. multiflora*, no pronounced diel changes of both CuZnSODs were found.

MnSOD is enriched in the mitochondrial fraction of both plants (Fig. 2). This fractions show also higher activity of COX - mitochondrial marker enzyme: 0.205 ± 0.014 and 0.171 ± 0.018 μmol(reduced cytochrome *c*) mg $^{-1}$(protein) min^{-1}, comparing to crude extract: 0.041 ± 0.008 and 0.039 ± 0.005 for *C. minor* and *C. multiflora*, respectively. These results indicate that MnSOD is located in mitochondria. In leaf extracts of *C. minor* growing under drought and high PFD the high activity of the MnSOD was mainly during the afternoon hours (Fig. 3*A*,*B*) and in well watered plants of *C. multiflora* under high PFD during afternoon hours and in the early dark period (Fig. 3*C*). It could be expected that activity of this mitochondrial enzyme reflects changes in general mitochondrial activity, as superoxide radicals are generated in mitochondria at several sites of the electron transport chain (Perl-Treves and Perl 2002). A high mitochondrial activity is also required for the repair of damage caused by different stresses (Volin and Reich 1996), and to dissipate excess of reducing equivalents such as malate (Van Lis and Atteia 2004). Conversely, it was shown that the activity of fumarase, which can be regarded as a mitochondrial marker enzyme, exhibits only a low activity in plants performing CAM (Miszalski *et al.* 2001). As malate is a metabolite of the TCA cycle, it is postulated that in CAM plants, a down regulation of the TCA cycle is necessary for an effective malate accumulation during the night (Miszalski *et al*. 2001). Our experiments show that in well watered plants of both the C_3-CAM intermediate *C. minor* and the obligate C_3 plant *C. multiflora*, a pronounced oscillation of the MnSOD activity does occur during the day/night cycle (Fig. 3*B*). Thus, the induction of its activity was not related to drought stress. Possibly, oscillations of MnSOD activity in our experiments can be explained by the engagement of mitochondria in the dissipation of an excess of reducing equivalents produced during afternoon.

In *C. minor* exposed to drought and irradiation stress, showing only weak CAM, a rapid decrease of MnSOD activity during the night was observed as activity in the early light period was not measured (Fig. 3*A*). Induction of the MnSOD in the light period was lower and appeared later in the light phase in comparison to well watered plants (Fig. 3*B*). This may imply that these changes are not typical for plants performing CAM.

To avoid overreduction plant cells use malate for dissipating excess energy (Van Lis and Atteia 2004). We

can expect that MnSOD is induced when respiration in mitochondria is stimulated and malate can be involved in the TCA cycle. In leaf extracts of plants collected during afternoon hours, independently on the applied conditions, mitochondrial MnSOD activity bands in both species (*C. minor* and *C. multiflora*) were well pronounced (Fig. 3*A*,*B*). MnSOD activity was also detected in extracts from *C. multiflora* leaves collected in the first part of the dark period, but in *C. minor* this enzyme was active only at the end of the light period. Thus, we may conclude that the inactivation of MnSOD during darkness was faster in the CAM plant compared to the C_3 plant. No MnSOD activities were measured at the beginning of the light period. It can be expected that in CAM plants, activity of mitochondria in removing excess reduction equivalents is not required when NADH is used for malate production. In experiments with four *Clusia* species (*C. venosa, C. minor, C. major, C. alata*) it was shown before that oxygen uptake rates measured with an oxygen electrode were lower at night (Franco *et al.* 1990) than during the day. This observation is in accordance with the MnSOD activity measurements shown in this paper. However, in contrast to our studies, Franco *et al.* (1990) found that respiration rates were low for C_3-like *Clusia* species. We suggest that this discrepancy may be the result of different sampling times. The respiration rate analyzed by Franco *et al.* (1990) could also have been affected by the experimental conditions using 0.1 M carbonate-bicarbonate buffer generating CO_2 which can influence NADH levels available for respiration.

The literature on the role of mitochondria in CAM is still scant. It is well known that during the dark period in CAM malic and citric acids pass the TCA in the mitochondria before nocturnal vacuolar storage (Osmond 1978, Olivares *et al.* 1993). In CAM, a down-regulation of mitochondrial activity in the dark period might be expected so that the organic acids are available for nocturnal storage, as discussed above. On the other hand, a high activity of a mitochondrial enzyme, such as MnSOD, is expected during the light period to deal with oxidative stress, as shown here. In conclusion, regulation of mitochondrial activity during CAM, which was only weakly expressed in *C. minor* under the conditions of the present study, must be rather complex and more work with more variable environmental conditions is required, which is under way in our laboratory.

References

Adams III, W.W., Demmig-Adams, B.: Operation of the xanthophylls cycle in higher plants in response to diurnal changes in incident sunlight. - Planta **186**: 390-398, 1992.

Bartosz, G.: Oxidative stress in plants. - Acta Physiol. Plant. **19**: 47-64, 1997.

Beauchamp, C., Fridovich, I.: Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. - Anal. Biochem. **44**: 276-287, 1971.

Borland, A.M., Griffiths, H.: A comparative study on the regulation of C_3 and C_4 carboxylation processes in the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* and the C_3-CAM intermediate species *Clusia minor*. - Planta **201**: 368-378, 1997.

Borland, A.M., Griffiths, H., Broadmeadow, M.S.J., Fordham, M.C., Maxwell, C.: Short term changes in carbon-isotope discrimination in the C_3/CAM intermediate *Clusia minor* L. growing in Trinidad. - Oecologia **95**: 444-453, 1993.

Borland, A.M., Griffiths, H., Maxwell, C., Fordham, M.C., Broadmeadow, M.S.J.: CAM induction in *Clusia minor* L. during the transition from wet to dry season in Trinidad: the role of organic acid speciation and decarboxylation. - Plant Cell Environ. **19**: 655-664, 1996.

Borland, A.M., Técsi, L.I., Leegood, R.C., Walker, R.P.: Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/biochemical characterisation and intercellular localisation of carboxylation and decarboxylation processes in three species which exhibit different degrees of CAM. - Planta **205**: 342-351, 1998.

Bradford, M.M.: A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Broetto, F., Lüttge, U., Ratajczak, R.: Influence of light intensity and salt-treatment on mode of photosynthesis and enzymes of the antioxidative response system of *Mesembryanthemum crystallinum*. - Funct. Plant Biol. **29**: 13-23, 2002.

Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D., Van Breusegem, F.: Dual action of active oxygen species during plant stress responses. - Cell mol. Life Sci. **57**: 779-795, 2000.

De Mattos, E.A., Herzog, B., Lüttge, U.: Chlorophyll fluorescence during CAM-phases in *Clusia minor* L. under drought stress. - J. exp. Bot. **50**: 253-261, 1999.

De Mattos, E.A., Lüttge, U.: Chlorophyll fluorescence and organic acid oscillations during transition from CAM to C_3-photosynthesis in *Clusia minor* L. (*Clusiaceae*). - Ann. Bot. **88**: 457-463, 2001.

Demmig-Adams, B., Adams III, W.W.: Xanthophyll cycle and light stress in nature: uniform response to excess direct sunlight among higher plant species. - Planta **198**: 460-470, 1996.

Dodd, A.N., Griffiths, H., Taybi, T., Cushman, J.C., Borland, A.M.: Integrated diel starch metabolism with the circadian and environmental regulation of Crassulacean acid metabolism in *Mesembryanthemum crystallinum.* - Planta **216**: 789-797, 2002.

Franco, A.C., Ball, E., Lüttge, U.: Patterns of gas exchange and organic acid oscillations in tropical trees of the genus *Clusia*. - Oecologia **85**: 108-114, 1990.

Franco, A.C., Lüttge, U.: Midday depression in savana trees: coordinated adjustments in photochemical efficiency, photorespiration, CO_2 assimilation and water use efficiency. - Oecologia **131**: 356-365, 2002.

Halliwell, B., Gutteridge, J.M.C.: Free Radicals in Biology and Medicine. - Clarendon Press, Oxford 1999.

Hernández, J.A., Escobar, C., Creissen, G., Mullineaux, P.M.: Antioxidant enzyme induction in pea plants under high irradiance. - Biol. Plant. **50**: 395-399, 2006.

Herzog, B., Hoffmann, S., Hartung, W., Lüttge, U.: Comparison

of photosynthetic responses of the sympatric tropical C_3 species *Clusia multiflora* H.B.K. and the C_3-CAM intermediate species *Clusia minor* L. to irradiance and drought stress in a phytotron. - Plant Biol. **1**: 460-470, 1999.

Holtum, J.A.M., Aranda, J., Virgo, A., Gehrig, H.H., Winter, K.: $\delta^{13}C$ values and crassulacean acid metabolism in *Clusia* species from Panama. - Trees **18**: 658-668, 2004.

Hong, H.T.K., Nose, A., Agarie, S., Oxidation of various substrates and effects of the inhibitors on purified mitochondria isolated from *Kalanchoë pinnata*. - Biol. Plant. **49**: 201-208, 2005.

Inzé, D., Van Montagu, M.: Oxidative stress in plants. - Curr. Opin. Biotechnol. **6**: 153-158, 1995.

Kato, J., Yamahara, T., Tanaka, K., Takio, S., Satoh, T.: Characterization of catalase from green algae *Chlamydomonas reinhardtii.* - J. Plant Physiol. **151**: 262-268, 1997.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head bacteriophage T4. - Nature **227**: 680-685, 1970.

Lüttge, U.: Nocturnal citrate accumulation and its response to environmental stress in the CAM plant *Kalanchoe pinnata* (Lam.) Pers. - Plant Cell Environ. **13**: 977-982, 1990.

Lüttge, U.: One morphotype, three physiotypes: sympatric species of *Clusia* with obligate C_3 photosynthesis, obligate CAM and C_3-CAM intermediate behaviour. - Plant Biol. **1**: 138-148, 1999.

Lüttge, U.: Light-stress and crassulacean acid metabolism. - Phyton (Austria) **40**: 65-82, 2000.

Lüttge, U.: CO_2-concentrating: consequences in crassulacean acid metabolism. - J. exp. Bot. **53**: 2131-2142, 2002.

Lüttge, U. Photosynthesis: CAM plants. - In: Thomas, B., Murphy, D.J., Murray, B.G. (ed.): Encyclopedia of Applied Plant Sciences. Pp. 688-704. Academic Press, Oxford 2003.

Maxwell, K., Borland, A.M., Haslam, R.P., Helliker, B.R., Roberts, A., Griffiths, H.: Modulation of Rubisco activity during the diurnal phases of the crassulacean acid metabolism plant *Kalanchoe daigremontiana*. - Plant Physiol. **121**: 849-856, 1999.

Miszalski, Z., Kluge, M., Ziegler, H.: Stimulatory effect of sulphite on photosynthetic $^{14}CO_2$-fixation in *Kalanchoe dagremontiana* Hamet et Perier. - J. Plant Physiol. **151**: 535-540, 1997.

Miszalski, Z., Niewiadomska, E., Ślesak, I., Lüttge, U., Kluge, M., Ratajczak, R.: The effect of irradiation on carboxylating/decarboxylating enzymes and fumarase activities in *Mesembryanthemum crystallinum* L., exposed to salinity stress. - Plant Biol. **3**: 17-23, 2001.

Miszalski, Z., Ślesak, I., Niewiadomska, E., Baczek-Kwinta, R., Lüttge, U., Ratajczak, R.: Subcellular localization and stress responses of superoxide dismutase isoforms from leaves in the C_3-CAM intermediate halophyte *Mesembryanthemum crystallinum* L. - Plant Cell Environ. **21**:169-179, 1998.

Mittler, R.: Oxidative stress, antioxidative stress tolerance. - Trends Plant Sci. **7**: 405-410, 2002.

Möllering, H.: Malat: Bestimmung mit Malat-Dehydrogenase und Glutamat-Oxalacetat-Transaminase. - In: Bergmeyer, H.U. (ed.): Methoden der Enzymologie. Pp. 1636-1639. Academic Press, New York 1974.

Möllering, H.: Citrate. Determination with citrate lyase, MDH and LDH. - In: Bergmeyer H.U. (ed.): Methods of Enzymatic Analysis. Pp. 2-12. Academic Press, New York 1985.

Niewiadomska, E., Karpinska, B., Romanowska, E., Ślesak, I., Karpinski, S.: A salinity-induced C_3-CAM transition increases energy conservation in the halophyte *Mesembryanthemum crystallinum* L. - Plant Cell Physiol. **45**: 789-794, 2004.

Niewiadomska, E., Miszalski, Z., Ślesak, I., Ratajczak, R.: Catalase activity during C_3-CAM transition in *Mesembryanthemum crystallinum* L. leaves. - Free Radical Res. **31**: S251-256, 1999.

Nyman, L.P., Benzing, D.H., Temple, P.J., Arditti, J.: Effects of ozone and sulfur dioxide on two epiphytic orchids. - Environ. exp. Bot. **30**, 207-213, 1990.

Olivares, E., Faist, K., Kluge, M., Lüttge, U.: $^{14}CO_2$ pulse-chase labelling in *Clusia minor* L. - J. exp. Bot. **45**: 1527-1533, 1993.

Olszyk, D.M., Bytnerowicz, A., Fox, C.A.: Sulfur dioxide effects on plants exhibiting Crassulacean acid metabolism. - Environ. Pollut. **43**: 47-62, 1987.

Osmond, C.B.: Crassulacean acid metabolism: a curiosity in context. - Annu. Rev. Plant Physiol. **29**: 379-414, 1978.

Perl-Treves, R., Perl, A.: Oxidative stress an introduction. - In: Inzé, D., Van Montagu, M. (ed.): Oxidative Stress in Plants. Pp. 1-32. Taylor and Francis, London 2002.

Roberts, A., Borland, A.M., Maxwell, K., Griffiths, H.: Ecophysiology of the C_3-CAM intermediate *Clusia minor* L. in Trinidad: seasonal and short-term photosynthetic characteristics of sun and shade leaves. - J. exp. Bot. **49**: 1563-1573, 1998.

Scandalios, J.G.: Oxygen stress and superoxide dismutases. - Plant Physiol. **101**: 7-12, 1993.

Ślesak, I., Miszalski, Z.: Superoxide dismutase-like protein from roots of the intermediate C_3-CAM plant *Mesembryanthemum crystalinum* L. in *in vitro* culture. - Plant Sci. **164**: 497-505, 2003.

Ślesak, I., Miszalski, Z., Karpinska, B., Niewiadomska, E., Ratajczak, R., Karpinski, S.: Redox control of oxidative stress responses in the C_3-CAM intermediate plant *Mesembryanthemum crystallinum*. - Plant Physiol. Biochem. **40**: 669-677, 2002.

Spalding, M.H., Stumpf, D.K., Ku, M.S.B., Burris, R.H., Edwards, G.E.: Crassulacean acid metabolism and diurnal variations of internal CO_2 and O_2 concentrations in *Sedum praealtum* DC. - Aust. J. Plant Physiol. **6**: 557-567, 1979.

Thayer, S.S., Björkman, O.: Leaf xanthophylls content and composition in sun and shade determinated by HPLC. - Photosynth. Res. **23**: 331-343,1990.

Van Lis, R., Atteia, A.: Control of mitochondrial function via photosynthetic redox signals. - Photosynth. Res. **79**:133-148, 2004.

Volin, J.C., Reich, P.B.: Interaction of elevated CO_2 and O_3 in growth, photosynthesis and respiration of three perennial species grown at low and high nitrogen. - Physiol. Plant. **97**: 674-684, 1996.